Universidad de Huelva

Departamento de Química "Profesor José Carlos Vílchez Martín

New tools for Genetic Engineering of Microalgae: Reprogramming the carotenoid biosynthetic pathway in the Chlorophyte Chlamydomonas reinhardtii

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Huelva, 2023

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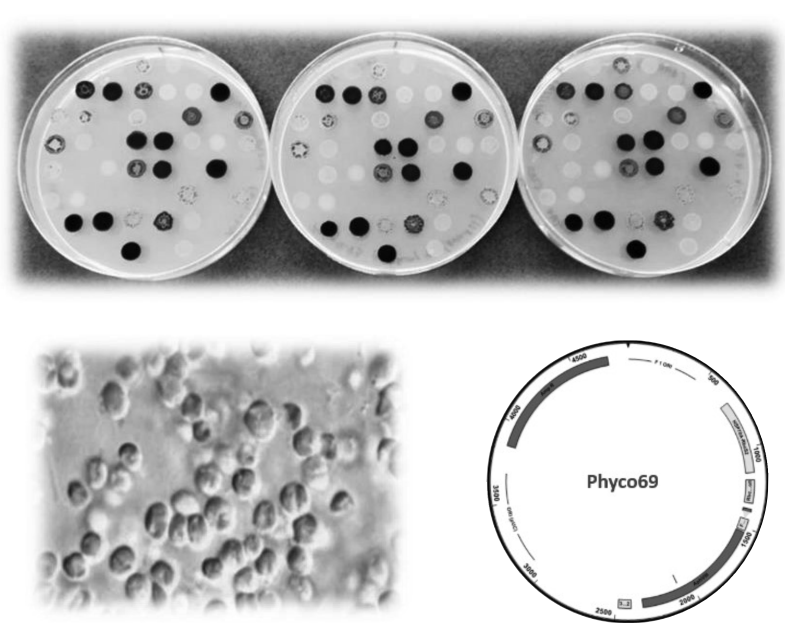

TESIS DOCTORAL

Ana María Molina Márquez

Huelva, 2023

UNIVERSIDAD DE HUELVA

FACULTAD DE CIENCIAS EXPERIMENTALES
DEPARTAMENTO DE QUÍMICA "PROFESOR JOSÉ CARLOS VÍLCHEZ MARTÍN"

TESIS DOCTORAL

"NUEVAS HERRAMIENTAS PARA LA INGENIERÍA GENÉTICA EN MICROALGAS: REPROGRAMANDO LA RUTA BIOSINTÉTICA DE LOS CAROTENOIDES EN LA CLOROFITA *CHLAMYDOMONAS REINHARDTII*"

"NEW TOOLS FOR GENETIC ENGINEERING OF MICROALGAE: REPROGRAMMING THE CAROTENOID BIOSYNTHETIC PATHWAY IN THE CHLOROPHYTE *CHLAMYDOMONAS REINHARDTII*"

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Ana María Molina Márquez

Trabajo presentado bajo la dirección de:

Dra. Rosa León Bañares
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A mi familia por su apoyo constante, especialmente a mis padres

Frase célebre

AGRADECIMIENTOS

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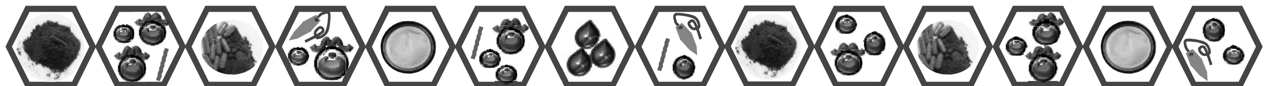

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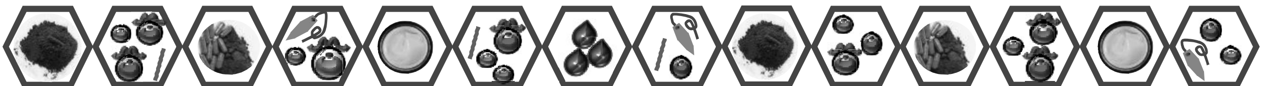
ABSTRACT



ABSTRACT

Microalgae comprise a ubiquitous group of microorganisms with photosynthetic capacity, which are essential for life on Earth. They produce about half of the atmospheric oxygen and fix atmospheric CO_2 into organic compounds, contributing to CO_2 mitigation, and can be found in a wide range of environmental conditions, from marine to freshwater or soil habitats, including extreme environments with high irradiance, low pH values or extreme temperatures. Microalgae have attracted the attention of the industry and, due to their biochemical and metabolic diversity, are considered a renewable and sustainable source of a large variety of high-value products, such as lipids, peptides, polysaccharides, recombinant proteins, or carotenoids, among others. Carotenoids are a diverse group of lipophilic isoprenoid compounds, appreciated by their pro-vitaminic, antioxidant, free radical-scavenger and colouring properties, playing an important role in the health of human and animals, which must include them in their diet.

The biotechnological potential of microalgae has postulated them as an alternative natural source of products with application in different sectors, such as in food-feed, pharmacology, cosmetic or energetic industry. However, many of the valuable compounds that microalgae produce are biosynthesized in small quantities, at the stationary phase, or under stressing culture conditions, which reduce their productivity. Genetic engineering is a potential tool to obtain enhanced microalgae with higher production efficiencies or able to accumulate recombinant proteins, such as vaccines and other therapeutic proteins. The discovery of new molecular tools, such as strong specific promoters or new genetic markers, and the design of efficient selective screening methods will help to accomplish the stable transformation of microalgae and will allow the easy expression of foreign DNA or the efficient silencing of specific endogenous genes.

The aim of this doctoral thesis is to develop genetic strategies and molecular tools to obtain enhanced microalgae, overcoming the limitations in the productivity of desirable compounds, such as carotenoids. In **Chapter 1**, the validation of a new multicistronic expression plasmid for the simultaneous expression of several genes

under the control of the same promoter, ensuring the selection and the stable expression of transgenes in microalgae is shown. In **Chapter 2**, a new efficient selectable marker gene has been tested in eukaryotic microalgae. This new marker gene is based in a bacterial phytoene desaturase gene and provides resistance to the herbicide norflurazon, offering an interesting alternative to the classical resistance to antibiotics. Finally, in **Chapter 3**, a trending genetic tool, CRISPR-Cas System has been implemented for microalgae as an efficient method to edit their genome. As a proof of concept, the system has been applied to the model chlorophyte *C. reinhardtii,* and enhanced cell lines able to accumulate the colourless carotenoid phytoene have been generated.

In **Chapter 1**, a new expression plasmid, named as Phyco69, is designed to express genes of interest (GOI) in microalgae. Phyco69 consists of the *APHVIII* gene sequence from *Streptomyces rimosus*, a marker gene that encodes the enzyme aminoglycoside 3'phosphotransferase and confers resistance to the antibiotic paromomycin, fused to a polylinker region through a short self-cleaving peptide (FMDV-2A). This peptide allows the expression of two genes under the control of the same promotor, since the polyprotein formed (GOI-FMDV-2A-APHVIII) is spontaneously split in the eukaryotic ribosome to generate two independent products. All these elements are under the control of a strong tandem quimeric promoter, formed by the fusion of Rubisco promoter and a fraction of the heat shock 70A promoter. In this work, Phyco69 has been validated as multicistronic plasmid for the expression of two genes, the GOI and the marker gene under the control of one promotor. As GOI, a second antibiotic resistance marker, the *ShBLE* gene, which confers phleomycin resistance has been used. RT-PCR and phenotypic studies have revealed a simple method to screen and select the transformant cells with the highest level of expression of both, the GOI and *APHVIII.* Immunodetection studies have shown that the multicistronic transcript generated from Phyco69 is correctly processed, producing independent proteins from a common promoter. Phyco69 offers an interesting strategy to guarantee the expression of the GOI, avoiding gene silencing as long as the microalga is maintained under the paromomycin pressure and, avoiding large time-consuming phenotypic screenings, since we have demonstrated that those transformants able to growth

under higher concentrations of paromomycin show higher expression levels of the GOI.

In **Chapter 2** a new selectable maker gene, *CRTIop,* is stated for the selection of microalgal transformant cells, based on the resistance to the bleaching herbicide norflurazon. Phytoene desaturase is a committed enzyme of the beginning of carotenoid biosynthesis pathway. This enzyme transforms the first colourless carotenoid, phytoene, into colour carotenoids using FAD as cofactor and it is essential for the synthesis of all carotenoids. In algae and plants, phytoene desaturase (PDS) is inhibited by the herbicide norflurazon. However, the bacterial phytoene desaturase (CRTI) is not sensitive to norflurazon and catalyses the conversion of phytoene into lycopene. In this chapter, a synthetic version of *CRTI* gene adapted to the codon usage of *C. reinhardtii* has been synthesized and has been demonstrated to be an efficient selectable marker gene in the microalga. The possible utilization of this gene as selective marker for other microalgae, both marine and fresh water, has also been demonstrated. Microalgae were transformed by the glass bead agitation method and selected in medium with norflurazon, obtaining an average transformation efficiency of 550 colonies per µg of DNA. All the transformants tested had incorporated the *CRTIop* gene in their genomes and were able to synthesize coloured carotenoids, demonstrating the efficiency of *CRTIop* gene as a selectable marker for microalgae.

In **Chapter 3**, the CRISPR-Cas9 System has been used to knock out the *PDS* gene in *Chlamydomonas reinhardtii*, obtaining cells that accumulate phytoene, the first carotenoid produced. In this work, two Cas9-Ribonucleoprotein complexes (RNP-PDS1, RNP-PDS2) were designed to target the first exon of the *PDS* gene assembled and tested *in vitro*, demonstrating its efficiency to cleave the target site in *PDS* gene. Subsequently, an *in vivo* experiment was performed, and 30 and 45 colonies per µg de DNA were obtained with the RNP-PDS1 and RNP-PDS2, respectively. Among the transformants obtained, 30 and 50% respectively appeared as white colonies, indicating the lack of coloured carotenoids. PCR and sequencing studies of the seven white microalgal colonies selected, revealed insertional mutations generating the knockout of *PDS* gene in the target site. HPLC analysis of the selected ΔE10 Knock out mutant showed that the only carotenoid synthetized by this mutant line was phytoene.

Abstract

Optical microscopy analysis revealed that both, parental line and ΔE10 Knock out mutant have similar shape. However, the transmission electron microscopy revealed in the mutant cells untypical chloroplast structure, with the presence of plastid membrane but not stacks of thylakoid membranes. Finally, the transcriptome analysis revealed clustering of the differentially expressed genes into functional groups related with protein and peptide synthesis, translation, ribosome biogenesis, photosynthesis and biosynthesis of thylakoids and photosystems components that affect other important cellular processes, such as CO_2 assimilation or flagella functionality.

RESUMEN

Las microalgas comprenden un amplio grupo de microorganismos con capacidad fotosintética, que es esencial para la vida en la Tierra. Producen alrededor de la mitad del oxígeno atmosférico y fijan CO_2 atmosférico metabolizándolos en compuestos orgánicos, contribuyendo a la mitigación de CO_2. Pueden encontrarse en diferentes habitats con diversas condiciones ambientales desde ambientes marinos, de agua dulce o suelos, incluyendo ambientes extremos con alta irradiancia, bajo pH o temperaturas extremas. Las microalgas han atraído la atención de la industria por su diversidad bioquímica y metabólica, son consideradas fuentes renovables y sostenibles de una amplia variedad de productos, como lípidos, péptidos, polisacáridos, proteínas recombinantes o carotenoides, entre otros. Los carotenoides son un grupo diverso de compuestos isoprenoides lipofílicos, apreciados por sus propiedades pro-vitamínicas, antioxidantes, secuestradores de radicales libres y colorantes, que juegan un importante rol in la salud humana y animal, quienes deben incluirlos en su dieta.

El potencial biotecnológico que tienen las microalgas las ha postulado como una fuente alternativa de productos con aplicaciones en diferentes sectores, como en la industria alimentaria, farmacológica, cosmética y energética. Sin embargo, muchos de los compuestos apreciados de las microalgas son producidos en pequeñas cantidades, en fase estacionaria o bajo condiciones estresantes de cultivo, que reducen su productividad. La ingeniería genética es una potencial herramienta para obtener microalgas mejoradas con más altas eficiencias de producción o que son capaces de acumular proteínas recombinantes, como vacunas y otras proteínas terapéuticas. El descubrimiento de nuevas herramientas moleculares, como promotores específicos fuertes o nuevos genes marcadores, y el diseño de métodos de selección efectivos de transformantes, ayudaría a lograr la transformación estable de microalgas y a obtener la expresión de ADN exógeno o el silenciamiento de genes endógenos específicos.

El objetivo de esta tesis doctoral es el desarrollo de estrategias genéticas y herramientas moleculares para la obtención de microalgas mejoradas genéticamente, superando las limitaciones en cuanto a la productividad de compuestos deseados

como los carotenoides. En el **Capítulo 1**, se muestra la validación de un nuevo plásmido de expresión multicistrónica que permite la expresión simultánea de varios genes bajo el control de un mismo promotor, asegurando la selección y expresión estable de transgenes en microalgas. En el **Capítulo 2**, se ha probado la eficiencia de un nuevo gen marcador en microalgas eucariotas. Este nuevo gen marcador se basa en el gen bacteriano que codifica para la fitoeno desaturasa y que confiere resistencia al herbicida norflurazon, ofreciendo una interesante alternativa a la clásica resistencia a antibióticos. Finalmente, en el **Capítulo 3**, se ha implementado en microalgas una herramienta genética muy popular, el sistema basado en CRISPR-Cas como un método eficiente para editar su genoma. Como prueba de concepto, el sistema ha sido aplicado en la microalga Clorofita *C. reinhardtii*, obteniendo líneas celulares capaces de acumular el carotenoide no coloreado fitoeno.

En el **Capítulo 1**¸ se ha diseñado un nuevo plásmido de expresión en microalgas, llamado Phyco69 que permite la expresión de genes de interés (GOI). Phyco69 está formado por la secuencia del gen *APHVIII* de *Streptomyces rimosus*, que codifica para la enzima aminoglicosidasa 3'fosfotransferasa y confiere resistencia al antibiótico paromomicina, fusionado a la región polilinker a través del pequeño péptido autohidrolizable (FMDV-2A). Este péptido permite la expresión de dos genes bajo el control del mismo promotor, ya que la poliproteína formada (GOI-FMDV-2A-APHVIII) es espontáneamente procesada en el ribosoma eucariótico generando dos productos independientes. Todos los elementos están bajo el control del promotor quimérico, constitutivo fuerte formado por la fusión del promotor Rubisco y una fracción del promotor 70A de choque térmico. En este trabajo Phyco69 ha sido validado como un plásmido multicistrónico para la expresión de dos genes, el GOI y el gen marcador bajo el control del mismo promotor. Como GOI se ha elegido un segundo gen que confiere resistencia al antibiótico fleomicina, el gen *shBLE*. Estudios de RT-PCR y fenotípicos revelaron un método simple para selección de células con mayores niveles de expresión de ambos genes, el GOI y el *APHVIII*. Los estudios de inmunodetección mostraron que el transcrito multicistrónico generado por Phyco69, es correctamente procesado generando proteínas independientes desde un promotor. Phyco69 ofrece una interesante estrategia para garantizar la expresión del GOI, evitando el

silenciamiento génico mientras la microalga se mantenga bajo presencia de paromomicina y, evita largas búsquedas fenotípicas, ya que se ha demostrado que aquellos transformantes capaces de crecer a mayores concentraciones de paromomicina muestran niveles más altos de expresión del GOI.

En el **Capítulo 2**¸ se presenta un nuevo gen marcador, fitoeno desaturasa (*CRTIop*,) para la selección de células transformantes de microalgas, basado en la resistencia al herbicida blanqueante norflurazon. La enzima fitoeno desaturasa es un paso limitante al principio de la ruta de biosíntesis de carotenoides. Esta enzima transforma el primer carotenoide no coloreado, el fitoeno, en un carotenoide coloreado utilizando FAD como cofactor, y siendo esencial para la síntesis de todos los carotenoides. En algas y plantas, la fitoeno desaturasa (PDS) es inhibida por el herbicida norflurazon. Sin embargo, la fitoeno desaturasa bacteriana (CRTI) no es sensible a norflurazon y cataliza la conversión del fitoeno a licopeno. En este capítulo, una versión sintética del gen *CRTI* ha sido sintetizada, adaptada al uso de codones de *C. reinhardtii* y ha demostrado ser un gen marcador eficiente en esta microalga. Se ha demostrado la posible utilización de este gen como un marcador selectivo para otras microalgas, tanto marinas como de agua dulce. Las microalgas fueron transformadas por agitación con perlas de vidrio y se seleccionaron en medio con norflurazon, obteniendo una media en la eficiencia de transformación de 550 colonias por µg de ADN. Todos los transformantes analizados incorporaron el gen *CRTIop* en su genoma y pudieron sintetizar carotenoides coloreados, demostrando la eficiencia del gen *CRTIop* como gen marcador en microalgas.

En el **Capítulo 3**, el sistema CRISPR-Cas9 se ha utilizado para realizar el knock out del gen *PDS* en *Chlamydomonas reinhardtii*, obteniendo células que acumulan fitoeno, el primer carotenoide producido. En este trabajo, se han diseñado dos complejos ribonucleoproteicos-Cas9 (RNO-PDS1, RNP-PDS2) para dirigirse al primer exón del gen *PDS*, ensamblándolos y probándolos *in vitro* y demostrando ser eficientes en la escisión del sitio diana en el gen *PDS.* A continuación, se realizó el ensayo *in vivo*, obteniendo 30 y 45 colonias por µg de ADN con el complejo RNP-PDS1 y RNP-PDS2, respectivamente. Entre los transformantes obtenidos, el 30 y el 50 % respectivamente, aparecieron como colonias blancas, indicando la falta de carotenoides coloreados. Se

seleccionaron 7 colonias de microalgas blancas para estudios de PCR y secuenciación, revelando mutaciones insercionales en el sitio diana que generaron knock out del gen *PDS*. Se seleccionó el mutante knock out ΔE10 para realizar ensayos de HPLC, mostrando que el carotenoide fitoeno fue el único detectado en esta línea mutante. Análisis de microscopía óptica mostraron que tanto la línea parental como la knock out ΔE10 tienen una forma similar. Sin embargo, la microscopía electrónica de transmisión reveló que las células mutantes presentaban una estructura atípica del cloroplasto, con la presencia de membrana plastidial pero no con apilamiento de membranas tilacoidales. Finalmente, el análisis transcriptómico reveló agrupación de genes diferencialmente expresados en grupos funcionales relacionados con la síntesis de proteínas y péptidos, la traducción, fotosíntesis y biosíntesis de tilacoides y componentes de fotosistemas que afectan a otros procesos celulares importantes, como la asimilación de CO_2 o la funcionalidad de los flagelos.

INTRODUCTION

INTRODUCTION

1. MICROALGAE IN BIOTECHNOLOGY

Microalgae are a diverse group of photosynthetic microorganisms, essential for life on Earth and based on the trophic chain. Through photosynthesis, they fix atmospheric CO_2 into organic compounds, using light energy and producing about half of the atmospheric oxygen. These ubiquitous microorganisms are adapted to cope with a wide range of environmental conditions, being able to grow in diverse aquatic, either marine or freshwater, and even soil habitats; and tolerate broad range of temperatures, salinities, pH values and light intensities. This versatility postulates microalgae as a novel source of natural bioactive compounds that can be adapted to diverse industrial process.

There is an increasing interest in microalgal biotechnology due to the numerous advantages of these microorganisms, that combine biochemical and metabolic diversity with the possibility to be renewable, sustainable and economical sources of valuable products, such us steroids, polysaccharides, oligopeptides, glycolipids, anti-cancer, anti-inflammatory, anti-fungal compounds, antioxidants, enzymes, vitamins, lipids and pigments, among others (Galasso et al., 2019; Vecchi et al., 2020, Coulombier 2021), which attracted great attention for commercial purposes (Figure 1).

The biotechnological potential of microalgae has made increase the interest in their mass cultivation, in the last decades. In addition, the production of microalgal biomass has important advantages in comparison to plant biomass, including higher light-use efficiency, more efficient carbon capture contributing to CO_2 mitigation, faster growth, and higher biomass productivity. Moreover, microalgae do not require fertile land and fresh water to grow, but they are able to grow in marginal land and wastewater, being possible their use in decontamination treatments, (Benedetti et al., 2018; Vecchi et al., 2020).

The implementation of microalgae in the biotechnological sector as source of demanding and renewable products is patented through the world (Table 1). Biofuels

produced by microalgae such as biodiesel and bioethanol can be eco-friendly alternative fuels with a lower contribution to environmental pollution and strong potential of fixing global CO_2 (Khan et al., 2018). *Botryococcus braunii, Dunaliella salina, Chlamydomonas reinhardtii*, *Scenedesmus* sp and *Chorella vulgaris* are considered carbohydrates-rich microalgae and potential source of economically viable bioethanol (Benedetti et al., 2018; Chng et al., 2017; Sivaramakrishnan & Incharoensakdi, 2018; Usher et al., 2014).

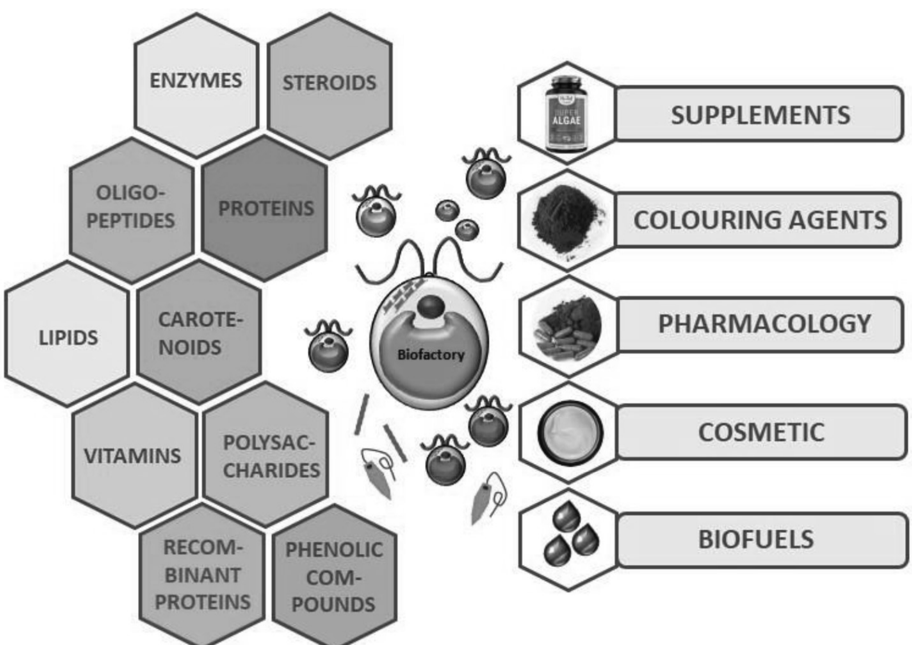


Figure 1. Microalgae as biofactory of high-value compounds and their applications.

Microalgae compounds have remarkable characteristics appreciate for food, feed, pharmacology, and cosmetic industry. They are also a rich source of protein, carbohydrates, vitamins, and lipids that highlight their role as a nutritional ingredient for humans and animals. In addition, their ability for the production of essential amino acids; polysaccharides such as starch, cellulose, hemicelluloses; vitamins like vitamin E, K or D; polyunsaturated fatty acids and pigments, all this makes microalgae a valuable source of natural health-promoting products, available both in pure form as

extracts, tablets or capsules orals and additives to several food products (Ferreira de Oliveira & Bragotto, 2022; Khan et al., 2018; Koyande et al., 2019). The therapeutic properties and the antioxidant activity of compounds such as the polyunsaturated acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and pigments, chlorophyll and carotenoids like β-carotene, astaxanthin, lutein or phytoene have demonstrated to control diabetes and obesity, prevent cardiovascular and ocular diseases, cancer, Parkinson's disease, atherosclerosis, asthma and arthritis (de Viteri et al., 2020; Khan et al., 2018; Meléndez-Martínez et al., 2022; Pal et al., 2020). Pigments, like β-carotene, are currently utilized in the industrial sector as colouring agents (Meléndez-Martínez et al., 2022). Microalgae are able to produce a wide range of other commercially valuable product, such as phenolic compounds with antioxidant activities, that neutralize reactive species of oxygens (ROS) primarily by hydrogen atom transfer (Khan et al., 2018) or alkaloids that seems to exhibit antimicrobial and anticancer properties and are synthesized by microalgae like *Scenedesmus* sps*, Arthrospira plantesis* and *Isochrysis galbana* (Sreenikethanam et al., 2022).

The main goal to achieve the competitive production of microalgae as feedstocks of desired metabolites is reducing operating cost, optimization of cultivation methods, improvement of processing technologies like harvesting or purification of the interesting compounds; and enhancing the productivity (Figure 2), (Benedetti et al., 2018; León-Bañares et al., 2004).

The improvement of microalgae by genetic modification is a valid approach to obtain enhanced strains with major production efficiencies or able to produce recombinant proteins, such as vaccines and other therapeutic proteins. Strategies for genetic manipulation of microalga includes overexpression of key gene/s involved in the biosynthesis of the valuable compounds, or target knock out of specific gene/s to block undesirable products, enhancing photosynthesis efficiency or development of other desired capacities, such as resistance to pathogens, or increase the tolerance to abiotic factors (Kumar et al., 2020) .

Industial and Medical Field	Cosmetics and food colorant	High-value metabolites	Biofuel	Dietary supplement and nutraceuticals
Polysaccharides	*Phycobiliproteins*	*Mycosporine-like amino acids*	*Oil to biodiesel*	*Polyunsaturated fatty acids (PUFAs)*
Chlorella spp. (*β-glucans, starch*)	*Arthrospyra platensis* (phycocyanin)	*Aphanizomenon flos-aquae*	*Botryococcus braunii*	*Parietochloris incise*
Porphyridium cruentum	*Carotenoids*	*Vitamins*	*Chlorella* spp.	*Porphyridium* spp. (arachidonic acid)
Netrium digitus	*Arthrospyra platensis*	*Euglena gracilis* (biotin, α-tocopherol)	*Dunaliella salina*	*Arthrospyra platensis*
Phycotoxins	*Chlorella vugaris*	*Prototheca moriformis* (ascorbic acid)	*Monoraphidium*	*Rhodomonas salina*
Amphidinium	*Haematococcus pluvialis*	*Arthrospyra platensis*	*contortum*	*Tetraselmis uecica* (α-linolenic acid)
Dinophysis	*Chlorella zofingiensis* (astaxanthin)	*Chlorella* spp.	*Scenedesmus* spp.	*Chlorella minutissima*
Prorocentrum	*Dunaliella salina* (β-carotene)	*Proteins*	*Carbohydrate to bioethanol*	*Monodosus* spp.
Phycobiliproteins		*Arthrospyra platensis*	*Spirogyra* spp.	*Nannochloropsis* spp.
Red algae (Phycoerythrin)		*Chlorella* spp.	*Chlorococum* spp.	*Neochloris oleoabundans*
			Bio-hydrogen	*Pavlova lutheri* (eicosapentaenoic acid)
			Chlamydomonas reinhardtii	*Crypthecodiuimu* spp.
				Isochrysis galbana
				Schizochytrium spp.
				Thalassiosira spp.
				Thraustochytrium spp. (docosahexaenoic acid)

Table 1. Biotechnological application of microalgae and their high-value metabolites. Adapted from (Benedetti et al., 2018)**.**

Several biotechnological applications of microalgae have been proposed at lab-scale, however only a few are commercially exploited. Examples of this are the production of carotenoids, namely astaxanthin by *H.pluvalis*, β-carotene by *D. salina* and in a small-scale cultivation *Chlorella* spp*.* o *Spirulina* spp*.* for the production of other high-value products, such as protein, vitamins or lipids for biofuel or food feed industry (Khan et al., 2018). Despite the potential of microalgae, many of the valuable compounds are biosynthesized at small quantities in the stationary phase; and in most of the cases, under stressing culture conditions such as nutrient starvation, high-intensity light or hight salt concentration, which cause a low productivity of the desired compounds.

The use of transgenic microalgae can be a step forward for their feasible utilization in many biotechnological applications, analogous to the progress achieved in agriculture by the widespread use of transgenic plants. Many of the crop's species currently cultivated are not natural because they have undergone selective breeding, interspecies crosses or mutagenesis and molecular genetic engineering (Molina-Márquez Ana et al., 2021).

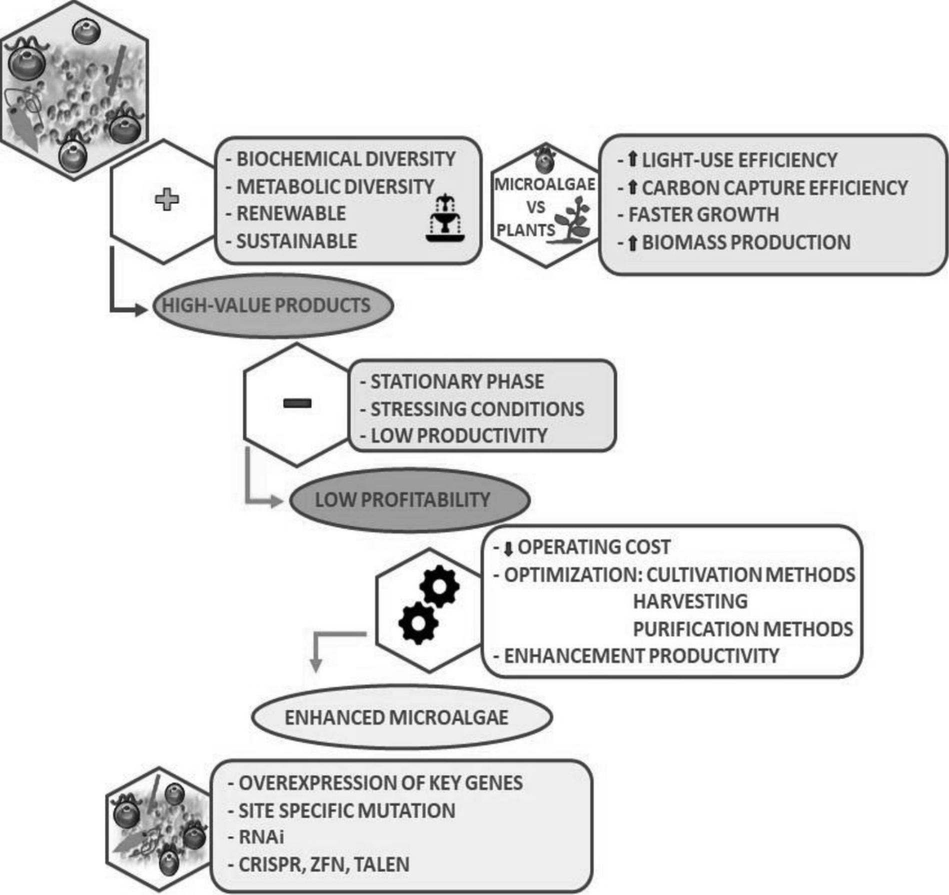


Figure 2. Benefit of microalgae as natural source of resources and opportunities for improvement. Detail of the main benefits of microalga (+), the difficuties of microalgae as feedstock (-) and the opportunities for improvement (⚙).

2. GENETIC ENGINEERING IN MICROALGAE

Genetic engineering is postulated as a potential tool to enhance microalgae, through the expression of foreign DNA or silencing of specific endogenous genes, to manipulate biosynthetic or regulatory sequences and increase the production of the desired metabolites (Chapter 1 and 3). However, microalgae have significantly less genomic resources, efficient genetic tools or well-stablished techniques than other

microorganisms, such as bacteria, yeasts or fungi and this limits the progress in microalgal bioengineering (Kumar et al., 2020).

Information about genome sequence or metabolic pathways, among other genetic and metabolic resources, that are key to identify target genes, is only available for a limited number of microalgal strains, mostly model. With the progress in the computational biology and bioinformatic technics to precise genome assembly and its annotation, in addition to the decrease of the price, many microalgal genome sequences will be study and the information will be release (Kumar et al., 2020). This information is essential to identify new target sequences in their genome involved in the desire compound synthesis pathway and generate new strategies to increase its accumulation in the enhancement microalgae strain. Progress in the molecular tools and genetic techniques besides the finding of appropriate gene markers, the design of efficient selective screening procedures and improving precision in gene targeting, will help to accomplish stable microalgal transformation and will increase the number of genetically modified microalga species, which to date remains low.

2.1. TRANSFORMATION TECHNOLOGIES

Microalgae can be transformed by different techniques, that allow the incorporation of external material, foreign DNA molecules or ribonucleoproteins into the cells, while preserving their viability (Figure 3). The choice of an adequate transformation method is essential to guarantee DNA delivery inside the cell and get its integration into the chromosome, allowing the successful genetic enhancement of microalgae. Usually, the technique should be optimized for each new species, this makes that many species remain untransformed, or the transformation efficiency rate is very low.

The model microalgae *Chlamydomonas reinhardtii* was the first genetically modified microalgae. In this case, a cell-wall deficient arginine auxotrophic mutant was transformed using polyethylene glycol (PEG) or poly-L-ornithine with *arg4* gene from yeast (J.-D. Rochaix & J. van Dillewjin, 1982). Agitation with glass beads in the presence of PEG is one of the most common transformation methods, because no specialized equipment is required, beside the fact that it is fast, cost-effective and

simple; however, wall-less cells or cells with very thin cell walls are needed and the efficiency transformation rate is low. *Chlamydomonas reinhardtii, Dunaliella salina* or *Chlorella ellipsoidea* are examples of *Chorophytes* that have been transformed by glass bead agitation technique (Molina-Márquez Ana et al., 2021). Agitation with silicon-carbide whiskers is also a popular method used to transform microalga, that can be used with walled-cells, like *Amphydinium* sp, *Symbiodinium microadriaticum* and *C. reinhardtii*, but the important drawback is the toxicity of the whiskers (Sreenikethanam et al., 2022). Electroporation is a simple and efficient transformation method where an electric shock is applied to the cells for a short period of time to generate temporarily pores on the cell membrane, through what the desired molecules penetrate. This method yields much higher transformation rates than glass bead agitation and can be successfully applied to both wall-less and walled strains, but the efficiency in walled cell is considerably lower and cell viability can be affected by the voltage applied. *Scendesmus obliquus, C. reinhardtii, Chlorella vulgaris* or *Nannochloropsis* sp. are example of microalgal species successfully transformed by this method (Molina-Márquez Ana et al., 2021). Microparticle bombardment or biolistic method consists in shooting small gold or tungsten particles coated with DNA into the target cells. Its efficiency for nuclear and chloroplast transformations, is high and can be used with microalgal cells, independently of their cell wall. This method is widely used in plants, diatoms and in walled chlorophytes with efficient results (Molina-Márquez Ana et al., 2021). Finally, transformation by co-infection with *Agrobacterium* allows transferring DNA molecules into the target cells by natural infection process. This technique is independent of cell wall and large fragments of DNA can be transferred and more stably integrated; however, it is needed to optimize culture conditions depending on the species. Among species successfully transformed by Agrobacterium co-infection technique are *Tetraselmis chuii, Chlorella sorokiniana, Chlamydomonas reinhardtii, Scenedesmus* or *Nannochloropsis gaditana* (Cha et al., 2011; Mary Sanitha et al., 2014; P. K. Sharma et al., 2021; Úbeda-Mínguez et al., 2015).

Despite the great effort and advancement in the transformation technologies of microalgae, several problems of such low efficiency, instability of the transformats or low expression of foreign genes are still pending issues that need to be improved.

2.2. EXPRESSION OF FOREING DNA SEQUENCES IN MICROALGAE

The optimization of the transformation method is essential to guarantee delivery of DNA into the cell nucleus, but other genetic strategies require attention to ensure the optimal expression of a foreign gene. Regulatory sequences, like promoter or untranslated region (UTR); adaptation of the codon usage of the transgene to that of host microalga or positional effects due to the integration site in the algal genome, are important to achieve the successful expression of foreign genes in microalga (Figure 3).

Microalgae, like *Chlamydomonas reinhardtii,* have been used as biofactory of recombinant proteins; however, the low expression level of the transgenes, and instability and variable nuclear transgene expression has limited their implementation on the industrial sector. Many studies have been done to identify cis-regulatory DNA sequences capable of driving high transgene expression, for example promoters or UTRs (Elizabeth A. Specht & Stephen P. Mayfield, 2013; Lodha et al., 2008; McQuillan et al., 2022; Schroda et al., 2000, 2002; Scranton et al., 2016). Schorda et al. 2000 demonstrated the highly efficiency of HSP70A promoter in *Chlamydomonas*. The significantly improve transgene expression levels was observed both fusing HSP70A sequence or only fusing two identified motifs within it, that are responsible of reducing the probability for the transgene being silenced, upstream to other promoter regulatory sequences, generating *HSP70A/RbcS2*, one of the strongest in microalgae (Schroda et al., 2002). Currently, the search of new potential promoter is leading to the generation of synthetic constructions that have demonstrated the capacity to drive the expression of exogenous genes at levels equal or above those achieved by the best native one, using a minimal sequence, of only about 70 bp, in contrast to the about 500 bp of the *HSP70A/RbcS2,* with a clear advantage in the size of the transformation cassette (Lodha et al., 2008; McQuillan et al., 2022; Scranton et al., 2016). An interesting proposal is the use of viral promoters that has been successfully utilized in plants but remain to be optimized in algal systems (Bolaños-Martínez et al., 2022; Díaz-Santos et al., 2013; Malla et al., 2021). Additionally, the insertion of introns like the first introns, of the ribulose-1,5-biphosphate carboxylase/oxygenase small subunit 2 (rbcS2), in the transgene or in the expression vector sequence, just as the

sequential insertions of introns has caused a significant and additive improvement of the transgene expression, strongly influence by the splicing signals location (Baier et al., 2018, 2020; Doron et al., 2016; Kumar et al., 2020).

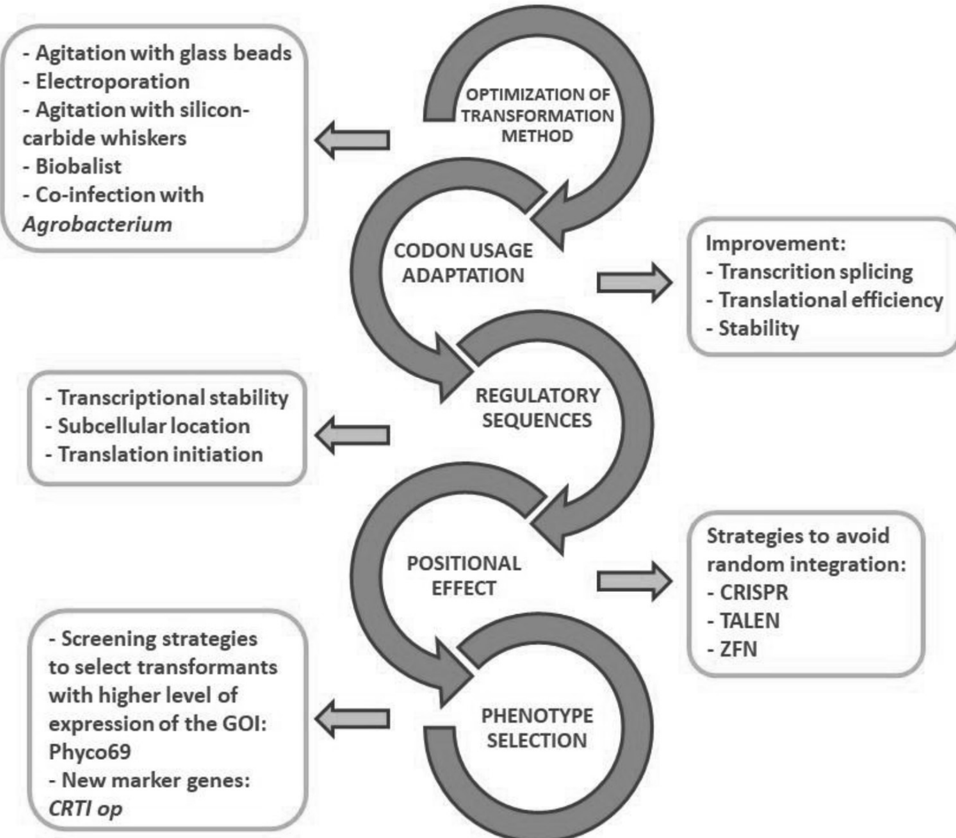


Figure 3. Genetic engineering in microalgae. Main strategies to tackle to genetic edition in microalgae**.**

Silencing mechanisms in microalgae make challenging the expression of transgenes and have hampered the use of microalgae as biofactories. Unusually tight chromatin or epigenetic events can effectively suppress the expression of foreign genes (Neupert et al., 2009, 2020). An approach successfully used to ensure the expression of the

transgenes, eluding the silencing mechanism of microalgae, is the fussing of the gene of interest (GOI) with an antibiotic resistance marker gene both, directly (Fuhrmann et al., 1999) or through a self-cleaving peptide, such as the 2A peptide derived from the foot-and-mouth disease virus (FMDV-2A) (Ryan et al., 1991), since the expression of the GOI is guaranteed as long as the cells are undergo to pressure of the selective antibiotic. In Chapter 1, we describe the validation of the expression vector Phyco69, in which a GOI can be linked to the selective *APHVIII* gene, that confers resistance to the antibiotic paromomycin, through the self-cleaving peptide FMDV-2A. Consequently, the transformants with higher tolerance to the selective antibiotic, also show the higher level of expression of the GOI. This plasmid makes easier the selection of the transformants with the highest expression level, by screening with increasing concentrations of the antibiotic avoiding time-consuming phenotypes search.

The integration mechanism of the expression vector on the microalgal genome is mainly produced via non-homologous recombination in apparently random loci (León-Bañares et al., 2004; Sizova et al., 2001), therefore strong influence in the level of expression of the GOI will be observed due to insertional point, the positional effect for the surrounding environment and the number of copies inserted on the genome. The new genome editing technologies can partly overcome these difficulties because they are based on engineered nucleases, that works as dubbed molecular scissors that allows the integration of transgene in a specific point on the genome. Engineered nucleases included zinc-finger nuclease (ZFN), TAL effector endonuclease (TALEN) and clustered regularly interspaced short palindromic repeat sequence (CRISPR) that recognize, and cleave specific sequences in the genome, producing double strand breaks (DSBs) (Jeon et al., 2017).

2.3. NUCLEAR SELECTABLE MARKER GENES AND SCREENING METHODOLOGIES

Even when the method of transformation is optimized, the codon usage has been adapted to that of the host microalga, the promotor works efficiently and the integration in the genome has been produced, the expression of foreign genes usually

results in a large variability among different clones. All this makes indispensable an efficient selectable marker to ensure stable transformation, and an appropriate screening methodology to avoid extensive time-consuming selection to identify the clone/s with higher levels of expression of the GOI, among a large number of clones (Chapter 1).

Selectable marker genes should allow the easy selection of clones with the GOI. The main selective approaches are based on three strategies, those that conferee antibiotic resistance, those that conferee herbicide resistance and those that rescue the wild type phenotype. The *NIT1*, *ARG7, NIC7, OEE1* genes follow the wild type of rescue-strategy and has been successfully used in *Chlamydomonas reinhardtii, Dunaliella salina, Volvox carteri, Chlorella ellipsoidea* and *Phaedactylum tricornutum* (Doron et al., 2016). For example, *NIT1, the* gene, encoding nitrate reductase, was used for the transformation of *Chlamydomonas* (Fernandez et al., 1989). This gene was introduced by nuclear transformation in a *NIT1* mutant of *Chlamydomonas reinhardtii strain*, enabling its growth with nitrate as a nitrogen source, and the rescue of wild type phenotype (Kindle et al., 1989). The selection of transformed cells based on the complementation of auxotrophic mutants can be considered ecofriendly and safe for human health, however the necessity to have a specific gene-defective mutant strains is a limitation. The antibiotic resistance strategy is the most widely used to select transformed cell and is routinely used in microalgae. Different antibiotic-resistance genes, such as *Ble, aphVIII, aadA, aph7, nptII, cato* or *CRY1-1* have been utilized in the nuclear transformation of microalgal species, such as *C. reinhartii, Nannochloropsis sp., Dunaliella salina, Volvox carteri, Haematococcus pluviales, Chlorella sp., Phaeodactylum tricorniatum* or *Amphidinium* sp. among others (Doron et al., 2016; Sizova et al., 2001). Despite the success of antibiotic resistance genes as selectable markers, their use has raised concerns regarding to human health and environment, due to the risk of horizontal gene transfer and the generation of antibiotic-resistant microorganisms. The selective strategy based on resistance to herbicides is a viable and attractive alternative for transformation of microalgae and is considered environmentally friendly (Doron et al., 2016). For example, *GAT, ALS*, *PDS1* gene have been demonstrated to be successful selective marker gene in

microalgae as *Chlamydomonas reinhardtii, Porphyridium* sp*. Haematococcus pluvialis, Chlorella sp. or Parietochloris incisa*, using resistance to glyphosate or sulfometuron methyl as selective agents. Moreover, in the present thesis we demonstrate the viable use of the gene *CRTI* and the selective herbicide norflurazon as strategy for the isolation of genetically modified microalgae (Chapter 2). *CRTI* gene isolated from the bacteria *Erwinia uredovora,* currently renamed as *Pantotea ananatis,* codes a bacterial phytoene desaturase enzyme that, catalyses the direct conversion of phytoene into all-trans lycopene and, unlike plant and algae phytoene desaturase (PDS), is not sensitive to norflurazon (Misawa et al., 1990). We have demonstrated its viability as selectable marker in the chlorophyte microalga *Chlamydomonas reihardtii,* using norflurazon as selective agent and propose their potential utilization as marker gene in other microalgal species, as more detailed explained in Chapter 2.

Selectable markers used in directed mutagenesis make easier to identify the desire mutants than in random mutagenesis. However, effective screening strategies should be designed to identify microalgal mutant colonies with higher level of expression of the GOI (Chapter 1) among variable expression of obtained mutant cells and consequently those with higher accumulation of the desire compounds. One of the reasons to the variability and even poor expression of the transgene is the efficient capacity of microalgae to silence foreign DNA by RNA interference (siRNA) mechanisms, as well as the insertional point, the rearrangement of transgenes, the number of copies inserted on the genome and the positional effect due to the surrounding environment where the transgene has been integrated mainly by non-homologous recombination (Molina-Márquez Ana et al., 2021).

3. STRATEGIES TO EDIT MICROALGAL GENOME

Different strategies can be used to edit microalgal genomes and obtain enhanced strains with the desired characteristics, such us robustness or higher productivity of valuable compounds, converting microalga into sustainable efficient biofactories (Figure 4).

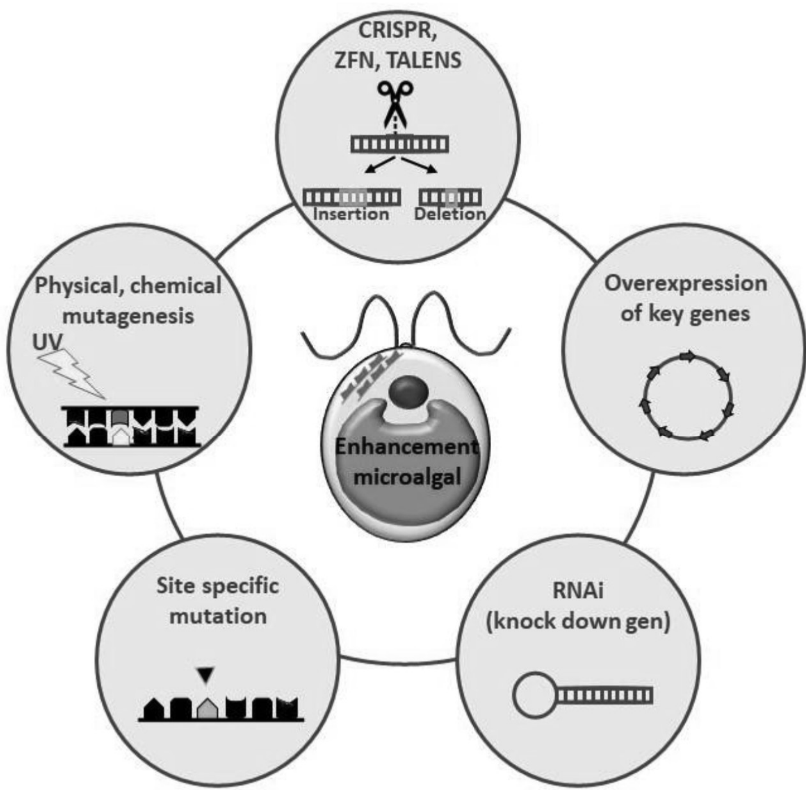


Figure 4. Examples of genetic engineering technics in microalgae

Other possibility is directed mutagenesis, based on reverse genetics, which consists of the modification of a specific targeted gene in the microalgal genome, and the observation of the phenotype provoked. The genetic modification involves knock-out, knock in, and in some occasions accompanied by the expression of new genes in the mutant cells (Bleisch et al., 2022). Unlike random mutagenesis, this technology requires deep knowledge of the target metabolic pathway and its regulation, as well as effective transformation protocols for each host microalgal strain. In microalgae, as happens in higher plants, foreign DNA is preferentially integrated by non-homologous recombination (Rosa León-Bañares et al., 2004). This impeded metabolic studies via reverse genomics, which are usual in other eukaryotes, until the discovery of RNA interference (iRNA). This technology originally described in the nematode *Caenorhabditis elegans*, allows the silencing, at least partially, of specific genes by

post-transcriptional silencing mechanisms (Cerutti et al., 2011). iRNA-mediated silencing of genes of the carotenogenic pathway has been reported in microalgae. Transformants of *C. reinhardtii* with low expression levels of phytoene desaturase (PDS) (Vila et al., 2008) and phytoene synthase (PSY) (Molnar et al., 2009) were obtained by RNA interference.

Other genetic tools available to edit algal genomes are zinc-finger nucleases (ZFN) or transcriptional activator like effector nucleases (TALENS) (Ferenczi, Pyott, Xipnitou, Molnar, et al., 2017; Hsu, 2014; W. Z. Jiang & Weeks, 2017; Nymark et al., 2016). These systems are based in the fusion protein of endonuclease domain of the bacterial restriction enzyme *FokI*, and site-specific DNA binding domains proteins. The DNA sequence that is recognized by Zinc finger nuclease is unique within genome of the host cell, cleaving it (Gupta & Musunuru, 2014). The DSBs generated can be repaired by two possible reparation mechanisms, the non-homologous end-joining or error prone (NHEJ) and homology-directed repair or error-free (HDR). Although HDR is preferred because generates knock out or knock in mutants with desire mutations, such as precise point mutations, sequence deletions or insertion of the donor DNA sequences; NHEJ widely occurs and are usual to produce out-of-frame mutation or premature stop codons, especially useful for studying knock out mutant cells (Molina-Márquez Ana et al., 2021). TALEN and ZFN, interestingly tends to promote HDR method of DNA reparation of the DBS (Greiner et al., 2017; Jeon et al., 2017). These methodologies have been successfully implemented to specific gene targeting in the genome of microalgae, like *Chlamydomonas reinhardtii* or *Nannochloropsis oceanica* (Greiner et al., 2017; Kurita et al., 2020, 2022). However, the recent development of clustered regularly interspaced short palindromic repeat sequence (CRISPR) technology and its application to microalgae, is postulated as an accurate and simple method to edit the algal genomes much more efficiently than the previous methods.

3.1. CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT SEQUENCE (CRISPR).

CRISPR technology has revolutionized genome editing field with its efficiency, accuracy and simplicity compared to previous nuclease-based editing methods, such as ZFNs and TALENs (Ferenczi, Pyott, Xipnitou, & Molnar, 2017; Hsu, 2014; W. Z. Jiang & Weeks, 2017; Nymark et al., 2016).

Originally, CRISPR system is part of the bacterial and archaeal adaptive immune system against invading viruses and conjugative plasmids (Francisco J.M. Mojica et al., 2005). CRISPR loci consists in array of spacer-interspacer sequences associated to a set of *CRISPR-Cas* nuclease genes. The interspacer comprises repetitive short palindromic sequences of 30 – 40 base pair (bp) length that are located between spacer sequences or crRNA. The crRNA comprises short viral or bacterial sequences of about 20 bp length to allow the microorganisms recognise the invader sequence or protospacer quickly by homology. Subsequent, invader DNA sequence is cleaved and acquires as new spacer sequences due to the action of CRISPR-Cas genes that encode for effector endonuclease, such as Cas9 and accessory enzymes that also process the pre-crRNA. An essential requirement for this system is the presence of the Protospacer-Adjunctive-Motif (PAM) upstream or downstream of the protospacer. PAM is a short sequence of 3-7 nucleotide essential to stablish the union between the CRISPR-Cas System and protospacer and it is specific for each effector nuclease (Hsu, 2014; W. Jiang et al., 2014).

The application of the CRISPR-Cas technology as genome editing tool is possible by simply designing a specific spacer and include them in a programmable, artificially designed guide RNA (gRNA), which leads the complex to its target in the genome (Hajiahmadi et al., 2019). The gRNA comprises two parts, crRNA or homologous spacer that is specifically designed to lead the complex at the target site in the genome of the microorganism and, the tracrRNA or trans-activating crRNA that specifically recognises the Cas nuclease, binding it and assembly the ribonucleoprotein complex. The major advantage of CRISPR system compared with ZFN and TALEN system resides in the fact that is the gRNA, and not the effector nuclease, that should be modified for

targeting specificity, which is much simpler and more economical (Jeon et al., 2017). Another benefit of this system is that the efficiency and the precision of CRISPR nuclease that seem to be higher than ZFN and TALEN nucleases, as well as the diversity of nucleases available to edit genomes, which opens up a variety of attractive possibilities (Jeon et al., 2017).

The diverse CRISPR systems have been classified by phylogenia and molecular mechanism of action into two classes and six types (Table 2) (Jeon et al., 2017). The type V (CRISPR-Cfp1 or Cas12) has been successfully used to edit *Chlamydomonas* genome (Ferenczi, Pyott, Xipnitou, & Molnar, 2017; Greiner et al., 2017). However, the most widely used is the type II nuclease Cas9, derived from Streptomyces pyogenes (spCas9), which PAM sequence is “NGG” and is located at the 3’ end of the protospacer (Hsu, 2014).

CLASS	PROTEIN	EFFECTOR COMPLEX	
I	I	Cas3	Multi-subunit
	III	Cas10	
	IV	Csf1	
II	II	Cas9	Single- subunit
	V	Cpf1 or Cas12a/b	
	VI	Cas13	

Table 2. Classification of the CRISPR-Cas system. Adapted from (Molina-Márquez Ana et al., 2021)

Two methods are used to delivery CRISPR-Cas editing systems, the plasmid-based or transgene method and the DNA-free genome editing method. The first one consists in co-expressed *Cas9* gen and gRNA in an expression vector in the microalgae genome (Greiner et al., 2017; W. Jiang et al., 2014) and the second one, consist in delivery Cas:gRNA ribonucleoprotein complex (DNA-free RNP complex method) to the microalgae cell.

CRISPR-Cas9 system has registered edition efficiencies as high as 60% (Slattery et al., 2018) and was the first CRISPR system used to edit successfully the first microalgae, *Chlamydomoanas reihardtii.* Microalgal cells were transformed by electroporation and transgene method delivered CRISPR-Cas9 system, obtaining target multiple genes in a single transformation, where three exogenous mutated genes *GFP, GUS* and *APHVIII* were restored and one endogenous gen *FK12B*, was mutated conferring resistant to rapamycine to the microalgae (W. Z. Jiang & Weeks, 2017). From then, other microalgae have been successfully edited by CRISPR-Cas system (Table 3), such as *Volvox carteri* (Ortega-Escalante, José a., Jasper & Miller, 2019), *Thalassiosira pseudonana* (Hopes et al., 2016), *Nannochloropsis* sp (Wang et al., 2016). *Coccomyxa* sp. (Yoshimitsu et al., 2018) or *Phaeodactylum tricorniatum* (Nymark et al., 2016).

Transgene method reported lower efficiencies due to the possible toxicity of the Cas9 protein in the *C. reinhardtii* cells (W. Jiang et al., 2014; W. Z. Jiang & Weeks, 2017; Shin et al., 2016). However, Greiner's experiments reported that knock out mutants of *PSY* integrated correctly the spCas9 (Cas 9 from *Streptococcus pyogenes*) and saCas9 (Cas9 from *Staphylococcus aureus)* nucleases gene in the genome of the mutant cells without toxicity evidence for *C. reihardtii* (Greiner et al., 2017) and the targeting frequencies registered were higher when was used SaCas9 as nuclease. In *volvox carteri*, transgene method was successfully used to obtain knock-in mutants of *GLSA* and *REGA* genes by biolistic and suggested that the constitutive expression of SpCas9 did not affect the viability of the mutant cells; similar results was observed in *P. tricornutum* (Nymark et al., 2016; Ortega-Escalante, J. A., Jasper & Miller, 2019; A. K. Sharma et al., 2018).

The DNA-free RNP-based method arises as an interesting alternative to avoid the possible toxicity effect of the accumulation of Cas9 nuclease in the cells and reduce

SPECIES	PROTEIN	TRANSFORMATION	METHOD	MUTATED GENES	REFERENCE
Chlamydomonas reinhardtii	SpCas9	Electroporation	Transgen	*aphVII, mGFP, tmGluc and FKB12*	Jiang et al., 2014
		Electroporation	Free-DNA	*CpFTSY and ZEP*	Baek et al., 2016
		Electroporation	Free-DNA	*MAA7, CpSRP43 and ChlM*	Shin et al., 2016
		Electroporation	Transgen	*ARGShBle, Ku70, fkb12, ALS*	Jiang & Weeks, 2017
	dCas9	Glass beads	Transgen	*rfp, CrPEPC1*	Kao & Ng, 2017
	SpCas9 and SaCas9	Electroporation	Free-DNA and Transgen	*PSY1, cHR2, COP1/2, COP5, PHOT, aphVIII and acry*	Greiner et al., 2017
	LbCpf1	Electroporation	Free-DNA	*FKB12, CpFTSY, CpSRP43, and PHT7*	Ferenczi et al., 2017
	SpCas9	Electroporation	Free-DNA	*CrFTSY*	Kim et al. , 2020
	SpCas9	Glass beads and Biolistic	Transgen-DNA	*apt9*	Guzmán-Zapata et al., 2019
Coccomyxa sp.	*SpCas9*	Electroporartion	Free-DNA	*KJFTSY*	Yoshimitsu et al., 2018
Volvox carteri	SpCas9	Bombardment	*Transgene*	*gslA, regA and invA APHVII; Bsr*	Ortega-Escalante et al., 2019
Nannochloropsis gaditana	SpCas9	Electroporation	*Transgene*	Nitrate reductase (g7988)	Wang et al., 2016
				ZnCys	Ajjawi et al., 2017
Phaeodactylum tricornutum	DiaCas9	Bombardment	Free-DNA	*PtUMPS, PtAPT and PtAureo1a*	Serif et al., 2018
		Conjugation	Transgene	*Urease gene*	Slattery et al., 2018
		Bombardment and Conjugation		*Myb*	Sharma et al., 2018
		Bombardment		*CpSRP54 gen*	Nymark et al., 2016
		Bombardment		*vtc2 and pho4*	Stukenberg et al., 2018
Thalassiosira pseudonana	SpCas9	Bombardment	Free-DNA	*Urease gene*	Hopes et al., 2016

Table 3. Microalgae genetically modified by CRISPR system. Abbreviations: SpCas9, Cas9 protein from *Streptoccocus pyogenes*; dCas9, "dead" version of Cas9 from *Streptoccocus pyogenes*; SaCas9, Cas9 protein from *Staphylococcus aureus*; LbCpf1, Cpf1 or Cas12 protein from *Lachnospiraceae* sp.; DiaCas9, a codon optimized version of Cas9 from *Streptoccocus pyogene*; APHVII, hygromacyn b resistance gene from *Streptomyces hygroscopicus*; *mGFP*, the mutant green fluorescent protein gene; *tmGluc*, the mutant luciferase gene from *Gaussia*; *FKB12*, peptidyl-prolyl cis-trans isomerase gene from *Chlamydomonas* sp; *CpFTSY*, β subunit of ATP synthase gene from *Chlamydomonas* sp.; *ZEP*, zeaxanthin epoxidase gene from *Chlamydomonas* sp.; *MAA7*, the beta subunit of tryptophan synthase gene from *Chlamydomonas* sp; *CpSRP43*, chloroplast *SRP4* gene from *Chlamydomonas* sp; and *ChlM*, Mg-protoporphyrin IX S-adenosyl methionine O-methyl transferase gene from *Chlamydomonas* sp; *ARG*, argininosuccinate lyase gene; *Shble*, bleomycin/zeocin resistance gene from *Streptoalloteichus hindustanus*; *Ku70*, ATP-dependent DNA helicase 2 subunit 1 gene from *Chlamydomonas* sp; *ALS*, acetolactate synthase gene from *Chlamydomonas* sp.; rfp, red fluorescent protein gene; *CrPEPC1*, phosphoenolpyruvate carboxylase isoform 1 gene from *Chlamydomonas* sp.; *PSY1*, phytoene synthase-1 gene from Chlamydomonas reinhardtii; Chr2 channel rhodopsin-2 gene from *Chlamydomonas* sp.; *COP1/2*, chlamyopsin-1/2 gene from *Chlamydomonas* sp. *COP5*, chlamyopsin-5 from *Chlamydomonas* sp.; *PHOT*, phototropin gene from *Chlamydomonas* sp.; *aphVIII,* paromomycin resistance gene from *Streptomyces rimosus*; acry, cryptochrom *acry* gene from *Chlamydomonas* sp.; *CpFTSY/CrFTSY*, Chloroplast SRP receptor gene from *Chlamydomonas* sp.; *CpSRP43*, Chloroplast SRP43 gene from *Chlamydomonas* sp.; *PHT7*, phosphate transporter gene from *Chlamydomonas reinhardtii* sp.; *apt9*, adenine phosphoribosyl transferase gene from *Chlamydomonas* sp.; *KjFTSY* chloroplast homolog of signal recognition particle receptor gene from *Coccomyxa* sp.; *gslA*, Asymmetric division protein GlsA gene from *Volvox* sp.; REGA, REGA protein gene from *Volvox* sp.; *INVA*, kinesin gene from Volvox sp.; ;Nitrate reductase (g7988), nitrate reductase gene from *Nannonchloropsis* sp.; *ZnCys*, transcriptional factor from *Nannochloropsis* sp.; *PtUMPS*, orotidine-5′-phosphate decarboxylase domain gene from *Phaeodactylum* sp.; *PtAPT*, APT gene from *Phaeodactylum* sp.; *PtAureo1a*, Aureochromes gene from *Phaeodactylum* sp.; Myb, transcriptional factor from *Phaeodactylum* sp.; *CpSRP54*, chloroplast signal recognition particle gene from *Phaeodactylum* sp.; *VTC2*; vacuolar protein gene from *Phaeodactylum* sp.; *PHO4*, phosphate transporter gene from *Phaeodactylum* sp. Adapted from (Molina-Márquez Ana et al., 2021)

the off-target effect, due to the transitory activity of the RNP complex (W. Jiang et al., 2014; Shin et al., 2016). With this strategy, restriction of the use of genetically modified organism (GMO) disappears, since any foreign DNA is inserted in the genome of the microorganisms and therefore, they can be implemented in the technological sector, for example, a double knock out of *ZEP* and *CPFTSY* genes in *C. reinhardtii* is able to induce the constitutively production of zeaxanthin, showing enhancement in the photosynthetic productivity and higher biomass accumulation under high lighting (Baek et al., 2016; Spicer & Molnar, 2018). However, European Union maintain the restrictions for the use of CRISPR technology in practical and commercial applications because gene-edited organisms are considered currently, as GMO organisms (Spicer & Molnar, 2018).

CRISPR technology has still some limitations, and improvements should be done to gain precision in genome engineering and, promote HDR mechanism of reparation of the double-strand breaks (DSB) in detriment of NEJH. HDR mechanism induces desired mutations and improves the precision, such as gene or base correction (Jeon et al., 2017). Advances in the transformation method and the mechanisms for delivery RNP-CRISPR complexes into a widely range of species of microalgae or minimize the off-target editing events in the algal genomes are also pending improvements for CRISPR methodology (Jeon et al., 2017; Molina-Márquez Ana et al., 2021).

The type of DSB generated using CRISPR-Cas systems depends on the Cas effector nuclease, for example Cas9 produce blunt double-strands cleavage, due to its two catalytic domains, RuvC and HNH, but CFP1 or Cas12 produce staggered cuts, due to the only one RuvC-like catalytic domain. Ideally, HDR is preferred to NHEJ as reparation mechanism for its precision. Great efforts have been done to enhance the reparation via HDR, for example, supplying a single or double donor template flanked by homologous sequences seems to promote the HDR as reparation mechanism in *C. reinhardtii* (Greiner et al., 2017; W. Z. Jiang & Weeks, 2017). However, when double stranded donor is supplied multiple insertions are common (Greiner et al., 2017; Zorin et al., 2005). The election of Cas protein seems to be important, for example CRISPR-Cpf1 RNPs and single-stranded oligodeoxynucleotides as template favour reparation via HDR (Ferenczi, Pyott, Xipnitou, & Molnar, 2017).

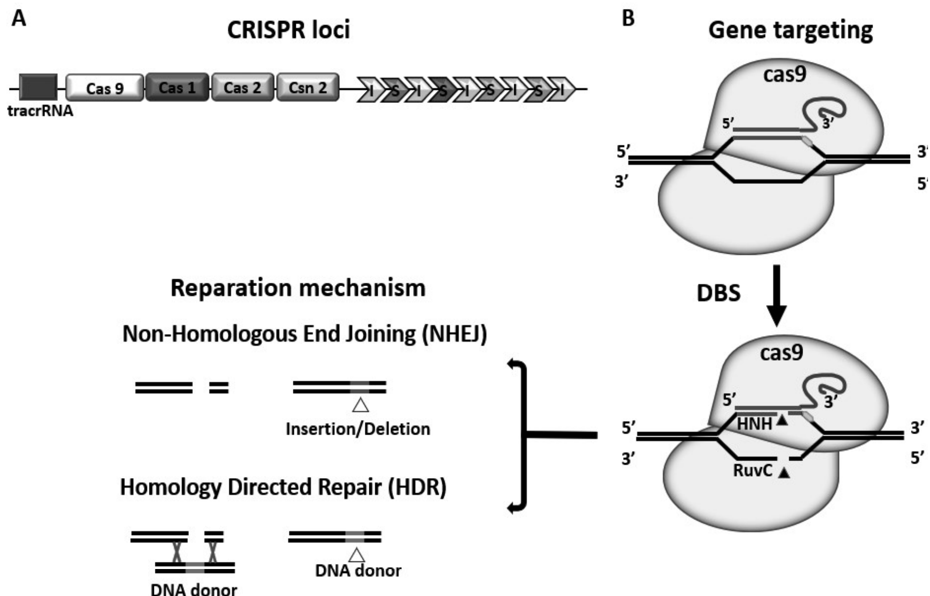


Figure 5. Type II CRISPR-Cas9 System. A: Detail of the CRISPR array. **B:** Detail of the editing mechanism of the CRISPR-Cas9 system. The Cas9 nuclease protein (grey) is bound to the guide RNA through tracrRNA (green). Subsequently, the complex recognizes the target site in the genome by homology with the specifically designed crRNA (red) and the PAM (yellow) and he Cas9 protein cleavages the targeted DNA generating a double-strand break (DSB) (Δ). The DBS is repaired by either the NHEJ (Non-Homologous End joining), or by HDR (Homology Directed Repair), being able to incorporate a homologous donor DNA at the breaking point.

Several approaches have been proposed to boost CRISPR-Cas technology and improve accuracy of genetic edition. For example, diaCas9 that has one of its two catalytic domains inactive and cleaves only one of the two strands, needing two gRNA to obtain staggered cuts (Kao & Ng, 2017; Nymark et al., 2016) or a “dead” version of Cas9 (dCas) with both catalytic domains mutated and without possibility to cleave DNA, which, however, can bind precisely to the target site and regulate the expression of the gene, activating or repressing the transcription processes (Gilbert et al., 2015).

Genetic edition of microalgae by CRISPR-Cas can contribute to increase the knowledge of their metabolism and to develop enhanced microalgal strains with biotechnological

potential and industrial applications, as alternative sustainable feedstock of valuable compounds. For example, CRISPR-Cas system has been reported as to increase the lipid productivity in the industrial microalgae *Coccomyxa* spp. (Yoshimitsu et al., 2018) and *Nannochloropsis* sp. (Ajjawi et al., 2017; Verruto et al., 2018). In chapter 3 CRISPR-Cas9 technology was used to knock out the phytoene desaturase gene (*PDS*), producing the accumulation of the carotenoid phytoene.

4. CAROTENOIDS

Carotenoids are a wide group of lipophilic isoprenoid molecules, produced by photosynthetic organisms and some non-photosynthetic fungi or bacteria. They have attractive properties, such as pro-vitamin A action, antioxidant activity or dye. These characteristics make carotenoids essential in the diet of organisms that cannot synthesize them. They are also very interesting for the industrial sector with multiple applications, such as natural colorants for food and beverages, or in animal feeding (Figure 5). For example, within aquaculture farming, ketocarotenoid are responsible for the red colour of the flesh of salmonoids (de Carvalho & Caramujo, 2017). Formally, carotenoids are divided into two groups, carotenes and xanthophylls, carotenes are pure hydrocarbons and xanthophylls are oxygenated derivatives carotenes.

Carotenoids are pigments industrially exploded for a long time, for instance β-carotene, lycopene, astaxanthin, zeaxanthin and lutein are well stablished and the demand of fucoxanthin, canthaxanthin and the colourless carotenoids phytoene and phytofluene is increasing in a growing market (Ali et al., 2022; Havas et al., 2018; Meléndez-Martínez et al., 2015; Olmedilla-Alonso et al., 2021; Ye & Huang, 2020). The global carotenoids market reached $1.44 billion in 2019 and had been projected to reach nearly $ 2 billion in 2026 with a compound annual growth rate of 4.5% (Igreja et al., 2021; Ren et al., 2021).

Commercial production of carotenoids is mainly based in the chemical synthesis, which reach between 80-90% of the global carotenoids market, in contrast about 10-

20% of the carotenoids that are obtained from natural resources, like plants or microalgae. Chemical synthesis is less expensive than biosynthesis from natural resources (Ren et al., 2021). For example, the production cost of astaxanthin through chemical mechanisms is about \$1000 Kg^{-1}, in contrast to the cost using the microalgae *Haematococcus pluviales* reaches \$2500-\$7000 Kg^{-1} (Igreja et al., 2021). However, natural carotenoids are highly appreciated, especially because the growing concern about chemical additives used in food, and the blue economy, sustainable and circular bioeconomy policies that encourage the pursuit of sustainable food and production systems (Araújo et al., 2021). Moreover, natural carotenoids exhibit better properties, for example chemical synthesis of β-carotene generates only all-trans isomers, whereas the β-carotene obtained by biosynthesis from microalgae is a mixture of all-trans and 9-cis isomers (Gong & Bassi, 2016).

Plant and microalgae are the main natural sources of these pigments. In plant, the cellular content of carotenoids is low, requiring not only large areas of cultivation to obtain high production, but also specific agriculture practices and seasonality influences that increase the cost. On the contrary, microalgae culture can be realized in controlled bioreactors independently from the weather; most species grow rapidly, and provide higher yields (Igreja et al., 2021). The global market of algae is growing, and it is expected to reach \$970 million at 2025. The global carotenoid market, on its hand, is expected to reach \$2 billion at 2026, focused mainly on food and beverage (23.5%), pharmaceuticals (9.2%), cosmetics (6.5%) and dietary supplements (23.5%) (Ren et al., 2021). Microalgae are Generally Recognized As Safe (GRAS) dependent on the policies, being European Union (E.U.) regulation more severe and restrictive than others, for example United State of America (U.S.A.) (European Algae Biomass Association, 2021). The most cultivated species in Europe are *Chlorella* spp., *Scenedesmus almeriensis, Nannochloropsis* spp., *Haematococcus pluvialis*, together with *Spirulina* currently renamed *Arthrospira* (Ferreira de Oliveira & Bragotto, 2022). Other species industrially cultivated are *Tetraselmis* sp., *Dunaliella salina, Porphyridium* sp., *Tisochrysis lutea*, formerly *Isochrysis galbana, Phaeodactylum tricornutum*, *Thalassiosira* sp., *Scenedesmus* sp., *Chaetoceros muelleri* and *Acutodesmus obliquus* (Araújo et al., 2021). *Dunaliella* is highly appreciated in health

food, and feed industry due to its capacity for the production of β-carotene, with provitamin A properties. Astaxanthin from *Haematococcus pluvialis* is demanded in aquaculture for colouring the flesh of fish, like salmon, as well as in nutraceutical industry because its antioxidant activity that seem to promote the strengthening of the immune system and protect against neurodegenerative conditions and cancer. Lutein is widely marketed as, pharmaceutical, nutraceutical, food colouring agent, and specially for its role to prevent age-related macular degeneration (Ali et al., 2022).

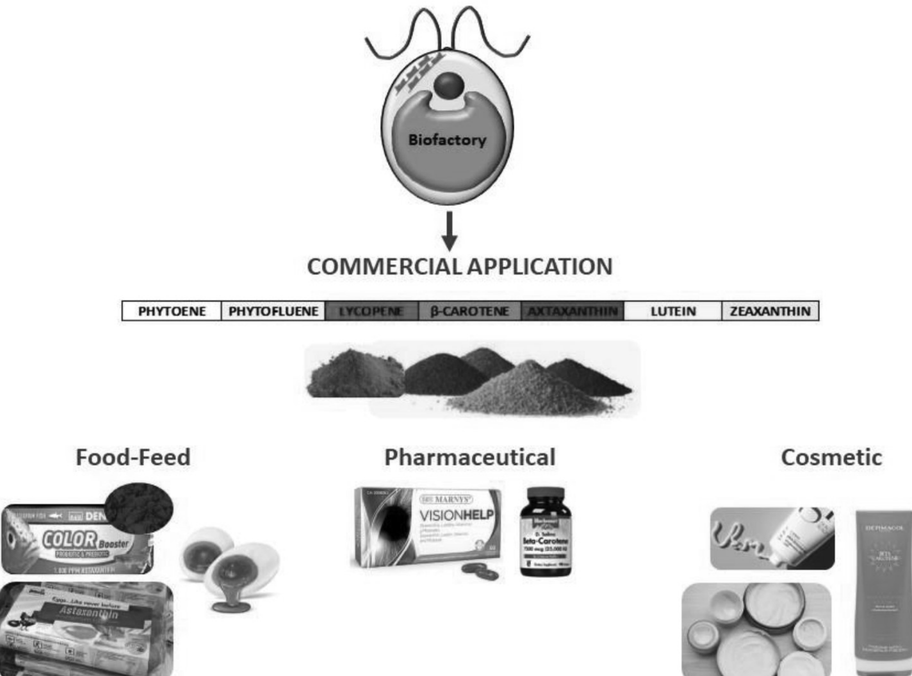


Figure 6. Main carotenoid produced by microalgae and their commercial applications.

4.1. PHYSIOLOGICAL FUNCTIONS OF CAROTENOIDS

Carotenoids are part of the light harvesting complexes (LHC) that are associated, together with chlorophylls, to harvesting light and reaction center proteins, constituting functional pigment-binding proteins embedded in the thylakoid membrane (Baroli et al., 2000). Carotenoids are essential in photosynthesis, acting as accessory light harvesting pigments that can absorb light, in a wide range of

wavelengths, ranging from violet to green-yellow light of the visible spectrum, and complementing the absorption range of chlorophylls. Subsequently, the energy absorbed is transferred to chlorophylls of the photoreaction centres initiating the photochemical reaction of photosynthesis (Polívka & Frank, 2010; Stamatakis et al., 2014). Strong evidence indicates the existence of a tightly regulated coordination in the biosynthesis of proteins, carotenoids and chlorophylls integrating the photosystems (Bouvier et al., 2005).

Another essential function of these molecules is the photoprotection of the photosynthetic apparatus against the photooxidative damage. Excessive light and other stressing conditions can provoke photoinhibition or inhibition of the photosynthesis. Under these circumstances, accumulation of triplet chlorophyll can cause the forming of singlet oxygen, the main responsible of the oxidative damage of the pigments, lipids, and proteins of the photosynthetic thylakoid membrane. Photoprotection consists in absorbing the damaging excess of light and avoiding the accumulation of reactive oxygen species (ROS), such as single oxygen ($^1O_2$), harmless chlorophyll reactive intermediates ($^1Chl^*$, $^3Chl^*$), hydrogen peroxide (H_2O_2), and free radicals ($OH^{\cdot}$, $O2^{-\cdot}$, $NO^{\cdot}$, $RS^{\cdot}$, $RO2^{\cdot}$) by quenching excess energy (Zareba et al., 2016).

Non-photochemical quenching (NPQ) is the most common photoprotective response and includes several components, such as state transition (qT), and thermal dissipation (qE), induced by a low pH in the thylakoid lumen. qE involves the direct quenching between carotenoid molecules and chlorophyll reactive intermediates, scavenging ROS like $^1O_2$ or free radicals. Therefore, the carotenoid molecules are excited to the triplet state thanks to the system of conjugated double bonds dissipating the excess of energy produced during photosynthesis as thermal energy and returning to the ground state (Baroli et al., 2000; Demmig-Adams & Iii, 1996; Zareba et al., 2016). Xanthophyll cycle is key in photoprotection by NPQ, under normal conditions violaxanthin is accumulated by the enzymatic action of zeaxanthin epoxidase (ZEP), however under light stress, pH of the lumen is acidified promoting the binding of violaxanthin de-epoxidase (VDE) to the thylakoid membrane and de-epoxidation of violaxanthin to zeaxanthin (Zea) with the intermediate antheraxanthin (Baroli et al., 2000; Grossman et al., 2004). The antioxidant activity and ROS-quenching

capacity of carotenoids is largely dependent on the number of the conjugated double bonds, the nature of the end groups, cyclic or acyclic, and the presence of substituents in the molecule. For example, Zea with eleven conjugated double bonds and two hydroxyl groups is an extremely efficient quencher, followed by β-carotene with eleven and lutein with ten (Zareba et al., 2016); astaxanthin with two carbonyl and two hydroxyl groups has been observed to be about ten times more effective than zeaxathin, lutein and β-carotene, all with fewer substituents (Mordi et al., 2020).

4.2. BIOTECHNOLOGICAL APLICATIONS OF CAROTENOIDS

Carotenoids are bioactive compounds that play an important role in human and animal health, mainly due to their important antioxidant, nutritional and colouring properties. Oxidative stress is a recurrent cause of several human diseases, including diverse type of cancer, cardiovascular and coronary diseases, neurodegenerative conditions and age-related macular degeneration and cataracts (Sharifi-Rad et al., 2020). It has been demonstrated that carotenoids have a health-promoting effect in human health and there is evidence of reduction the risks of cancer, anti-inflammatory and obesity disorders, reparation of damaged retina and reduction of free radicals (Ren et al., 2021).

β-carotene was the first high-value compound commercialized from microalgae, mainly produced by *D. salina* that can accumulate 13% of dry weight (DW), but other microalgae as *chlorella zofingiensis, spirulina platensis* and *Caulerpa taxifolia* can yield of 0.1-2% DW. An interesting *C. zofingiensis bkt1* strain enhance by genetic engineering can accumulate lutein in 13.81 mg g^{-1} DW, β-carotene 7.18 mg g^{-1} DW and zeaxanthin 7.00 mg g^{-1} DW under high light and salt stress (Ren et al., 2021). β-carotene has been revealed to produce a significant reduction of the incidence of skin tumours induced by UV light and/or chemical carcinogens, prevention of lung cancer and may also protect against stomach cancer and inhibition of ovarian, cervix and breast cancer, as well as it has been attributed significant reduction of the severity of psoriasis and photoprotective effect on the skin (Mordi et al., 2020). Nevertheless,

some studies have shown discrepancies, that indicate that β-carotene supplementation can exacerbate UV-carcinogenic expression (Black et al., 2020).

Lutein is a natural antioxidant with beneficial properties tested in human health, such as scavenging free radical for skin health, preventing age-related macular degeneration and Alzhemier's disease (Ren et al., 2021). Microalgae are potential source of lutein, and some strains has been investigated with this propose, among them *Scenedesmus, Chlorella, coccomyxa, Parachlorella* and *Tetraselmis* that can reach values of 7.39 mg/g DW in *C. sorokiniana*¸ or 11,87 mg/g DW in *Parachlorella* sp. (Ren et al., 2021).

Zeaxanthin together with lutein are macular pigments that function as blue-light filters protecting the retinal membrane from blue light and improving visual capacity. It has been demonstrated that theses pigments have antioxidant and anti-inflammatory activities and promote the prevention of neurological disease and macular degeneration. Microalgae are appreciated to produce zeaxanthin due to the high productivity and bioavailability, reaching values of 7.00 mg g^{-1} DW under high light in *C. zofingiensis bkt1* and 5.9 mg g^{-1} DW in a mutant of *D. salinzaea1* (Ren et al., 2021).

Phytoene and phytofluene with three and five conjugated-double bonds, respectively, are colourless carotenoids, with an essential role as precursors of the rest of the carotenoids. Unlike others, they absorb in UV region, phytoene absorbs maximally in the UVB region (280-320 nm) and phytofluene in the UVA region (320-400 nm) (Meléndez-Martínez et al., 2019). They have been largely ignored possibly due to the lack of colour, which made their detection challenging, but currently, they have been demonstrated to be present in several foods, such as watermelon, apricots or tomato, and to have health-promoting properties in humans (Olmedilla-Alonso et al., 2021). The minor extension of conjugated double bonds system confers these carotenoids less rigidity and less aggregatation tendency, which results in higher bioaccessibility than other carotenoids. The capacity of these pigments to absorb strong UV radiation provide them attributes adequate for their use as cosmetics and health-promoters. Besides, they are attractive nutriocosmetics that can be used as whitening agents to treat dark spots nutricosmetic (Meléndez-Martínez et al., 2019).

Carotenoid	Colour	D.b (in rings)	Absortion maxima (nm)		
Canthaxanthin	Red	13 (4)		472	
Astaxanthin	Red	14 (4)		468	
Lycopene	Pink	11 (0)	446	474	504
β-carotene	Orange	11 (2)		454	480
Zeaxanthin	Orange	11 (2)			
Lutein	Yellow	10 (1)	424	448	476
Phytofluene	Colourless	5 (0)	331	348	367
Phytoene	Colourless	3 (0)		286	

Table 4. Main commercially carotenoids and detail of the absorption maxima in acetone or petroleum ether, number of double bonds (D.b.) and colour. Adapted from (Meléndez-Martínez et al., 2019).

4.3. CAROTENOIS BYOSINTHESIS PATHWAY

Isoprenoid biosynthetic pathway, the precursors for carotenoid

Carotenoids are terpenoid pigments with C40 backbones formed by condensation of 5C building block of isopentenyl pyrophosphate (IPP), precursor of all isoprenoids. IPP can be synthetized via the well-known mevalonate pathway (MVA), through the condensation of three acetyl-CoA molecules, or via non-mevalonate, 1-deoxy-D-xyloluse 5-phospate (DOXP) or 2-C-methyl-D-erythriol 4-phospate (MEP) pathway through the condensation of a pyruvate and two D-glyceraldehyde-3-phospate molecules (Ramos-Valdivia et al., 1997) (Figure 7).

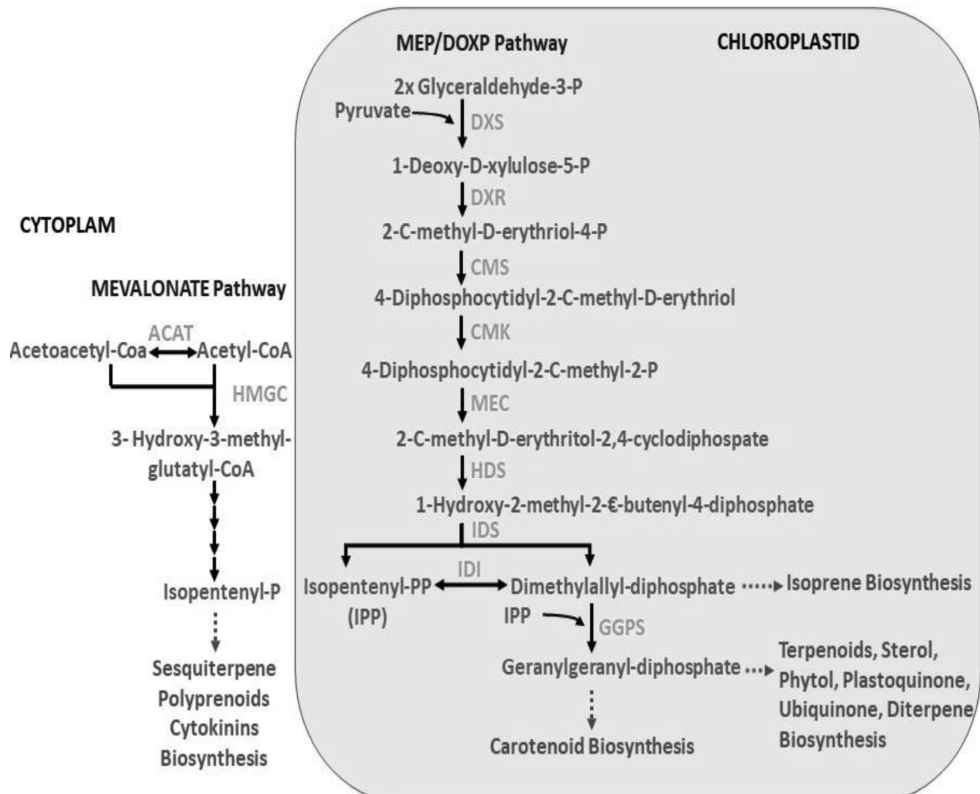


Figure 7. Isoprenoid biosynthetic pathway. Abbreviations: DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MCS, 4-diphosphocytidyl-2,4- cyclodiphosphate synthase; HDS, 1-Hydroxy-2-mehtyl-2-(E)-butenyl-4-diphosphate synthase; IDS, Isopentenyl diphosphate: dimethiylallyl diphosphate synthase; IDI, dimethiylallyl diphosphate isomerase; GGPPS, Geranylgeranyl pyrophosphate synthase; ACAT, Acetyl-CoA acethyltransferase; HMGC, 3-hydroxy-3-methylglutaryl-CoA synthase.

In higher plants the MVA pathway, located in the cytoplasm, mediates the synthesis of IPP as precursor for sesquiterpene, polyprenoids and cytokinins biosynthesis, while the MEP pathway is in the chloroplasts and catalyses the conversion of IPP into terpenoids, sterol, phytol, plastoquinone, ubiquinone, diterpene or carotenoids. In most chlorophyte microalgae the cytosolic MVA pathway appears to be absent (Capa-Robles et al., 2009) and IPP is synthetized exclusively through the MEP pathway,

according to the pattern of incorporation of [1-^{13}C] glucose in isoprenoids (Disch et al., 1998; Grossman et al., 2004). Although, *3-hydroxy-3-methylglutaryl-CoA synthase* gene (*(HMG)-CoA*), that is involved in the first step of the MVA pathway, has been detected in the genome C.*reinhardtii* no other genes of the pathway, such as *HMG-CoA reductase* or *mevalonate kinase*, have been registered (Grossman et al., 2004). The MVA pathway seem to have been lost during evolution process in Chlorophyta like *C. reihardtii* or *Chlorella* sp. and in Eustigmatophycease like *Nannochloropsis* sp. and maintained in diatoms, Glaucophytes and some Rhodophyta (P.W. Huang et al., 2021; Lauersen, 2019; Lohr et al., 2012). However, according to the transcriptome analysis realized by Huang and coworkers, the microalga *Haematococcus pluviales* JNU35 seems to synthesize IPP by MVA pathway because the expression of six genes implicated in the pathway have been detected, but IPP synthesis via MEP pathway is predominant (L. Huang et al., 2019).

Carotenoids are synthesized in the chloroplast by the action of a series of nuclear-encoded membrane-associated proteins, which have amine-terminal extensions that target them to the chloroplasts. Carotenoids are mainly located in the thylakoid membranes, being part of the core complexes, or of the light-harvesting antenna systems of photosystem I and II. In addition, carotenoids can be found in other structures, such as the eyespot of some microalgae or the plastoglobules of many plant plastids (Grossman et al., 2004)

The biosynthesis of carotenoids has been exhaustively reviewed by Cunningham and Gantt (Cunningham & Gantt, 1998) and more recently by Botella-Pavía (Botella-Pavía et al., 2004). Some reviews are specifically focussed on the carotenoid biosynthesis in algae (Lohr, 2009; Varela et al., 2015).

Condensation

IPP and its isomer dimethylallyl-pyrophosphate (DMAPP) are the backbone for the synthesis of carotenoids (Figure 8). Three molecules of IPP (C5) are sequentially added to a DMAPP molecule by the enzyme geranylgeranyl pyrophosphate (GGPP) synthase resulting in the formation of C20-compound GGPP.

Phytoene synthase catalyses the first committed step in carotenogenesis, the condensation of two molecules of GGPP to yield the first uncoloured carotenoid, phytoene (40C) and is an important regulatory step, as evidenced in the studies where *PSY* gene from *Dunaliella salina* or from *Chlorella zofingiensis* was overexpressed in the microalgae *C. reinhardtii* leading to increase the carotenoid contents (Cordero et al., 2011; Couso et al., 2011) or its silencing by miRNA in *Phaeodactylum tricornutum* leaded to reduce the accumulation of carotenoids (Kaur & Spillane, 2015).

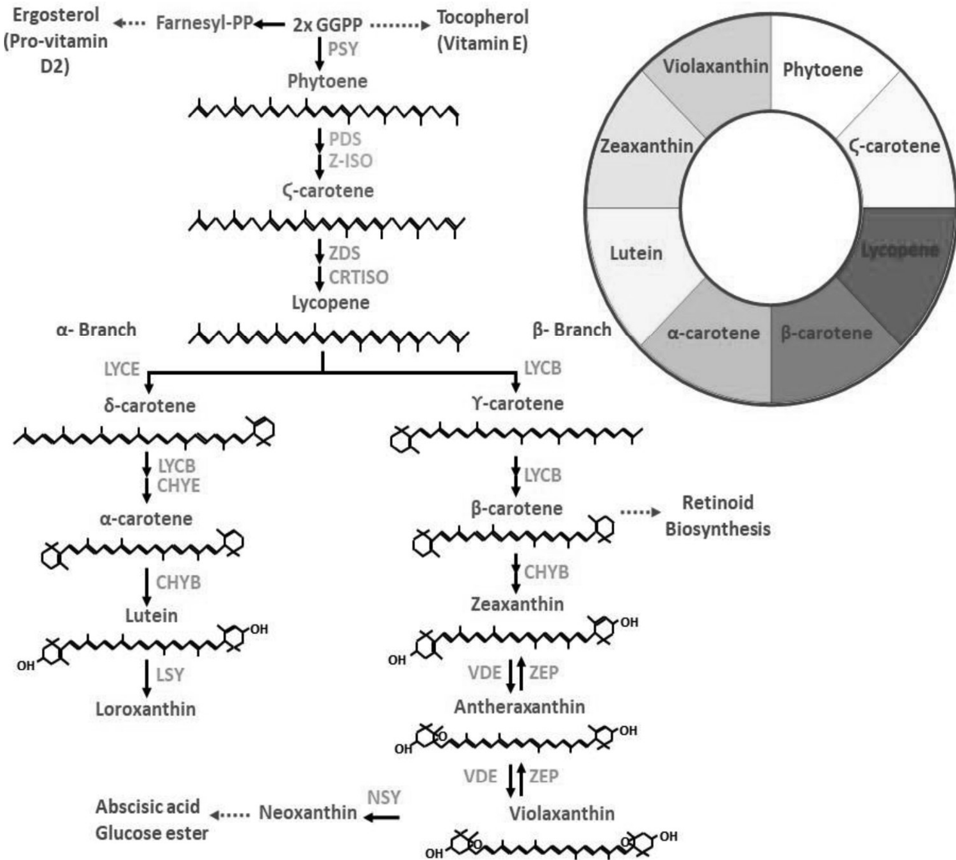


Figure 8. Carotenoid biosinthetic pathway. Abbreviations: GGPP, Geranylgeranyl pyrophosphate; PSY, Phytoene synthase; PDS, phytoene desaturase; Z-ISO, ς-carotene isomerase; ZDS, ς-carotene desaturase; CRTISO; Carotenoid isomerase; LCYE, Lycopene ε-cyclase; LCYB, Lycopene β-cyclase; CHYE, Carotene ε-hydroxylase; CHYB, Carotene β-

hydroxylase; ZEP, Zeaxanthin epoxidase; VDE, Violoxanthin de-epoxidase; NSY, Neoxanthin synthase; LSY, Loroxanthin synthase.

Desaturation and isomerization

Phytoene with three conjugated double bonds and without absorbance in the visible spectrum undergoes two subsequently desaturations and two isomerations. The first one introduces two conjugated double bonds within phytoene thanks to the enzyme phytoene desaturase (PDS), generating ξ-carotene with the intermediate phytophluene. The enzyme Z-ISO catalyses the isomerization of 9,15,9`-tri-cis-ξ-carotene to 9,9`-di-cis-ξ-carotene, substrate to the second desaturase enzyme. The second desaturation step introduces two additional conjugated double bonds into ξ-carotene by ξ-carotene desaturase (ZDS), resulting in prolycopene with the intermediate neurosporene. Prolycopene is isomerised to all-trans lycopene by the enzyme carotenoid isomerase (CRTISO) (Cunningham & Gantt, 1998; Varela et al., 2015).

Cyclization

From Lycopene, the carotenogenesis pathway is split into two branches, α and β branches (Figure 8). In the α branch, the enzyme epsilon lycopene cyclase (LCYE) introduce a ε-ring into lycopene to yield delta carotene (ƺ-carotene) and subsequently, the enzyme beta lycopene cyclase (LCYB) catalyzes the addition of a β-ring into ƺ-carotene to produce α-carotene (β,ε-carotene). In the β branch, LCYB introduce subsequently two β-ring, generating β-carotene (β, β-carotene) via the intermediate gamma carotene (γ-carotene) (Lohr et al., 2005; Varela et al., 2015).

Introduction of oxygen functions

α-carotene and β-carotene can be further hydroxylated or/and epoxidated. In both branches, the C3 atom of the ε- ring or β-ring are hydroxylated, producing lutein (β,ε-carotene-3,3′-diol) or zeaxanthin (β,β-carotene-3,3′-diol), respectively. The hydroxylation of α-carotene and β-carotene is catalysed by carotenoid hydroxylase (CHY) that comprises two subfamilies of enzymes with different structures, BCH that are non-hemo di-iron protein (CHYB) and CYP97 that are heme-containing cytochrome

P450 hydroxylases (CHYE or LUT1). CHYE oxidate ε-rings and both subfamilies can hydroxylate β-rings (Varela et al., 2015).

In green algae has been found genes of *BCH* (*BCH1, BCH2*) and *cyp97a/b/c* genes (*CYP97A3, CYP97A5* and *CY97C1,* CYP97C3) (Velmurugan Aswini et al., 2022). In *C. reinhardtii* lutein is hydroxylated in the methyl group of C9, producing Loroxanthin by a putative loroxanthin synthase (LSY) not known (Grossman et al., 2004; Varela et al., 2015)

Zeaxanthin is epoxidized in two sequential steps by the action of enzyme zeaxanthin epoxidase (ZEP) that introduce two epoxy-groups at C5, C6 double bonds of the two β-ring, via the intermediate antheraxanthin. In plants, the enzyme violaxanthin de-epoxidase (VDE) catalyses two sequential de-epoxidation from violaxanthin to zeaxanthin with the intermediate antheraxanthin in a cycle named as xanthophyll cycle involved in NPQ. In *C. reinhardtii* a mutant of *vde* gene (npq1) was isolated during the screening of mutants with reduced NPQ (Anwaruzzaman et al., 2004; Niyogi, 1997). The VDE encoded by *Chlamydomonas* resulted to be an atypical VDE, not homologous to plant VDE, but related to a photosynthetic bacteria lycopene. This VDE is in the stromal side of the membrane, instead in the thylakoid lumen, as the rest of plant-type (Li et al., 2016).

Neoxanthin is produced from violaxanthin by an enzyme called neoxanthin synthase (NSY). The first putative NSY sequence genes reported were in tomato and potato, together with a paralogous lycopene cyclase (LCY) gene that apart the convert lycopene to β-carotene, can synthesized violaxanthin to neoxanthin. In *C. reinhardtii* no orthologous sequence to NSY has been detected, therefore neoxanthin synthesis could be produced by a bifunctional LCY or by a NSY with different DNA sequence (Giossi et al., 2020).

4.4. PHYTOENE DESATURASE ENZYMES

Phytoene desaturase (PDS) is a membrane-associated protein involved in the conversion of phytoene (7,8,11,12,7’,8’,11’,12’-octahydro-Ψ,Ψ-carotene) into the

first coloured carotenoid ξ-carotene (7,8,7',8'-tetrahydro- Ψ,Ψ -carotene) by two desaturations, introducing two double bonds into C11, C12 and C11', C12' of the polyene chain through the intermediate phytophluene (7,8,11,12,7',8'-hexahydro-Ψ,Ψ-carotene) (Cunningham & Gantt, 1998; Varela et al., 2015).

PDS is an oxidoreductase enzyme that contains a dinucleotide binding domain of flavin adenine dinucleotide (FAD), which is required as a redox cofactor. FAD is reduced during desaturation and transfers de electron to plastoquinone (PQ), producing plastoquinol (PQH_2), which transfers them to the plastid terminal oxidase (PTOX) or to the Cytb6f, connecting the carotenoid biosynthesis pathway with the electronic transport chain (Foudree et al., 2012; Varela et al., 2015; Velmurugan Aswini et al., 2022).

PDS play a crucial role in carotenoid biosynthesis and has been targeted by blenching herbicides, such as norflurazon, which can inhibit the activity of PDS enzyme through competition with its cofactors. This blocks the coloured carotenoid biosynthesis, producing the phytoene accumulation and the degradation of the chlorophyll, therefore collapsing photosystem II and causing cellular whitening (León et al., 2005; Velmurugan Aswini et al., 2022). Some authors have demonstrated that the modification of key amino acids in the FAD binding domain of PDS protein generates norflurazon-resistant mutants (Varela et al., 2015; Velmurugan Aswini et al., 2022).

In bacteria and fungi, phytoene desaturase (CRTI), unlike plant and algae PDS can convert phytoene into lycopene without the participation of any other enzyme. The low sequence homology between *CRTI* and *PDS* suggests that they arose independently in the evolution. CRTI does not exhibit sensitivity to bleaching herbicides, like norflurazon as will be demonstrated in Chapter 2.

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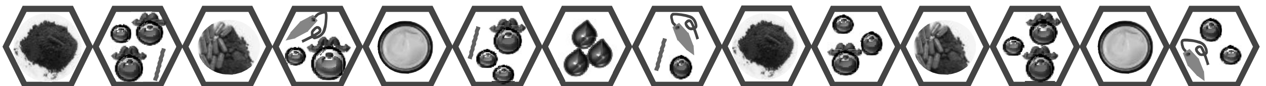
OBJECTIVES



OBJECTIVES

1. Generate a new multicistronic expression plasmid for the simultaneous expression of two genes under the control of one promotor in microalgae (**Chapter 1**).

2. Design a simple and efficient screening method to obtain transformant microalgae with high level of expression of the gen of interest (**Chapter 1**).

3. Synthetize a new selectable marker gene, *CRTIop,* a codon-adapted version of the bacterial *CRTI* gene, fused to a chloroplastic transit peptide encoding DNA fragment (**Chapter 2**).

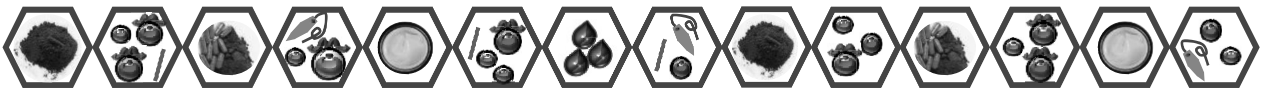
4. Demonstrate the ability of the new tp-CRTIop marker gene for the selection of microalgal transformants, using the herbicide norflurazon as selective agent **(chapter2)**

5. Validate a clustered regularly interspaced short palindromic repeats associated to endonuclease Cas9 (CRISPR-Cas9) system for the edition of specific genes in *Chlamydomonas reinhardtii* (**Chapter 3**).

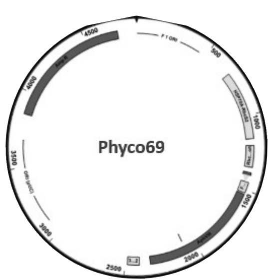
6. Generate, by Cas9-Ribonucleoprotein complexes, a phytoene desaturase knock-out microalga mutant strain able to accumulate phytoene as the only carotenoid and study the effects of lacking carotenoids on the metabolism and the physiology of the microalga (**Chapter 3**).

RESULTS AND DISCUSSION

CHAPTER 1



Validation of a New Multicistronic Plasmid for the Efficient and Stable Expression of Transgenes in Microalgae



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Article

Validation of a New Multicistronic Plasmid for the Efficient and Stable Expression of Transgenes in Microalgae

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Abstract: Low stability of transgenes and high variability of their expression levels among the obtained transformants are still pending challenges in the nuclear genetic transformation of microalgae. We have generated a new multicistronic microalgal expression plasmid, called Phyco69, to make easier the large phenotypic screening usually necessary for the selection of high-expression stable clones. This plasmid contains a polylinker region (PLK) where any gene of interest (*GOI*) can be inserted and get linked, through a short viral self-cleaving peptide to the amino terminus of the aminoglycoside 3′-phosphotransferase (APHVIII) from *Streptomyces rimosus*, which confers resistance to the antibiotic paromomycin. The plasmid has been validated by expressing a second antibiotic resistance marker, the *ShBLE* gene, which confers resistance to phleomycin. It has been shown, by RT-PCR and by phenotypic studies, that the fusion of the *GOI* to the selective marker gene *APHVIII* provides a simple method to screen and select the transformants with the highest level of expression of both the *APHVIII* gene and the *GOI* among the obtained transformants. Immunodetection studies have shown that the multicistronic transcript generated from Phyco69 is correctly processed, producing independent gene products from a common promoter.

Keywords: 2A; microalgae transformation; paromomycin; multicistronic transcript

1. Introduction

Microalgae are a heterogeneous group of photosynthetic microorganisms highly attractive for the production of many different interesting metabolites. However, the high cost of microalgal culture systems and the low productivity of many of these compounds have hampered their commercial applications. Currently, only high added value compounds, such as pharmaceuticals, cosmetics, or nutraceuticals, are produced from microalgae at industrial scale [1]. In other fields, competition with synthetic products or with those obtained from other sources makes the production of microalgal-based compounds non-viable from the economical point of view [2–6]. As a consequence, more attention is being paid to the genetic engineering of microalgae as a potential tool to achieve economically feasible production of bulk materials and to enhance the productivity of the high added value ones [7–9]. Although in recent years, an increasing number of microalgal species have been successfully transformed and important goals have been accomplished, the genetic transformation of microalgae

has still important pending challenges. Both, the low stability of the transgenes and the high variability of their expression levels among the obtained transformants, make necessary large screenings to select high-expression stable clones [10–12]. In fact, low nuclear expression of transgenes in microalgae has hampered the efficient and stable engineering of metabolic pathways and has limited the use of these microorganisms for the commercial production of recombinant proteins. Until now, the expression of heterologous genes in chloroplasts is the only approach that has led to protein accumulation at economically viable levels [8,13,14]. However, nuclear expression provides the recombinant proteins with desirable characteristics, which cannot be acquired if they are expressed in the chloroplasts, such as post-translational modifications or targeting of the protein products to different organelles [15,16].

The nuclear transformation of microalgae takes place mainly by random integration. This means that transgenes are randomly inserted in the genome, and consequently their expression level will largely depend on a series of factors such as the insertion site, the environment genome surrounding of the inserted transgene, the number of copies inserted or differences with the codon usage of the host [10]. Random insertion followed by an exhaustive phenotypic screening of a large number of clones continues being the most usual approach for the selection of transformants with good levels of expression and stability [17,18]. However, different imaginative solutions have been proposed to overcome these time-consuming screenings and to get high expression of the transgenes. Improvement of promoters and regulatory regions (HSP70A, RbcS2 intron) [19–23], inclusion of additional introns [24] or adaptation of the codon usage of the transgene to that of the host microalga [25–27] have shown to improve expression and stability of marker genes such as GFP and luciferase in *Chlamydomonas reinhardtii*. We have previously demonstrated that fusing the gene of interest without promoter to an antibiotic resistance gene can lead to high expression [28]. Neupert and coworkers described high accumulation of proteins encoded by nuclear transgenes (about 0.2% of total soluble proteins) by UV induced mutagenesis of the obtained transformants followed by screening to isolate defective mutants in transgene suppression mechanisms [29]. A similar strategy was proposed by Kong and coworkers, who studied expression of proteins in a mutant of *Chlamydomonas* affected in a methyltransferase involved in transcriptional gene silencing [30].

A further improvement has been achieved by including the self-cleaving 2A peptide derived from the foot-and-mouth disease virus (FMDV-2A) between the *GOI* and the selectable gene. This peptide is processed in the eukaryotic ribosome due to a ribosomal skip between Gly23 and Pro24 amino acids [31]. The use of this small peptide for the co-expression of several genes under the same promoter and then, thanks to its co-translational cleavage ability obtain the independent gene products, has been applied to a wide range of eukaryotic systems, such as mammalian cells [32], fungi [33] and plants [34]. This strategy has also been shown to work in microalgae [30,35]. Mayfield and coworkers described the fusion of the bleomycin resistance gene (*ShBLE*) with the xylanase gene of *Trichoderma reesei* [35] and several fluorescent marker genes [16] through the small self-cleaving 2A peptide and demonstrated the obtaining of high yields of the independent proteins from a common transcript [36]. However, the high mutagenic effect of bleomycin and related antibiotics [37,38] and the need of extending the number of selective agents available have led us to propose an alternative gene fusion strategy to improve transgenes expression in microalgae based on the *APHVIII* resistance gene. This gene encodes the enzyme aminoglycoside 3′-phosphotransferase from *Streptomyces rimosus* and confers resistance to the antibiotic paromomycin [39]. We have generated different multicistronic expression plasmids in which the polylinker region is fused through the short self-cleaving 2A peptide to both termini of the selective *APHVIII* gene and evaluated their efficiency for the genetic transformation of the microalgae *Chlamydomonas reinhardtii*. Moreover, we have demonstrated that screening of the transformants with increasing amounts of the antibiotic paromomycin provides a simple method for the selection of clones with the highest level of expression of *APHVIII* gene, and consequently of the gene of interest, among the obtained transformants.

2. Results and Discussion

2.1. Construction and Transformation Efficiency of Several Multicistronic APHVIII-based Fusion Plasmids

Three different plasmids containing the paromomycin resistance gene, *APHVIII*, fused to a polylinker region through the foot-and-mouth disease virus self-cleaving 2A peptide sequence (FMDV-2A) have been constructed. In the three plasmids the *APHVIII* gene is placed under the control of the heat shock protein 70A/ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2 tandem chimeric promoter (HSP70A-RBCS2) and terminated by the 3'RBCS2 untranslated region [19]. In addition, in the three plasmids the first intron of the Rubisco small subunit, which has shown to increase transformation efficiencies, has been included after the promoters and immediately before the corresponding marker gene or polylinker region, as detailed in Figure 1A. Plasmid pSI103 described by Sizova was used as a control plasmid [40]. The first construct encodes for the APHVIII protein linked through its carboxyl end (C-end) to the FMDV-2A peptide, which is followed by the polylinker region. The resulting plasmid was called PhycoC67. In the second construct, the C-end of the *APHVIII* sequence was linked to the self-cleaving 2A peptide through a flexible peptide sequence (GASGQGASGADIGASGQGASDA), this plasmid was denoted as PhycoC67FL. In the third construct, the same elements used for plasmid PhycoC67 were placed in inverse order, so that the PLK region followed by the self-cleaving 2A peptide is fused to the amino-end (N-end) of the *APHVIII* gene, this last plasmid was named Phyco69. The detailed schematic diagrams of this construct are shown in Figure 1A, and the sequences of the proteins obtained from their expression are shown in Figure 1B.

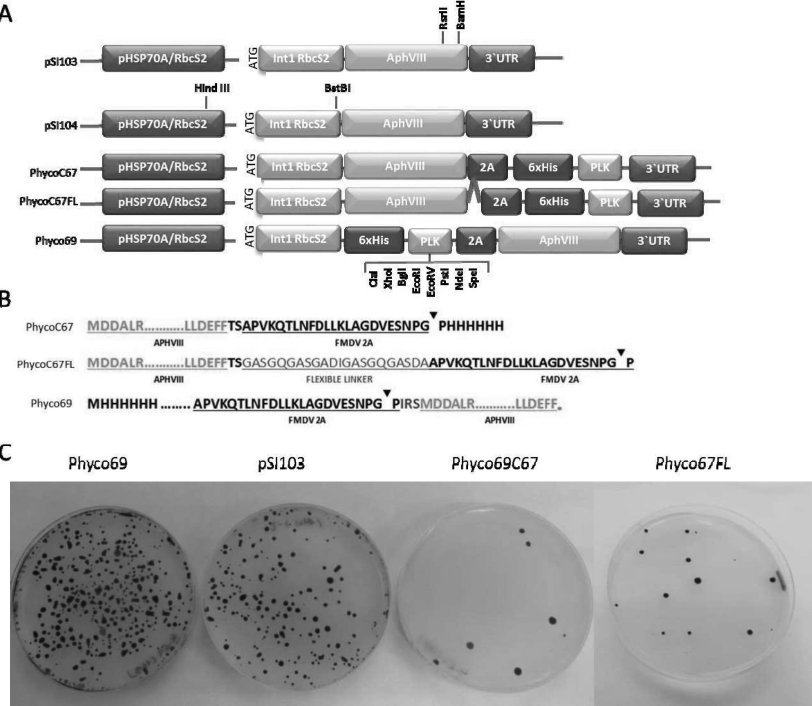

Figure 1. Schematic diagram of the main elements of the three new plasmids generated in this work in comparison with the control pSI103 plasmid (**A**) and of the translation products resulting from them (**B**). The blue line "¯" represents the flexible peptide sequence GASGQGASGADIGASGQGASDA. "v" denotes the hydrolysis point in the self-cleaving peptide FMDV-2A. Representative examples of the nuclear transformation of *Chlamydomonas reinhardtii* with the three newly generated plasmids (Phyco69, PhycoC67, and PhycoC67FL) and the control pSI103 plasmid are also shown (**C**).

The 2A peptide allows that the chimeric transcripts generated from these constructions are processed upon translation in the ribosome, generating independent gene products, the APHVIII, and the one corresponding to the gene inserted in the polylinker region. The ribosomal skip takes place between the two final C-terminal amino acids of the 2A peptide, glycine, and proline, and consequently an extra peptidic sequence will be added to the C-end of the APHVIII when it is situated upstream the 2A peptide, as it happens in plasmids PhycoC67 or PhycoC67FL. However, in the case of plasmid Phyco69, the *APHVIII* gene is located downstream the 2A peptide, and consequently, a single proline amino acid will be added to its N-terminal.

The model microalga *Chlamydomonas reinhardtii* was transformed by the glass beads agitation method with the three plasmids, PhycoC67, PhycoC67FL, and Phyco69, as described in Section 3.3 Materials and Methods. The efficiency of these transformations was compared with that obtained with the control plasmid pSI103 [40]. As shown in Figure 1C, the transformation efficiency of PhycoC67 was extremely low, showing that even small peptides such as the FMDV-2A self-cleaving peptide interfere with the functionality of the aminoglycoside 3′-phosphotransferase if these are bound to its carboxyl terminal end. Protein linkers imitate the linkers naturally occurring in multi-domain proteins and usually avoid misfolding or impaired bioactivity of the fusion proteins, however, the flexible peptide used in PhycoC67FL did not offer any appreciable advantage. By contrast, Phyco69 plasmid, in which the FMDV-2A is linked to the amino terminal of the APHVIII protein, provided the highest transformation efficiencies with around 420 transformants μg^{-1} of DNA. This is three-fold the mean transformation efficiency obtained with the control pSI103 plasmid designed by Sizova [39], which is about 130 transformants μg^{-1} of DNA (Table 1).

Table 1. Transformation efficiency of *Chlamydomonas reinhardtii* with increasing quantities of the indicated plasmids. All values are the average of between three and five repetitions ± SD. The mean number of transformants μg^{-1} of DNA is also indicated.

Plasmid	200 ng	500 ng	1 μg	2 μg	Transformants μg^{-1} DNA
Phyco69	98 ± 22	215 ± 38	380 ± 58	820 ± 70	427
pSI103	28 ± 12	60 ± 21	140 ± 32	250 ± 45	131
PhycoC67	0	5 ± 2	7 ± 4	18 ± 6	6.5
PhycoC67FL	0		8 ± 4	12 ± 7	3.5

We concluded that the fusion of peptides through the amino-end does not affect the functionality of the aminoglycoside 3′-phosphotransferase, however, the fusion to the carboxyl-end of even small peptides such as the 2A peptide, interferes with its function. Although there is certain variability in the number of transformants obtained with these plasmids, we systematically obtained much higher transformation efficiencies with the plasmid Phyco69, around three times higher than with the control plasmid pSI103. After a careful analysis, we found that the increase in the transformation efficiency with the new Phyco69 plasmid is due to the fact that in Phyco69 the *APHVIII* gene is in the same reading frame than the initiation codon, which is immediately before the RbcS2 intron. Whereas in pSI103 the initiation codon does not match with the open reading frame of the APHVIII protein. Detailed sequences of the products obtained after the elimination of the RbcS2 intron in pSI103 and Phyco69 are shown in Figure A1. In pSI103 a mix of transcripts with different reading frames must be produced and the selective pressure of the antibiotic makes possible that those transformants in which the correct APHVIII protein is synthesized survive. The detailed map of the Phyco69 plasmid, the one chosen for further studies, can be found on PhycoGenetics website (http://phycogenetics.com/products/).

This plasmid allows higher transformation efficiencies than other plasmids with the same promoter. In addition, selection based on the antibiotic paromomycin is fast and clean, without spontaneous resistant clones or mutagenic effects [10]. The placement of the polyclonal region upstream the aminoglycoside 3′-phosphotransferase solves the problems generated when additional peptide sequences are fused to the carboxyl-end of this protein. In this case, the desired genes have to

be deprived of their stop codon before being cloned in the Phyco69 plasmid to allow the transcription of the whole transcription unit. However, this apparent disadvantage ensures that all the transformants surviving in the presence of paromomycin are also expressing the *GOI*, which is placed upstream the *APHVIII*. If the *GOI* was placed downstream the resistance gene, some of the transformants could contain truncated transcripts in which the *GOI* is not transcribed. Since nuclear DNA insertion in microalgae is usually accompanied of deletions or truncations both in the genomic and insert DNA [41], ensuring the expression of the target gene by placing it upstream the selectable marker can be very useful.

Generation of fusion recombinant proteins or even the addition of short protein sequences to the ends of a protein can interfere with its structure and function. The possible influence of the remnants from the short 2A peptide in each protein of interest has to be investigated for each case. In the case of the APHVIII protein, we have demonstrated that the addition of even a few amino acidic units to its carboxylic-end affects its functionality. The few cases found in the literature, which describe the fusion of APHVIII to other proteins, involve its link through the amino end [18,40]. The structure of the APHVIII protein has been determined by X-ray crystallography [42] and is available in the protein data bank (PDB 4H05). APHVIII is a dimeric protein, each monomer consists of two lobes, the N-terminal domain with a β-strand motif of five anti-parallel sheets and two α-helices, and the catalytic C-domain with six α-helices. The three-dimensional structure of the APHVIII protein confirmed the conclusions previously established on the basis of sequence comparisons and point mutations, that pointed the final amino acid Phe267, as one of the conserved residues essential for the direct interaction with the aminoglycoside substrate [40]. This can explain why plasmid PhycoC67, in which the polylinker region is downstream the *APHVIII* sequence fused to its carboxylic-end, shows such a low transformation efficiency.

2.2. Phyco69 Enables the Selection of Transformants with High Expression Levels of the Gene of Interest

Our hypothesis is that by subjecting the transformants obtained with the plasmid Phyco69 to screening with increasing concentrations of the antibiotic paromomycin, it will be possible to select those clones which accumulate high concentrations of the aminoglycoside 3'-phosphotransferase protein and consequently of our protein of interest. Furthermore, both the protein of interest and the protein conferring resistance to the antibiotic are processed from the same multicistronic transcript, and if the transformants are maintained under selective conditions, the synthesis of the protein of interest is guaranteed to enhance in this way the stability of the transgenes. To validate this hypothesis, we cloned the antibiotic-resistance *ShBLE* gene, which confers resistance to antibiotics of the bleomycin family in the polylinker region of Phyco69 plasmid. The *ShBLE* gene with the first intron of RbcS2, which has shown to increase stability and transformation efficiency of the *ShBLE* gene [19,43], was PCR-amplified and inserted in the polylinker of Phyco69. The resulting plasmid, Phyco69BLE was used for the genetic transformation of *Chlamydomonas*. The layout of the experiment is depicted in Figure 2. Initial transformants selected in the presence of 30 $\mu g \cdot mL^{-1}$ of paromomycin appeared after four or five days and were screened with increasing concentrations of paromomycin (300, 400, 500 $\mu g \cdot mL^{-1}$) and phleomycin (0.5, 1 $\mu g\ mL^{-1}$). One hundred transformants were cultured in 2 mL of liquid TAP medium with paromomycin 15 $\mu g\ mL^{-1}$ during 48 h. The cellular density of all the cultures was then adjusted to the same value, and a drop of each transformant culture was symmetrically spotted in an ordered array on TAP agar plates supplemented with 300, 400 or 500 $\mu g \cdot mL^{-1}$ of paromomycin or with 0.5 or 1 $\mu g\ mL^{-1}$ of phleomycin, where they were grown for several days. All experiments were carried out in triplicate.

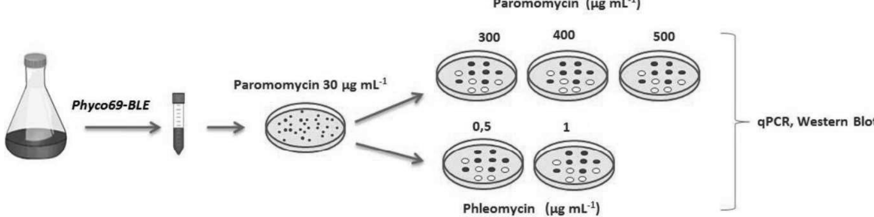

Figure 2. Schematic diagram of the selective strategy used to choose *Chlamydomonas* transformants with high levels of expression of genes *APHVIII* and *ShBLE*.

The average survival rate after transference to 500 µg mL^{-1} of paromomycin was 55%, very similar to the average survival rate in the presence of 1 µg mL^{-1} phleomycin, which was about 57% (Figure A2). We observed that practically all the transformed clones able to grow in the presence of high concentrations of paromomycin (500 µg mL^{-1}) are also able to grow vigorously with phleomycin (1 µg mL^{-1}). This indicates that these clones have high levels of expression of both *APHVIII* and *ShBLE* genes because it has been previously reported that high levels of the bleomycin binding protein, encoded by the *ShBLE* gene, are necessary to show a phleomycin resistance phenotype [35,43].

To confirm this, we studied the expression levels of *APHVIII* and *ShBLE* in a series of transformed clones obtained with the Phyco69BLE plasmid (Figure 3A). The correct simultaneous insertion of both, *APHVIII* and *ShBLE* genes, in the genome of the selected clones, was checked by PCR with specific primers that anneal at the end of the *ShBLE* gene and at the beginning of the *APHVIII* gene. All the clones checked had incorporated both genes correctly in their genomes (Figure 3C).

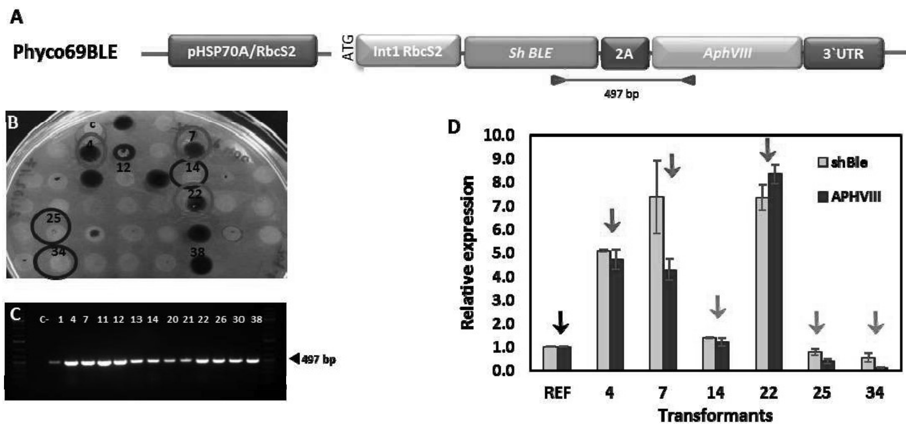

Figure 3. Relative expression of genes *ShBLE* and *APHVIII* in a series of *Chlamydomonas* transformants resistant to paromomycin. *Chlamydomonas* was transformed with the plasmid Phyco69BLE (**A**), and a selection of clones resistant to paromomycin and phleomycin (**B**) were analyzed by PCR using genomic DNA as target to test correct insertion of the *BLE-2A-APHVIII* cassette (**C**), and by real-time PCR (**D**) to test the relative expression level of the genes *ShBLE* (■) and *APHVIII* (■). Red arrows indicate the clones which show an important increase (>5 times) in the expression levels of *APHVIII* and *ShBLE* in relation with the reference transformed clone (↓), and correspond to the clones circled with a red line in panel B. Blue arrows denote the clones with a small difference in the expression of these genes in relation with the reference transformed clone and correspond to the clones circled with a blue line in panel (**B**). The expression of all genes was normalized to the ubiquitin ligase (*UBQL*) house-keeping gene and presented as fold-change relative to the transcript level of the reference transformed clone, which was chosen among those transformants which show low expression levels. Values are the average of three replicates, and bars indicate standard deviation.

On the one hand, we studied three PAR^R, BLE^R-transformants that could grow in 30 μg mL^{-1} paromomycin but were not able to survive neither in paromomycin 500 μg mL^{-1} nor in phleomycin 1 μg mL^{-1} (#14, #25, #34). On the other hand, we selected three transformants able to grow vigorously both in paromomycin 500 μg mL^{-1} and in phleomycin 1 μg mL^{-1} (#4, #7, #22). The transcripts levels corresponding to both genes were determined by real-time PCR using specific primers for each gene and the ubiquitin ligase gene (*UBQL*) as housekeeping gene (Figure 3D).

As shown in Figure 3D, the *APHVIII* and *ShBLE* transcript levels in the transformants which tolerate high concentrations of paromomycin (500 μg mL^{-1}) are between five and eight times the transcript levels found in the transformants isolated in 30 μg mL^{-1} paromomycin but unable to survive at higher concentrations of paromomycin (Figure 3B). This confirms that the selective pressure with paromomycin provides a fast method for the selection of the transformants with the highest levels of expression of the desired gene.

The self-cleaving peptide FMDV-2A has been successfully used in several eukaryotic systems [34]. Mayfield and coworkers have demonstrated in the microalga *Chlamydomonas reinhardtii*, that the FMDV-2A approach can be successfully used to fuse the desired gene to the *ShBLE* gene, which provides resistance to antibiotics of the bleomycin family [36]. The mechanism of action of BLE, which relies on antibiotic sequestration rather than enzymatic inactivation, ensures that the transformants surviving in these antibiotics have high levels of expression [43]. However, it is desirable to extend the palette of selectable markers available to be used with the FMDV-2A system because the selection of some microalgae must be done with other antibiotics to which they are more susceptible. In addition, bleomycin is highly mutagenic [37,44], and this is an undesirable effect, especially when an unequivocal relation between the obtained phenotype and the expressed gene must be established.

2.3. Phyco69 Enables the Simultaneous Expression of Two Genes, Which are Efficiently Processed Generating Independent Proteins

Although the utility of the 2A peptide to generate independent mature proteins from a common RNA transcript has been well established, the cleavage efficiency of the 2A sequences can be influenced by the C-terminal of the protein upstream the 2A. In the present work, immunodetection studies using anti-APHVIII antibodies on the transformants obtained with Phyco69BLE plasmid showed a 29.2 KDa-band, which corresponds to the size of the APHVIII protein, while no band is detected at 42.9 KDa, the size expected for the fusion Ble-2A-APHVIII protein product (Figure 4, Figure A3).

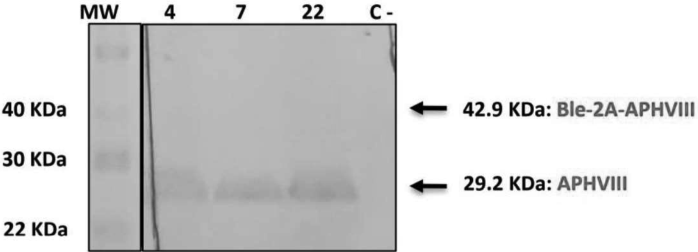

Figure 4. Immunoblot analysis showing the APHVIII protein product from *C. reinhardtii* cells transformed with the plasmid Phyco69BLE. Total soluble cell protein extracts (60 μg) of three *Chlamydomonas* transformants (#4, #7, #22) and one control untransformed *Chlamydomonas* culture (C-) were fractionated with SDS-PAGE, transferred to a PVDF membrane and probed with rabbit anti-APHVIII polyclonal antibodies and alkaline phosphatase-conjugated goat anti-rabbit IgG, as indicated in materials and methods. MW lane: Protein molecular weight markers.

This demonstrates that the multicistronic transcript is correctly processed generating independent proteins and no detectable fusion product. The 2A system has previously shown to improve expression

of the squalene synthase from *Botryococcus braunii* [30] and several selectable marker proteins in *Chlamydomonas* [35,45]. In all these cases, the efficient generation of two independent protein products through the 2A peptide has been reported. Furthermore, this auto-cleaving peptide has allowed the successful expression of four independent proteins from a single vector in *Chlamydomonas* [18] and up to six different genes in human cell lines [46]. Other viral sequences similar to the foot-and-mouth disease 2A have been reported to undergo similar translational self-cleaving with different efficiencies [47]. Moreover, some authors have even reported the efficient expression of two genes from a single bicistronic mRNA by simply joining the two genes of interest by a short stretch of unstructured junction sequences, without the need of viral sequences [48]. Although further insights are necessary to clarify the precise mechanism of the 2A induced ribosome skip, multicistronic plasmids have important advantages over co-transformation approaches in which the selective marker and the gene of interest are in independent expression cassettes and usually result in low percentages of genome insertion of the desired gene.

3. Materials and Methods

3.1. Strains and Culture Conditions

Chlamydomonas reinhardtii 704 strain (Cw15, Arg7^{+}, mt^{+}) was kindly donated by Dr. Roland Loppes (University of Liège, Sart Tilman, Belgium) [49], and cultured photomixotrophically in liquid or agar solidified Tris-acetate phosphate (TAP) medium under continuous white light irradiation (50 µE m^{-2} s^{-1} photosynthetically active radiation) at 25 °C in a culture chamber. The DH5α *Escherichia coli* strain, used for in vivo amplification of DNA, was cultured in an LB medium.

3.2. Plasmids Constructions

A synthetic DNA fragment (Genscript, Co, Piscataway, NJ, USA) flanked by the RsrII and the BamHI recognition sequences and consisting of 170 final nucleotides of the *APHVIII* gene without the stop codon, followed by the FMDV-2A, the 6 histidines encoding sequences and an artificial polylinker sequence, was cloned between the RsrII / BamHI sites of the plasmid pSI104 to obtain plasmid PhycoC67. Plasmid pSI104 [50] is a modification of plasmid pS103 [40] obtained from the *Chlamydomonas* Resource Center US (https://www.Chlamycollection.org). *APHVIII* encodes the enzyme aminoglycoside 3′-phosphotransferase *Streptomyces rimosus* and confers resistance to the antibiotic paromomycin. The FMDV-2A is a small self-cleaving peptide derived from the foot-and-mouth disease virus. Plasmid PhycoC67FL was prepared by including a synthetic DNA sequence that encodes the flexible peptide GASGQGASGADIGASGQGASDA between the *APHVIII* and the FMDV-2A sequences of plasmid PhycoC67. Plasmid Phyco69 available from PhycoGenetics SL., (Huelva, Spain) (http://www.phycogenetics.com) was constructed by including in front of the *APHVIII* gene of plasmid pSI104 a synthetic DNA fragment (Genscript, Co) consisting of the final nucleotides of the 5′ regulatory region of RbcS2, followed by the 6 histidines, the FMDV-2A encoding sequences, and an artificial polylinker. This DNA fragment was inserted between the HindIII and BstBI restriction sites of pSI104 (Figure 1). Phyco69BLE plasmid was obtained by inserting between the ClaI and SpeI restriction sites of plasmid Phyco69 a stop codon-less version of *Streptoalloteichus hindustanus BLE* gene. This version of the *ShBLE* gene contains the first intron of the *RBCS2* gene and confers resistance to bleomycin and related glycopeptide antibiotics, such as zeocin and phleomycin, upon binding to them.

3.3. *Chlamydomonas* Nuclear Transformation

The nuclear transformation of *C. reinhardtii* was carried out using the glass beads method of Kindle [51] with minor modifications. *Chlamydomonas* cultures were grown to a cell density of about 10^7 cells mL^{-1}, afterwards they were centrifugated, spinned down, and resuspended to get a final 100 fold concentrated cell suspension. 0.3 g of sterile glass beads (0.4–0.6 mm Ø) were added to 0.6 mL of concentrated cell suspension, 0.2 mL of 20% polyethylene glycol (MW8000), and the indicated quantity

of the desired plasmid. This mixture was agitated during 10 s, and then cells were resuspended in 50 mL of fresh TAP medium and left in dim light overnight. After this incubation, cells were spread onto the selective solid medium carrying 30 μg mL^{-1} of paromomycin. Transformed colonies were visible after four or five days.

3.4. Real Time PCR

qPCR experiments were performed on the Mx3000P Multiplex Quantitative PCR System (Agilent Technology, La Jolla, CA, USA) using as template 1 μL of the cDNA, synthesized from total RNA according to the Invitrogen SuperScript II RNase-reverse transcriptase manual (Life Technologies Corporation, Carlsbad, CA, USA) and Brilliant SYBR® Green QPCR Master Mix (Agilent Technologies, La Jolla, CA, USA). Cycling conditions were: 10 min at 95 °C for activation of the Hot start Taq polymerase and 40 cycles for the melting (30 s at 95 °C), annealing (30 s at 61 °C), and extension (30 s at 72 °C). Each qPCR measurement was carried out in triplicate using specific primers for either *ShBLE* or *APHVIII* (Table A1). The *UBC8* gene, encoding a ubiquitin ligase polypeptide (XM_001697453), whose expression was previously shown to be constitutive under the different conditions used [52,53] and was used as a housekeeping gene to normalize mRNA abundance. $2^{-\Delta\Delta CT}$ approach was used to calculate fold change relative to the expression level of a reference clone, which was chosen among the transformants which showed the lowest values [54].

3.5. Western Blot

For Western blot analysis, proteins, separated by denaturing SDS-PAGE as previously described [55], were transferred from acrylamide gel onto a polyvinylidene difluoride membrane (PVDF) using the Mini Trans-Blot system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. PVDF membrane was washed with TBS and blocked overnight with 2.5% nonfat-dry milk containing 0.2% Tween 20. Immunodetection of APHVIIII in *C. reinhardtii* crude extracts was performed using anti-APHVIII protein antibodies, raised in rabbit immunized with the recombinant purified APHVIII protein (500 mg), as primary antibodies at 1:8000 dilution in TBS (0.5% nonfat-dry milk, 0.2% Tween 20) and alkaline phosphatase (AP)-conjugated goat-anti-rabbit IgG (SIGMA) at 1:20,000 dilution as secondary antibody.

4. Conclusions

Great efforts are being made to increase the level of expression of heterologous genes in the microalgal nucleus. The fusion of the genes of interest through the self-cleaving 2A peptide to a selectable marker is a good strategy for expression of foreign genes in microalgae, which to date has never been implemented using the versatile and efficient paromomycin resistant gene, *APHVIII*. Here we demonstrate the suitability of a plasmid containing this antibiotic-resistant marker to express independent proteins linked through the FMDV-2A peptide, as long as they are fused through its N-terminal. Paromomycin is a stable and reliable antibiotic which lacks the mutagenic effects of other antibiotics, such as bleomycin or related antibiotics, and allows quick isolation of transformants without spontaneous resistance. Plasmid Phyco69, designed to contain any gene of interest linked through the 2A fragment upstream of the *APHVIII* gene has been validated using the *ShBLE*, which encodes the bleomycin resistant protein as a gene of interest. Immunodetection studies showed that the multicistronic transcript generated from Phyco69 plasmid is correctly processed, generating independent gene products. Phenotypic studies and RT-PCR analysis have shown that screening the transformants with increasing amounts of the antibiotic paromomycin provides a simple method for the selection of clones with the highest level of expression of *APHVIII* gene, and consequently of the gene of interest. This plasmid can be used to express different exogenous proteins of interest or overexpress homologous proteins in this and other related microalgae.

Author Contributions: Conceptualization, E.F, F.G.-M.; methodology, R.L, J.V.; validation A.M-M., R.R. and M.V.; formal analysis, A.M.-M. and R.L.; investigation, R.L and A.M.-M.; writing original-draft preparation R.L.; writing review and editing R.L; A.M.-M.; R.R. and M.V. All authors contributed to analysis of results and corrected the written paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

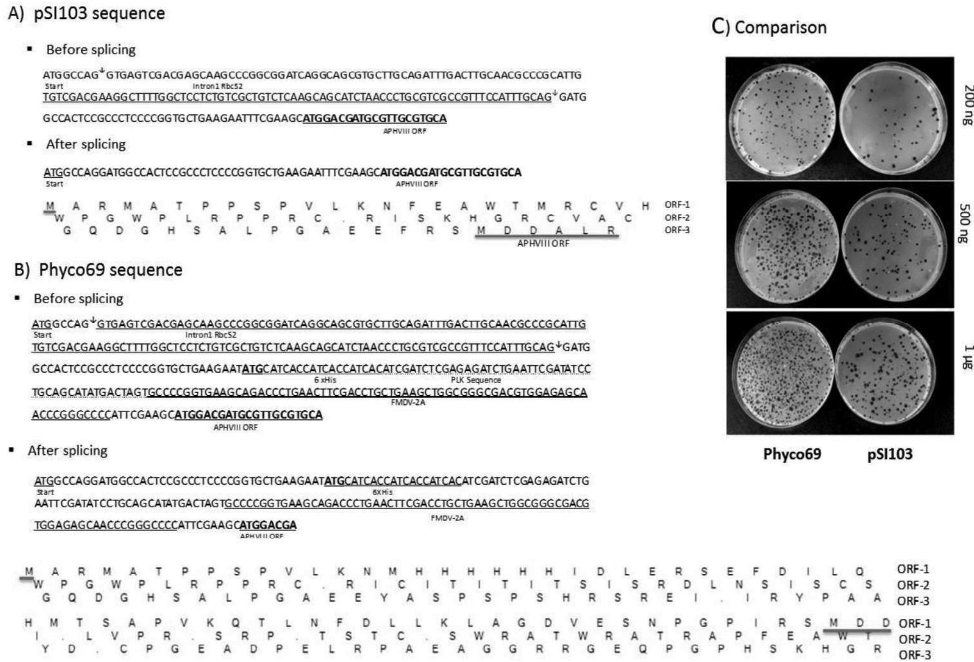

Figure A1. Detailed sequences flanking the start codon of the *APHVIII* gene before and after splicing of the first RbcS2 intron in the control pSI103 (**A**) and Phyco69 (**B**) plasmids and amino acidic sequence of the proteins generated in the three possible reading frames. A comparison of the efficiency of the transformation of *Chlamydomonas* with different quantities of each plasmid is also shown (**C**).

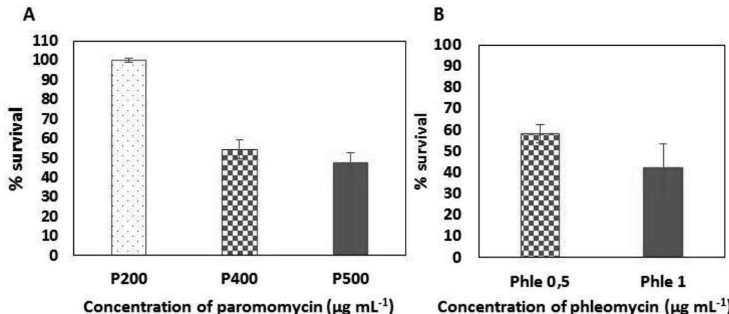

Figure A2. Average survival *Chlamydomonas* clones transformed with the plasmid Phyco69BLE and cultured with increasing concentrations of paromomycin (**A**) or phleomycin (**B**). Values are the average of at least three replicates and bars represent the standard deviation.

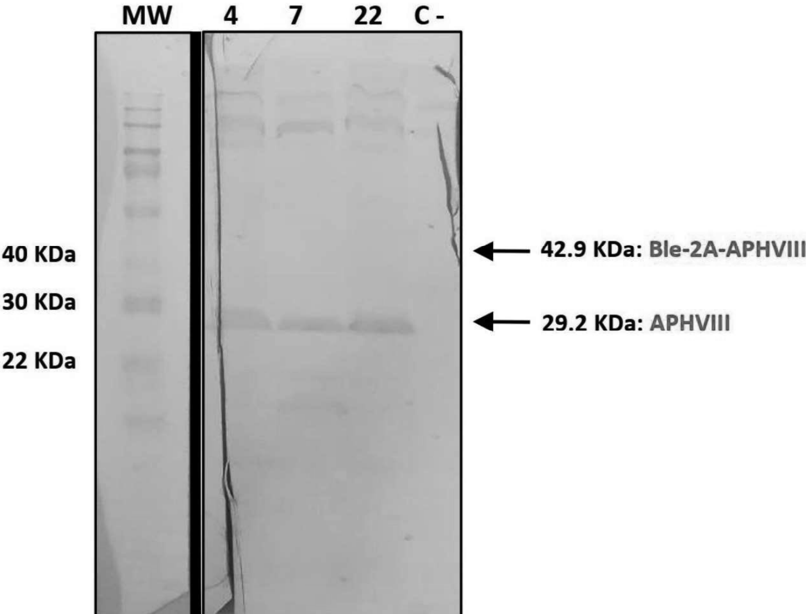

Figure A3. Inmunoblot analysis showing the APHVIII protein in the full-length blot. Total protein extracts (60 µg) were fractionated with SDS-PAGE, transferred to a PVDF membrane and probed with rabbit anti-APHVIII polyclonal antibodies and alkaline phosphatase-conjugated goat anti-rabbit IgG, as indicated in 3.4 materials and methods. MW, contains molecular weight markers; #4, #7 and #22 corresponding to three *Chlamydomonas* transformants and C-, one control untransformed *Chlamydomonas* culture.

Table A1. Oligonucleotides used in this work for quantitative RT-PCR experiments.

Target Gene	Primer Name	Sequence
BLE	qBLE-F	CGACTTCGCCGGTGTGGTC
	qBLE-R	CACGAAGTGCACGCAGTTGC
APHVIII	q*APHVIII*F	GAGGATCTGGACGAGGAGCGGAA
	q*APHVIII*R	CCCTCAGAAGAACTCGTCCAACAGC
UBC8	qUBC8	GTACAGCGGCGGCTAGAGGCAC
	qUBC8	AGCGTCAGCGGCGGTTGCAGGTATCT

BLE, bleomycin resistance protein of *Streptoalloteichus hindustanus* (1BYL-A); *APHVIII*, aminoglycoside 3'-phosphotransferase from *Streptomyces rimosus* (NG_047446.1); *UBC8*, Ubiquitin protein ligase from *Chlamydomonas reinhardtii* (XM_001697453.1).

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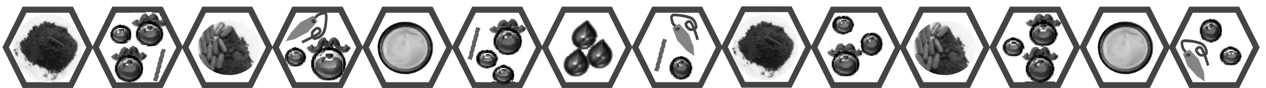
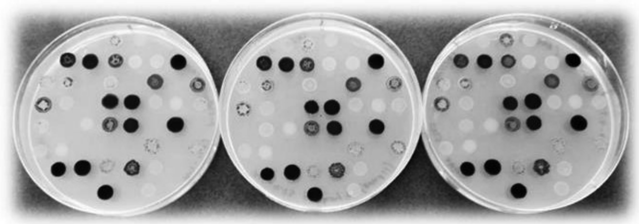
CHAPTER 2



The Bacterial Phytoene Desaturase-Encoding Gene (CRTI) is an Efficient Selectable Marker for the Genetic Transformation of Eukaryotic Microalgae



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Communication

The Bacterial Phytoene Desaturase-Encoding Gene (*CRTI*) is an Efficient Selectable Marker for the Genetic Transformation of Eukaryotic Microalgae

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Abstract: Genetic manipulation shows great promise to further boost the productivity of microalgae-based compounds. However, selection of microalgal transformants depends mainly on the use of antibiotics, which have raised concerns about their potential impacts on human health and the environment. We propose the use of a synthetic phytoene desaturase-encoding gene (*CRTIop*) as a selectable marker and the bleaching herbicide norflurazon as a selective agent for the genetic transformation of microalgae. Bacterial phytoene desaturase (CRTI), which, unlike plant and algae phytoene desaturase (PDS), is not sensitive to norflurazon, catalyzes the conversion of the colorless carotenoid phytoene into lycopene. Although the expression of *CRTI* has been described to increase the carotenoid content in plant cells, its use as a selectable marker has never been testedin algae or in plants. In this study, a version of the *CRTI* gene adapted to the codon usage of *Chlamydomonas* has been synthesized, and its suitability to be used as selectable marker has been shown. The microalgae were transformed by the glass bead agitation method and selected in the presence of norflurazon. Average transformation efficiencies of 550 colonies μg^{-1} DNA were obtained. All the transformants tested had incorporated the *CRTIop* gene in their genomes and were able to synthesize colored carotenoids.

Keywords: microalgae; *Chlamydomonas reinhardtii*; genetic transformation; carotenoid; *CRTI*; phytoene desaturase

1. Introduction

Microalgae have attracted considerable interest for the production of a wide range of compounds of applied interest due to their easy growth, their ability to fix atmospheric CO_2, and the valuable metabolites that some species can produce, which include pigments, food supplements, vitamins, antioxidants, polysaccharides, lipids, and other bioactive products [1–3]. Moreover, in the last years, there has been an increasing interest in microalgae as a potential source of biofuels [4,5]. However, despite the expectations generated, the production of biofuels and other useful compounds from microalgae will not be economically feasible unless the cost of microalgae cultivation and harvesting is lowered and the productivity increased. Genetic manipulation and synthetic biology show great promise to further boost the productivity of microalgae-based compounds [6–9]. Significant advances have been achieved in the development of molecular tools for genetic manipulation of microalgae. However, important challenges remain. One important issue is the fact that the selection of microalgal transformants depends mainly on the use of antibiotics as selective agents [10]. Antibiotic resistance genes continue to be the most commonly used selectable markers for the genetic manipulation of

algae and plants. However, the risk of horizontal gene transfer has raised concerns about their potential impacts on human health and the environment, and has encouraged the search for new non-antibiotic-based selection procedures [11].

Herbicide resistance genes are a good alternative for the selection of genetically modified plant cells. Examples of this are the glyphosate aminotransferase [12] or the acetolacetate synthase genes [13], which confer resistance to glyphosate and sulfomturon methyl herbicides, respectively, and have been used as reporter genes in the transformation of the unicellular chlorophyte *Chlamydomonas reinhardtii* and other microalgae, such as *Porphyridium* sp. [14] or *Parietochloris incisa* [15]. An interesting herbicide-based selective strategy is the use of mutated versions of the phytoene desaturase gene (*PDS*) resistant to bleaching herbicides such as norflurazon [16,17]. Phytoene desaturase (PDS) catalyzes the conversion of the colorless phytoene into ζ-carotene, which is converted to lycopene by ζ-carotene desaturase (ZDS) and carotene isomerase (CRTISO). PDS is a membrane-associated protein that uses the flavin adenine dinucleotide (FAD) as a redox cofactor, through which electrons are transferred to the plastoquinone, thereby connecting the desaturation of carotenoids with the photosynthetic electronic transport chain [1]. Treatment with norflurazon causes inhibition of phytoene desaturase by competition with its cofactors, resulting in suppression of carotenoid synthesis and cellular whitening [18]. By modifying key amino acids in the FAD binding domain, some authors have obtained mutated norflurazon-resistant versions of PDS and setup genetic selective procedures based on norflurazon as a selective agent [17,19].

In bacteria and fungi, however, the three reactions that convert phytoene into lycopene are carried out by a single enzyme, bacterial phytoene desaturase (CRTI), which presents a low degree of homology with the corresponding plant phytoene desaturase [20]. The *CRTI* gene seems to have emerged independently in evolution and, unlike plant and algae PDS, is not sensitive to norflurazon [21]. The *CRTI* gene was first identified and functionally analyzed by Misawa and coworkers [22] from the soil bacteria *Erwinia uredovora*, currently renamed as *Pantotea ananatis*. The pioneering work of Sandman's group showed that expression of this bacterial *CRTI* gene in tobacco plants enhanced the production of β-carotene. Furthermore, they observed higher resistance to the herbicide norflurazon in transgenic *CRTI*-expressing plants [23]. Several subsequent studies reported the expression of the bacterial carotenoid gene cluster from *Pantotea*, including the *CRTI* gene, to increase the carotenoid content in higher plants such as rice [24], tomato [25], or potato [26]. However, the possible use of *CRTI* as a selectable marker gene has never been investigated in algae or in plants. Poor expression of bacterial *CRTI* in microalgae, beside potential silencing and lack of stability of the transgene in algae, has withdrawn its use as a selectable marker. In the present study, we have synthesized a codon-adapted version of the bacterial *CRTI* gene and showed its suitability to be used as selectable marker gene in the genetic transformation of the model microalga *Chlamydomonas reinhardtii*.

2. Results and Discussion

2.1. Construction of Plasmid pSI06PLK-CRTIop

A synthetic *CRTI* gene with the codon usage adapted to *C. reinhardtii* was designed and synthesized by Genescript Co (Piscataway, NJ, USA). The mean difference between codon usage of the wild type *CRTI* gene and the genome of *C. reinhardtii* was 24%, as calculated using the graphical codon usage analyser v. 2.0 (http://gcua.schoedl.de/index.html). This difference was reduced to only 13% for the synthetic codon-optimized version. The *CRTIop* gene was fused to a DNA fragment which encoded the chloroplast transit peptide of the RuBisCo small subunit, and this tp*CRTIop* fusion product was cloned between the *BstBI*/*BamHI* restriction sites of the p106PLK plasmid, described in Section 3.2, generating the expression cassette outlined in Figure 1.

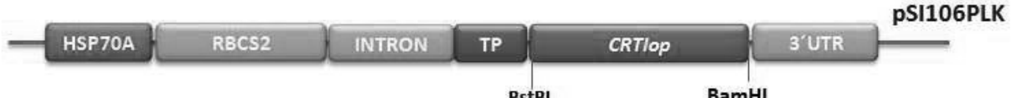

Figure 1. Expression cassette of the pSI106PLK-tp*CRTIop* plasmid.Abbreviations: HSP70A, heat shock protein 70A promoter; RBCS2, ribulose 1,5-biphosphate carboxylase small subunit promoter; TP, chloroplastic transit peptide; *CRTIop*, codon-optimized bacterial phytoene desaturase; 3′UTR terRBCS2, ribulose 1,5-biphosphate carboxylase small subunit terminator region.

2.2. *Sensitivity of the Chlorophyte Microalgae to the Bleaching Herbicide Norflurazon*

The ability of *CRTI* to act as a selectable marker for microalgae transformation is based on the sensitivity of the target microalgae to herbicides which inhibit phytoene desaturase (PDS). We have tested the sensitivity of the model chlorophyte *Chlamydomonas reinhardtii* to the bleaching herbicide norflurazon by culturing it with growing concentrations of the herbicide and determining the lethal doses (Figure 2). Samples of control non-transformed *C. reinhardtii* cultures were harvested at the exponential phase of growth and 100-fold concentrated by centrifugation, and 10 µL drops of the concentrated suspension were spotted on multi-well plates with growing concentrations (0–25 µg mL^{-1}) of the herbicide norflurazon. Concentration of the culture was done to mimic the conditions in which the transformation experiments are done (see Section 3.3). The minimal inhibitory norflurazon concentration for *C. reinhardtii* was 1.5 µg mL^{-1} after 15 days of incubation in the presence of norflurazon, as can be observed in Figure 2B. It is interesting to note that at shorter times, herbicide concentrations as low as 0.5 µg mL^{-1} seemed to inhibit *C. reinhardtii* growth. However, the microalgaeare able to survive and grow after an adaptation period. To establish the real lethal dose and avoid false negatives, in the subsequent transformation experiments it is necessary to follow the inhibitory effect of norflurazon for a long time period. All *C. reinardtii* transformants were selected at norflurazon concentrations ≥1.5 µg mL^{-1}.

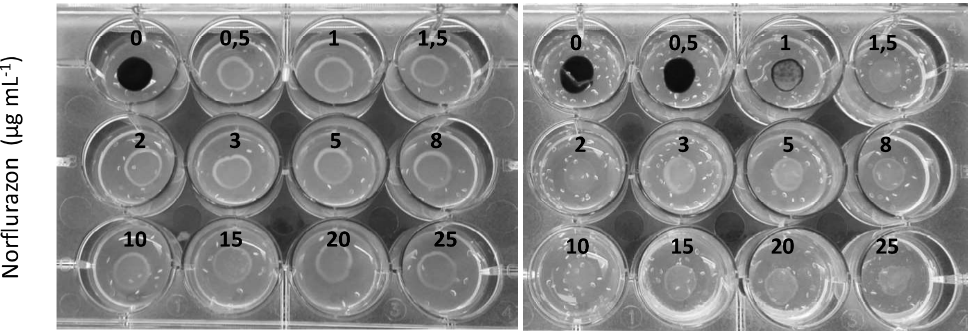

Figure 2. Norflurazon sensitivity test for the clorophyceae microalga *Chlamydomonas reinhardtii*. Drops (10 µL) of a 100-fold concentrated *C. reinhardtii* control untransformed culture were spotted on agar-solidified Tris-acetate phosphate (TAP) culture medium with increasing concentrations of the bleaching herbicide norflurazon, and their growth was evaluated 4 (**A**) and 15 (**B**) days after inoculation.

Furthermore, sensitivity experiments were carried out for other microalgal species, such as the freshwater trebouxiophyceae *Chlorella sorokiniana*, the marine prasinophyceae *Tetraselmis suecica*, and the halophilic chlorophyceae *Dunaliella salina* and *Dunaliella bardawill*. These studies revealed that the inhibitory norflurazon concentration dose was between 0.5 and 2 µg mL^{-1} for all the tested species, and that norflurazon can be an adequate selective agent for many different microalgal species,

including marine microalgae, for which traditional selective antibiotic agents are usually inefficient due to interference with the saline concentration of the medium (Figure S1).

2.3. Transformation of Chlamydomonas reinhardtii with the Plasmid pSI106PLK-tpCRTIop and Selection of Norflurazon-Resistant Norf^R-Chlamydomonas Transformants

Chlamydomonas reinhardtii cells were transformed by the glass beads agitation method with the plasmid pSI106PLK-tp*CRTIop* and selected in Tris-acetate phosphate (TAP) medium with norflurazon (1.5 μg mL^{-1}). Transformation efficiencies of 550 colonies $μg^{-1}$ DNA were obtained (Figure 3A). This transformation efficiency is of the same order as that usually obtained for transformations with paromomycin as the selective agent [27]. A randomly selected group of the obtained transformants were cultured in 2 mL of liquid TAP medium with norflurazon for 48h, and their cellular density was then adjusted to the same value. Drops of each transformed culture were spotted on TAP agar plates with a higher concentration of norflurazon (4 μg mL^{-1}), and those which grew at this concentration of the herbicide were further checked by PCR using the specific primers CRTIopFor (CAGCCGCGCCGTGTTCAAAGAG) and CRTIpoRev (CAGCAGGTCGCGGTAGGTGTG), as illustrated in Figure 3B. It is necessary to consider that nuclear transformations of microalgae take place by heterologous recombination. This means that the *CRTIop* gene is randomly inserted into the algal genome, and its expression level and stability largely depend on the insertion point. The two-round selection strategy with increasing concentrations of norflurazon allows the selection of the transformants with the highest resistance to the herbicide. Insertion of the *CRTIop* into the *C. reinhardtii* nuclear genome was confirmed in all the transformants checked (Figure 3C).

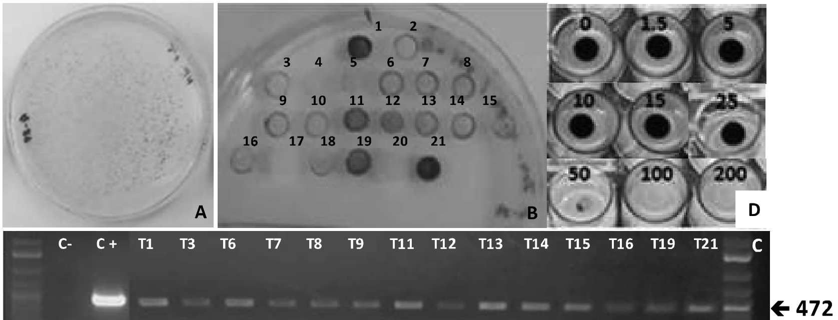

Figure 3. Molecular and phenotypic analysis of Norf^R-*Chlamydomonas* transformants. *Chlamydomonas* transformants selected in TAP with 1.5 μg mL^{-1} of norflurazon (**A**) were cultured in TAP agar plates with 4 μg mL^{-1} of norflurazon for 10 days (**B**). A band of the expected size (472 bp) corresponding to the *CRTIop* amplicon was shown in all the transformants tested (**C**). A norflurazon sensitivity test for the selected transformant T21 (**D**) was carried out as described in the Figure 2 legend with the indicated concentrations of norflurazon (from 0 to 200 μg mL^{-1}).

The transformants which grew more vigorously were subjected to a norflurazon sensitivity test as described in Figure 1. *C. reinhardtii* transformed with *CRTIop* showed a 33-fold increase in their tolerance to norflurazon, with an inhibitory dose of 50 μg mL^{-1}, equivalent to 160 μM, as shown for transformant T21 (Figure 3D) and for the other Norf^R-transformants selected (Figure S2). The synthetic tp*CRTIop* gene allowed for norflurazon-based selection of transformants with no background of spontaneous herbicide-resistant clones. The level of resistance to norflurazon acquired by the *C. reinhardtii* transformed with *CRTIop* is of the same order as the resistance reported by Suarez et al. [28] or Bruggeman and coworkers [12], who found 30- and 40-fold increases, respectively, in the tolerance to norflurazon for transgenic *Chlamydomonas* harbouring modified versions of its own *PDS* gene. Similar strategies using mutated PDS versions and norflurazon have been successfully

used for the selection of other transformed microalgae species, such as *Haematococcuspluvialis* [29,30], *Chlorella zofingiensis* [31,32], or *Isocrhrysis* [33].

2.4. Carotenoid Composition of Norflurazon-Resistant Norf^R-Chlamydomonas Transformants

The phenotypic characteristics of the selected transformants were further studied by chromatographic analysis. First, we compared the phytoene contents in the Norf^R-transformants with that of the control cells grown in the presence and in the absence of norflurazon (Table 1). As expected, in *C. reinhardtii* cultures grown without the herbicide, only trace levels of phytoene were found. After 24h of growth in the presence of norflurazon (1.5 μg mL^{-1}), the phytoene contents in control untransformed cells reached 4.4 mg g^{-1} DW. However, the phytoene intracellular concentrations of Norf^R-transformants grown with and without norflurazon ranged between 1.5 mg g^{-1} DW, for transformant T21, to 2.1 mg g^{-1} DW, for transformant T11. This is far from the intracellular level of phytoene in the control, which is between two and three times higher. This shows that the *CRTIop* gene is correctly expressed in the transformed microalgae and is able to convert phytoene into the downstream carotenoids. However, the presence of certain contents of phytoene in all the transformants tested indicates that the foreign CRTI is less efficient than the endogenous PDS in the absence of the herbicide.

Table 1. Concentration of the colorless carotenoid phytoene in control cells (C2) and Norf^R-transformants (T), grown for 24h with norflurazon. Control cells non-treated with norflurazon (C1) have also been included as a reference. Values are the average of three biological replications. Standard deviation is indicated.

Strain	C1	C2	T1	T11	T19	T21
Norflurazon (μg mL^{-1})	-	1.5	1.5	1.5	1.5	1.5
Phytoene (μg mL^{-1})	0.1 ± 0.5	4.4 ± 0.4	1.8 ± 0.1	2.1 ± 0.2	2 ± 0.2	1.5 ± 0.1

A representative Norf^R-transformant (T21) and the control untransformed strain were grown in TAP medium with norflurazon (1.5 μg mL^{-1}) for a complete analysis of their carotenoid profiles along the time. Samples were withdrawn every 24h, and pigments were extracted and analyzed (Figure 4). Typical chromatograms of the pigment extracts from Control (C2) and Norf^R-transformant cells registered at 288 and 450 nm, and are shown in the Supplementary Material (Figure S3).

In control parental *Chlamydomonas* treated with norflurazon, there was a reduction of all the colored carotenoids, excepting zeaxanthin, and an important accumulation of phytoene, due to the inhibitory effect of norflurazon. By contrast, in transformant cells treated with norflurazon, the reduction of the colored carotenoids and the accumulation of phytoene was much lower. After 24 h of incubation with norflurazon, the content of lutein in the Norf^R-transfomant was 50% higher than in the controls, while the content in β-carotene and violaxanthin was 2.5 times the level of control untransformed cells. These differences were even more acute for longer periods of incubation with norflurazon. The level of phytoene, on the contrary, was between three and five times higher in the norflurazon-treated control cells, reaching intracelullar levels of 4.4 mg g^{-1} DW at 24 h and 6.7 mg g^{-1} DW at 48h. This confirms that the *CRTIop* gene was working in the transformant, allowing the conversion of phytoene into lycopene and the subsequent carotenoids in the presence of norflurazon, which inhibits the endogenous PDS. Zeaxanthin was the only colored carotenoid which increased in the control untransformed cells, indicating a higher level of fotooxidative stress in these cells. This induces the xantophyll cycle, which catalyzes the conversion of violaxanthin into zeaxanthin. In the Norf^R-transfomants, the levels of zeaxanthin were inappreciable during the first 48 h. Only after 72 h of growth, when cultures started to be nutrient-limited, there was certain synthesis of this xanthophyll.

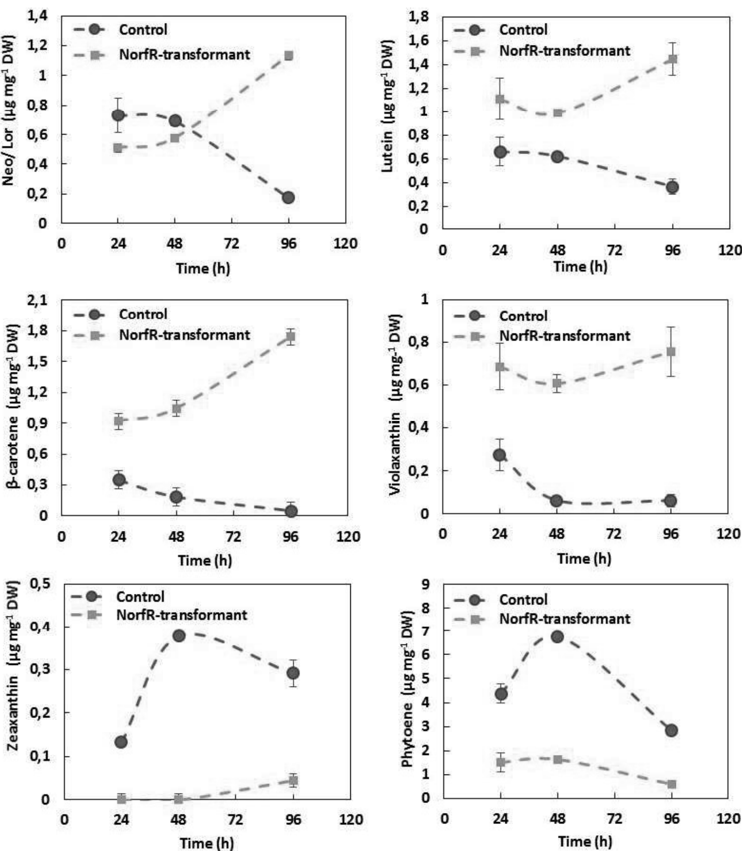

Figure 4. Time-course evolution of the main carotenoid pigments in *C. reinhardtii* control (●) and Norf^R-transformant (■) cells incubated with norflurazon (1.5 µg mL^{-1}). Values are the average of three biological replicates and bars indicate standard deviation. Neoxanthin and Loroxanthin (Neo/Lor) are expressed as unique values since they are not resolved in the analytical conditions used.

In the presence of norflurazon, the growth of the parental strain was severely affected. Meanwhile, the growth rate of the Norf^R-transformantswas similar with and without norflurazon, and was slightly higher than the growth rate of the control wild type without herbicide (Figure S4A,B). The carotenoid content of the Norf^R-transformants grown without herbicide was also checked and resulted to be very similar to that of control cells (Figure S4C,D). This means that the transformants selected in the presence of norflurazon with *CRTI* as a marker gene can grow at a normal rate and have practically normal contents of carotenoids [19,20]. It could be expected that the expression of an exogenous phytoene desaturase caused an increase in the contents of carotenoids. The fact that the *CRTIop* transformants studied had intracellular levels similar to that of the control cells in the absence of norflurazon could be due to a limitation in the supply of precursors from the previous step catalyzed by the Phytoene synthase (PSY). However, further studies should be done to confirm this issue.

PDS is the second step in carotenoid biosynthesis and an important regulatory point of the pathway [19,20]. The inhibitory effect of norflurazon is well known and has been widely used to study the physiological consequences resulting from the lack of carotenoids in higher plants and microalgae [34]. We have corroborated that blocking PDS activity by chemical inhibition with norflurazon impedes the formation of downstream carotenoids in *Chlamydomonas* (Figure 4), which is in agreement with what Nigoyi's group observed by mutagenesis-induced PDS inactivation [34]. Furthermore, we demonstrated that the expression of the foreign *CRTIop*, which is not affected by the

herbicide, allows bypass, at least partially, of the norflurazon-blocked step and enables the synthesis of colored carotenoids. Similar conclusions were reported by Liu and coworkers [35] or Steinbrenner and Sandmann [29], who used a modified *PDS* gene as a selectable marker for the genetic transformation of *Chlorella zofingiensis* and as *H. pluvialis*, respectively, and found that the transformants had the same or even higher carotenoid content than the untransformed controls.

3. Materials and Methods

3.1. Strains and Culture Conditions

Chlamydomonas reinhardtii 704 strain (Cw15, Arg7, mt+) was kindly donated by Dr. R. Loppes and cultured photomixotrophically in liquid or agar-solidified Tris-acetate phosphate (TAP) medium [36]. *Tetraselmissuecica*, kindly provided by IFAPA-Aguas del Pino station (Huelva, Spain), was cultured in F/2 medium in filtered sea water at pH 8, as reported by Guillard and Ryther [37]. *Dunaliella salina* (CCAP 19/18) was obtained from the culture collection of algae and protozoa (Scotland, UK) and grown in the culture medium described by Johnson and coworkers [38]. All were grown in a culture chamber at 25 °C under continuous white light irradiation (50 µE m^{-2} s^{-1} PAR).

3.2. Microalgal Expression of pSI106PLK Plasmid

Plasmid pSI106PLK is a renewed version of plasmid pSI104PLK [39]. It contains an expression cassette in which a multiple cloning site is preceded by the strong chimeric fusion promoter *HSP70A:RBCS2*, designed by Sizova [40], and the first intron of the *RBCS2* gene, and is terminated by the 3′ untranslated region of *RBCS2* (Figure 1).

3.3. Chlamydomonas Nuclear Transformation

Nuclear transformation of *C. reinhardtii* was carried out using the glass beads method [41] with minor modifications. *Chlamydomonas* cultures were grown as described in Section 3.1. to a cell density of 5×10^6 cells mL^{-1}, and resuspended to get a 100-fold concentrated cell suspension. 0.3 g of sterile glass beads (0.4–0.6mm Ø), were added to 0.6 mL of concentrated cell suspension with 0.2 mL of 20% PEG (MW8000) and about 1 µg of the desired plasmid (pSI106PLK-tpCRTIop). Negative controls, done in the same conditions with the empty plasmid (pSI106PLK), were included in all the transformation reactions. This mixture was agitated for 10s, resuspended in fresh TAP medium, and spread onto the selective solid medium with the indicated concentration of norflurazon. Transformed colonies were visible after 4 or 5 days.

3.4. Determination of Carotenoids

Samples from *C. reinhardtii* cultures, grown in TAP liquid medium supplemented with 1.5 µg mL of norflurazon in the same conditions described in Section 3.1., were used for the extraction of carotenoids with methanol as described by Linchtehaler [42]. The chromatographic analysis was performed in a Merck Hitachi HPLC equipped with a diode array detector as described by Young and coworkers [28] using an RP-18 column, a flow rate of 1mL min^{-1}, and a final injection volume of 100 µL. Two mobile phases were used: Solvent A (ethyl acetate 100%) and solvent B (acetonitrile:H_2O; 9:1 v/v). The gradients applied were: 0–16 min 0–60% A; 16–30 min 60% A; and 30–35 min 100% A. Standards were supplied by DHI (Hoersholm, Denmark). All experiments were done in triplicate, and the average values and standard deviation are represented. The significant differences have been analysed by a t-student test with a confidence level of 95%.

3.5. Dry Weight Determination

Dry weight was determined by filtering an exact volume of microalgae culture (30 mL) on pre-tared glass-fiber filters (GF/F Whatman). The filter was washed with a solution of ammonium formate (0.5 M) to remove salts and dried at 100 °C for 24 h. The dried filters were weighed in

an analytical balance and the dry weight calculated by the difference. Values are the average of three measurements.

3.6. Herbicide Sensitivity Test

Norflurazon sensitivity was assayed on multi-well plates with the corresponding agar-solidified medium supplemented with the indicated concentrations of the herbicide.

4. Conclusions

The use of a synthetic codon-adapted *CRTIop* gene fused to a chloroplastic transit peptide as a selectable marker for the genetic transformation of *Chlamydomonas reinhardtii* has been shown to be a reliable and efficient approach for the selection of transformants, contributing to increase the non-antibiotic-dependent markers available for microalgae and plant cells. *C. reinhardtii* transformants selected on norflurazon have been shown to have the *CRTIop* gene correctly inserted into their genomes to acquire a 33-fold increased resistance to norflurazon and be stable for long periods of time. This is a good alternative to genetic markers based on resistance to antibiotics, which can be very useful to establish genetic transformations systems for new microalgal species which are recalcitrant to inhibition with traditional antibiotics, as usually happens with marine and halophyllic microalgae. It can also be an interesting alternative for the selection of higher plants transformants without using antibiotics.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/9/3/49/s1. Figure S1: Norflurazon sensitivity test for several microalgae species. Drops (10 μL) of concentrated cultures of the microalgae *Tetraselmis suecica*, *Dunaliella salina*, *Dunaliella bardawill* and *Chlorella sorokiniana* were spotted on agar-solidified culture medium with increasing concentrations of the bleaching herbicide norflurazon (from 0 to 5 μg mL^{-1}) and their growth was evaluated 10 days after inoculation. The culture media used was that described in Materials and Methods for each species, excepting that the concentration of NaCl was reduced to half the salinity of the seawater for *T. suecica* and to 0.5M for *D. salina* and *D. bardawill.* Figure S2: Norflurazon sensitivity test for different Nor^R-transformants of the microalga *Chlamydomonas reinhardtii*. Drops (10 μL) of concentrated cultures of the transformants T1, T11 and T19 obtained as described in the legend of Figure 2, were spotted on agar-solidified TAP culture medium with increasing concentrations of the bleaching herbicide norflurazon (from 0 to 200 μg mL^{-1}) and their growth was evaluated 10 days after inoculation. Figure S3: Chromatographic analysis of pigments extracts from control and $Norf^R$-*Chlamydomonas* transfomants grown in the presence of norflurazon (1.5 μg mL^{-1}). Conditions for chromatographic separation are described in Materials and Methods. Peaks were identified as: neoxanthin/loroxanthin (1), violaxanthin (2), antheraxanthin (3), lutein (4), zeaxanthin (5), chlorophyll b (6), chlorophyll a (7) and β-carotene (8) and phytoene (9). Figure S4: Growth curves (A,B) and carotenoid content (C,D) of untransformed (Control) and transformed ($Norf^R$) strains of *Chlamydomonas reinhardtii* cultured without and with increasing concentrations of the herbicide norflurazon. Cultures of the control untransformed cells and of the $Norf^R$-transformant T21 were harvested at the beginning of exponential phase of growth and resuspended in fresh TAP medium without norflurazon or supplemented with 1.5, 8 and 20 μg mL^{-1} of norflurazon. Dry weight (DW) was determined every 24h. In addition, the carotenoid content of control and transformed ($Norf^R$) cells grown for 24h without or with 1.5 μg mL^{-1} of norflurazon were also determined. Neoxanthin and loroxanthin (Neo/Lor) are expressed a unique value since they are not resolved in the analytical conditions used. All the values are the average of three biological replicates and bars represent standard deviation.

Author Contributions: All authors have contributed to this research and agree to authorship, and submit this manuscript for its revision and publication. Conceptualization, R.L. and A.M.-M.; methodology, A.M.-M., A.B., and M.V.; software, A.M.-M.; investigation, A.M.-M. and A.B.; data curation, J.V. and M.V.; Writing—Original Draft preparation, R.L.; Writing—Review Andediting, A.M.-M., M.V., J.V., and R.L.; supervision, R.L. and J.V.; project administration, R.L. and J.V.; funding acquisition, R.L. and J.V.

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Conflicts of Interest: The authors declare no conflict of interest.

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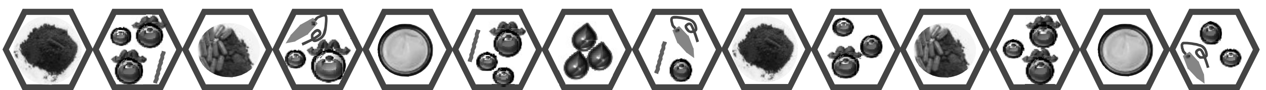
CHAPTER 2

Supplementary Material



SUPPLEMENTARY MATERIAL

Figure S1. Norflurazon sensitivity test for several microalgae species. Drops (10 µL) of concentrated cultures of the microalgae *Tetraselmis suecica, Dunaliella salina, Dunaliella bardawill,* and *Chlorella sorokiniana* were spotted on agar-solidified culture medium with increasing concentrations of the bleaching herbicide norflurazon (from 0 to 5 µg mL^{-1}), and their growths were evaluated for 10 days after inoculation. The culture media used was that described in the Materials and Methods for each species, excepting that the concentration of NaCl was reduced to half the salinity of the seawater for *T. suecica* and to 0.5M for *D. salina* and *D. bardawill.*

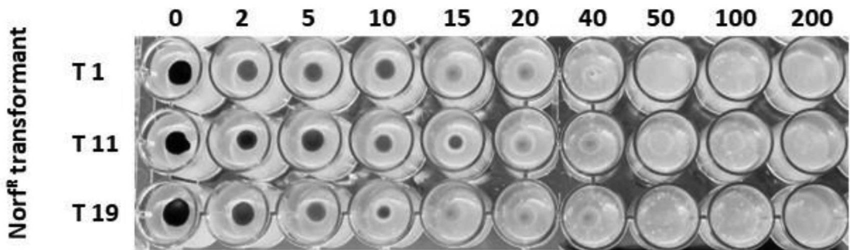


Figure S2. Norflurazon sensitivity test for different Nor^{R}-transformants of the microalga *Chlamydomonas reinhardtii*. Drops (10 µL) of concentrated cultures of the transformants T1, T11, and T19, obtained as described in the legend of Figure 2, were spotted on agar-solidified TAP culture medium with increasing concentrations of the bleaching herbicide norflurazon (from 0 to 200 µg mL^{-1}), and their growths were evaluated 10 days after inoculation.

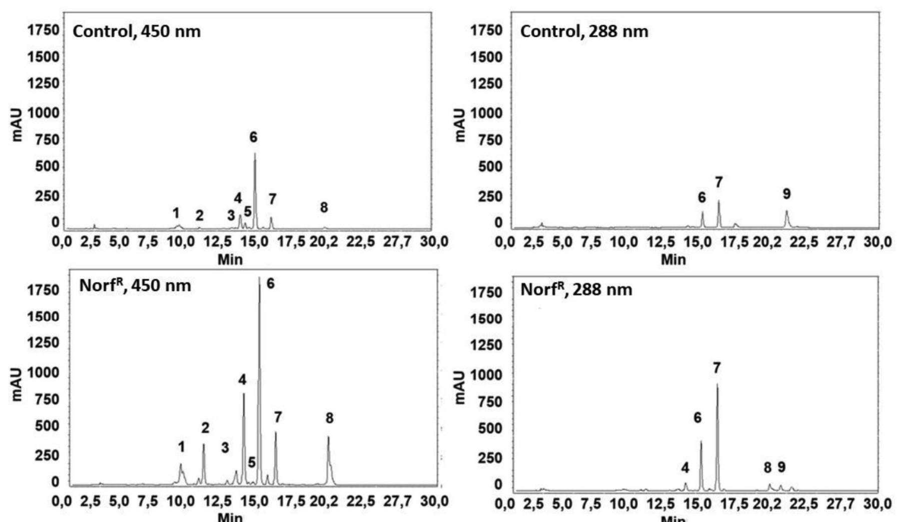


Figure S3. Chromatographic analysis of pigment extracts from control and Norf^{R}-*Chlamydomonas* transfomants grown in the presence of norflurazon (1.5 µg mL^{-1}). Conditions for chromatographic separation are described in the Materials and Methods. Peaks were identified as: Neoxanthin/loroxanthin (1), violaxanthin (2), antheraxanthin (3), lutein (4), zeaxanthin (5), chlorophyll b (6), chlorophyll a (7), β-carotene (8), and phytoene (9).

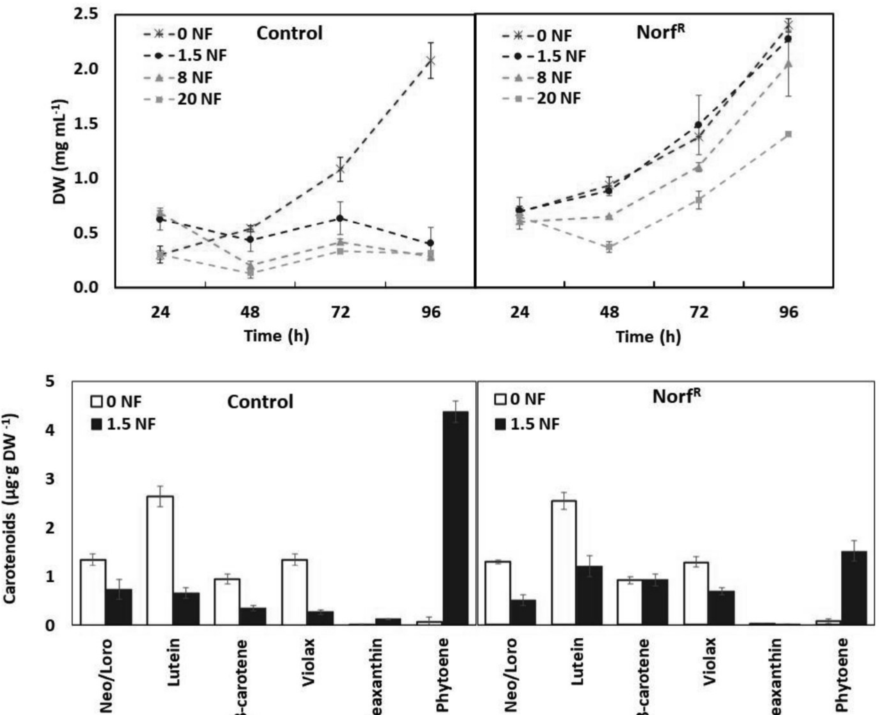
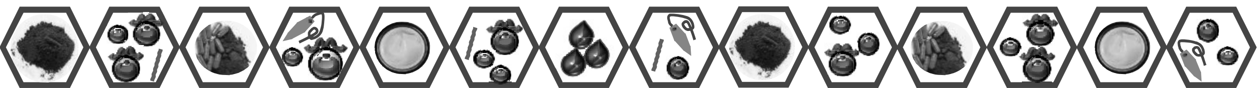
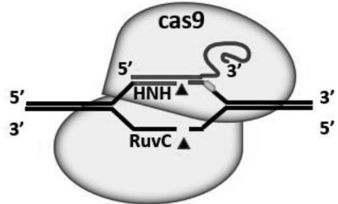


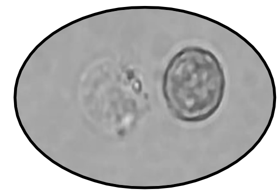
Figure S4. Growth curves (A, B) and carotenoid contents (C, D) of untransformed (Control) and transformed (Norf^R) strains of *Chlamydomonas reinhardtii,* cultured with and without increasing concentrations of the herbicide norflurazon. Cultures of the control untransformed cells and of the Norf^R-transformant T21 were harvested at the beginning of the exponential phase of growth and resuspended in fresh TAP medium without norflurazon or supplemented with 1.5, 8, and 20 µg mL^{-1} of norflurazon. Dry weight (DW) was determined every 24h. In addition, the carotenoid content of control and transformed (Norf^R) cells grown for 24 h with or without 1.5 µg mL^{-1} of norflurazon were also determined. Neoxanthin and Loroxanthin (Neo/Lor) are expressed as unique values since they were not resolved in the analytical conditions used. All the values are the average of three biological replicates and bars represent standard deviation.

CHAPTER 3



Characterization of a Phytoene Desaturase Knock-out Carotenoid-deficient Microalga Mutant Generated by Cas9-Ribonucleoprotein Complexes





CHAPTER 3

Debido a restricciones relativas a derechos de autor, el artículo en proceso de publicación del apartado Chapter 3 “Characterization of a phytoene desaturase knock-out carotenoid-deficient microalga mutant generated by Cas9-ribonucleoprotein complexes” ha sido retirado de la tesis. En sustitución del documento ofrecemos la siguiente información: resumen y palabras claves.

Abstract

Phytoene desaturase (PDS) is a key enzyme in the biosynthesis of carotenoids that catalyzes the desaturation of phytoene, the first colourless carotenoid. Initial difficulties to obtain PDS knock-out (KO) mutants were attributed the potential toxicity of phytoene or the possible existence of other enzymes with the same function, besides the extreme light sensitivity of carotenoid-less mutants.

In this study, PDS knock-out transformants have been obtained by a reverse genomic approach, using the clustered regularly interspaced short palindromic repeat (CRISPR) associated to Cas9 system. Single guide RNAs (sgRNA) were designed to target the first exon of *PDS* and the assembled ribonucleoproteins (RNP) were delivered into the cell by electroporation. Several white PDS-KO transformants which completely lacked coloured carotenoids and accumulated phytoene were obtained by this approach. The molecular analysis of these transformants revealed that all had been cleaved in the target site and the mutations generated consist mainly in the insertion of plasmid sequences of between 47 to at least 2078 nucleotide. ΔE10 PDS-KO was selected for further analysis and co-cultivated with non PDS-KO cells, since it seems to beneficiate the growth of PDS-KO transformants. A separation step by a fluorescence-activated cell sorting (FACS) allowed its study by HPLC-DAD, transmission electron microscopy (TEM) and transcriptomic analysis. The chromatographic separation of pigments showed that phytoene was the only carotenoid accumulated by this transformant, TEM revealed that the ultrastructure of *Chlamydomonas* ΔE10 PDS-KO mutant is completely altered, with an undeveloped chloroplast and other structures absent of severely modified. And finally, the transcriptomic analysis confirmed the degeneration of the thylakoids membranes and the affection of other important cellular processes. Conversely, those genes most clearly upregulated in the PDS-Knock out transformant encode regulatory stress-related proteins, corroborating the essential role of carotenoid in the metabolism of microalgae.

Key Words: *Chlamydomonas reinhardtii*, carotenoids, transcriptomics, phytoene desaturase, PDS, CRISPR, algae

CONCLUSIONS

CONCLUSIONS

CHAPTER 1

1. Phyco69 has been constructed and validated as a new expression plasmid for microalgae, which contains a polylinker region (PLK) linked through the short self-cleaving FMDV-2A peptide encoding region to the N terminus of the selective, aminoglycoside-3`phosphotransferase gene from *Streptomyces rimosus* (*APHVIII),* which confers resistance to the antibiotic paromomycin.

2. The fusion of the gene of interest to the selective marker *APHVIII* gene in Phyco69 plasmid provides a simple and fast screening method for the selection of transformant microalgae with the highest level of expression of both, the *APHVIII* gene and the gene of interest.

3. By Immunodetection studies we have shown that the multicistronic transcripts generated from Phyco69 plasmid under the control of an unique common promoter, are correctly processed in the eukaryotic ribosome, producing independent gene products.

4. Phyco69 ensures the stable expression of the transgenes, avoiding the silencing events, as long as the transformant cells are maintained under selective pressure.

CHAPTER 2

1. A codon-optimized version of the bacterial phytoene desaturase gene, *CRTIop,* has been demonstrated to be a reliable selective marker for the genetic transformation of eukaryotic microalgae, such as *Chlamydomonas reinhardtii*.

2. *CRTIop* provides resistance to the bleaching herbicide norflurazon and supposes an interesting alternative to antibiotic-based selection, opening the possibilities for its potential use as an universal marker gene for the genetic transformation of microalgae, including species which show no susceptibility to antibiotics.

3. CRTI-transformant microalgal cells have normal pigments profiles in the presence of norflurazon, while the control untransformed cells in the same conditions exhibited a dramatic reduction in the content of coloured carotenoids and an accumulation of phytoene.

CHAPTER 3

1. CRISPR-Cas9 ribonucleoproteins complexes have demonstrated to be efficient and easy systems for genome edition in the green microalga *Chlamydomonas reinhardtii*.

2. During the reparation of blunt double-stranded breaks, which mainly takes place by non-homologous end-joining (NHEJ), the incorporation of plasmid sequences and sequences of unknown origin has been observed, but none of the microalgal transformant tested incorporated the short donor DR sequence (36 bp) provided.

3. The targeted knock-out of the phytoene desaturase (*PDS*) gene by CRISPR-Cas9 RNPs complexes generates white, extremely light-sensitive transformants which accumulate phytoene and lack coloured carotenoids and chlorophylls.

4. Transmission electron microscopy reveals that the absence of coloured carotenoids and chlorophylls causes a complete disorganization of the chloroplast that exhibit an aberrant structure with no stacks of thylakoid

membranes, besides the lack of pyrenoid, the disorganization of the eyespot, the presence of abundant autophagic vacuoles and starch granules.

5. Differential Transcriptome analysis of the PDS knock-out transformants shows that the absence of coloured carotenoids affects diverse metabolic pathways, such as carbon fixation, photosynthesis protein synthesis and gene regulation in *Chlamydomonas*.

6. Strong downregulation of genes encoding different ribosomal and photosystems components, biosynthesis of carotenoids and chlorophylls, Rubisco subunit 1 and 2 (RBCS1 and PBCS2) is observed. Conversely, those genes most clearly upregulated encode regulatory stress-related proteins, one component of photosystem II and other non-functionally assigned genes, which role in the response to the lack of carotenoids is yet to be elucidated.

CONCLUSIONES

CAPÍTULO 1

1. Phyco69 ha sido construido y validado como un nuevo plásmido de expresión para microalgas, que contiene una región polilinker (PLK) unida a través de la región que codifica para el péptido autohidrolizable FMDV-2A con la región N terminal del gen selectivo aminoglicosidasa-3'-fosfotransferasa de *Streptomyces rimosus* (*APHVIII*), que confiere resistencia al antibiótico paromomicina.

2. La fusión del gen de interés al gen marcador selectivo *APHVIII* en el plásmido Phyco69 provee un método de selección simple y rápido para seleccionar las microalgas transformantes con mayor nivel de expresión de ambos genes, *APHIII* y el gen de interés.

3. Mediante estudios de inmunodeteción hemos mostrado que el transcrito multicistrónico generado al transcribir la secuencia del plásmido Phyco69 bajo el control de un único promotor común, es correctamente procesado en el ribosoma eucariótico, produciendo productos génicos independientes.

4. Phyco69 asegura la expresión estable de los transgenes, evitando eventos de silenciamiento, siempre que las células transformantes sean mantenidas bajo presión selectiva.

CAPÍTULO 2

1. Una versión optimizada al uso de codones de la microalga del gen bacteriano fitoeno desaturasa, *CRTIop*, ha demostrado ser un marcador selectivo fiable para la transformación genética de microalgas eucariotas, como *Chlamydomonas reinhardtii*.

2. *CRTIop* provee resistencia al herbicida norflurazon y supone una alternativa interesante a la selección basada en antibiótico, abriendo posibilidades para su potencial uso como gen marcador universal para la transformación genética de microalgas, incluyendo especies que no muestran susceptibilidad a los antibióticos.

3. Las células microalgales transformantes de CRTI mostraron un perfil de pigmentos normal in presencia de norflurazon, mientras que las células control no transformadas exhibieron una reducción dramática en el contenido de carotenoides coloreados y acumulación de fitoene.

CAPÍTULO 3

1. El complejo ribonucleoproteico CRISPR-Cas9 ha demostrado ser un sistema fácil y eficiente para la edición del genoma de la microalga *Chlamydomonas reinhardtii.*

2. Durante la reparación del corte de extremos romos generado en la doble hebra de ADN, que es principalmente reparada por el mecanismo de finales no homólogos (NHEJ), se observó la incorporación de secuencias plasmídicas y secuencias de origen desconocido, pero en ninguna microalga transformante analizada se ha incorporado la secuencia corta donada DR (36 pb) que fue añadida.

3. La pérdida de función del gen de la fitoeno desaturasa (*PDS*) producido mediante los complejos RNPs CRISPR-Cas9 generan transformantes blancos, extremadamente sensibles a las luz, que acumulan fitoene y presentan ausencia de carotenoides no coloreados y clorofilas.

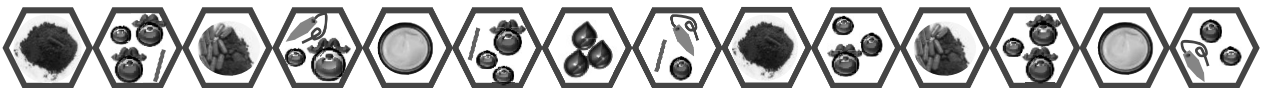
4. La microscopía electrónica de transmisión revela que la ausencia de carotenoides coloreados y clorofilas produce una completa desorganización del cloroplasto que exhibe estructuras aberrantes con la ausencia de

apilamiento de las membranas tilacoidales, además de la falta del pirenoide, la desorganización del eyespot, la presencia de abundantes vacuolas autofágicas y gránulos de almidón.

5. El análisis transcriptómico diferencial del transformantes knock-out de PDS muestra que la ausencia de carotenoides coloreados afecta a diversas rutas metabólicas, como la fijación de carbono, la síntesis de proteínas y la regulación génica en *Chlamydomonas*.

6. Se observa fuerte represión de genes que codifican diferentes componentes ribosomales y fotosintéticos, biosíntesis de carotenoides y clorofilas, así como las subunidades 1 y 2 de la RuBisCo (RBCS1 y RBCS2). En cambio, los genes que se encuentran más claramente sobreexpresados codifican para proteínas relacionadas con el estrés, un componente del fotosistema II y otros genes que no tiene asignada su función y cuyo rol ante la falta de carotenoides aún no se ha elucidado.

ABBREVIATIONS



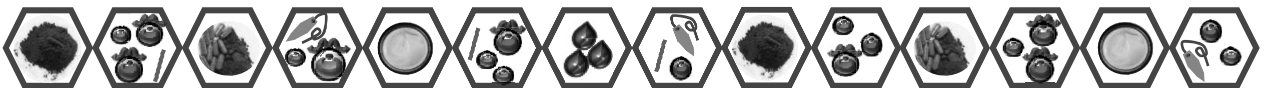
ABREVIATIONS

aadA	Aminoglycoside3′-adenylyltransferase gene
ALS	Acetolacetate synthase gene
APHVIII/aphVIII	Aminoglycoside 3′-phosphotransferase gene
aph7	Aminoglycoside phosphotransferase gene
Arg4	Argininosuccinate lyase gene
ARG7	Argininosuccinate lyase gene
BKT1	β-carotene quetolase
Ble	Phleomycin-binding protein gene
Cas	CRISPR associated protein
cat	Chloramphenicol acetyltransferase gene
$^{1}Chl^{*}$	Excited singlet Chl state
$^{3}Chl^{*}$	Excited triplet Chl state
CPFTSY	β subunit of ATP synthase gene
crRNA	CRISPR RNA
CRTI	Bacterial phytoene desaturase gene
CRY1-1	Cytosolic ribosomal protein S14 gene
Csf1	CRISPR associated protein subtype in Acidithiobacillus ferrooxidans
FKB12	Peptidyl-prolyl cis-trans isomerase
GAT	Glyphosate aminotransferase gene
GFP	Green fluorescence protein
GUS	β-glucuronidase
HSP70A/RbcS2	Heat shock protein 70A /Ribulose-1,5 bisphosphate carboxylase/oxigenase small subunit 2
NIC7	Quinolinatesynthetase gene
NIT1	Nitrate reductase gene
$NO^{\cdot}$	Nytroxyl radical

Abbreviation

NptII	Neomycin phosphotransferase gene
OEE1	Oxygen-evolvingenhancer protein 1 gene
$OH^{\cdot}$	Hydroxyl radical
$O2^{-\cdot}$	Superoxide radical
PDS1	Phytoene desaturase gene
$RS^{\cdot}$	Thiyl radical
$RO2^{\cdot}$	Peroxyl radical
ZEP	Zeaxanthin epoxidase gene

CURRICULUM VITAE



CURRICULUM VITAE

1. PUBLICATIONS

- Pérez, D., Moyá, M.L., Bautista, M., León, Rosa; **Molina-Márquez, Ana**; Vila, Marta; Romero-Azogil, Lucía; Benito, Elena; de Gracia García-Martín, María; Moreno-Gordillo, Paula; Rosado, Iván; Balestra, Fernando R.e. A novel biocompatible polymer derived from D-mannitol used as a vector in the field of genetic engineering of eukaryotic cells. *Colloids and Surfaces* B: Biointerfaces, 2023, 224, 113219. April 2023. Doi: 10.1016/j.colsurfb.2023.113219.

- Ariel Chazan, Ishita Das, Takayoshi Fujiwara, Shunya Murakoshi, Andrey Rozenberg, **Ana Molina-Márquez**, Fumiya K. Sano, Tatsuki Tanaka, Patricia Gómez-Villegas, Shirley Larom, Alina Pushkarev, Partha Malakar, Masumi Hasegawa, Yuya Tsukamoto, Tomohiro Ishizuka, Masae Konno, Takashi Nagata, Yosuke Mizuno, Kota Katayama, Rei Abe-Yoshizumi, Sanford Ruhman, Keiichi Inoue, Hideki Kandori, Rosa León, Wataru Shihoya, Susumu Yoshizawa, Mordechai Sheves2, Osamu Nureki4 &Oded Béjà. Phototrophy by antenna-containing rhodopsin pumps in aquatic environments. ***Nature.*** March 2023. Doi: 10.1038/s41586-023-05774-6.

- López-Sanmartín, M., Rengel, R., López-López, M., Lebrón, José A.; **Molina-Márquez, Ana**; de la Rosa, Ignacio; López-Cornejo, Pilar; Cuesta, Alberto; Vigara, J., León, R. D-amino acid peptides as antimicrobial agents against vibrio-associated diseases in aquaculture**.** *Aquaculture*, 2023, 569, 739362. February 2023. Doi:10.1016/j.aquaculture.2023.739362.

- A.Cuesta; M.Vila; **A.Molina-Marquez**; C.Carbajo; J.A.Lebron; M.López-López; P.LópezCornejo; M.L.Moyá; M.López-Sanmartín; J.R.López; J.I.Navas; J.Vigara; R.León. Influence of adding terminal tags on the structural and antimicrobial properties of the peptide caerin 1.1. *Aquaculture*. V 532; 736035. February 2021. Doi:10.1016/j.aquaculture.2020.736035.

- Schüler LM, Gangadhar KN, Duarte P, Placines C, **Molina-Márquez AM**, LéonBañares R, Sousa VS, Varela J, Barreira. Improvement of carotenoid extraction from a recently isolated, robust microalga, Tetraselmis sp. CTP4 (*chlorophyta*). *Bioprocess and Biosystems Engineering*. (5):785-796. May 2020. Doi: 10.1007/s00449-019-02273-9.

- **Molina-Márquez A,** Vila M, Rengel R, Fernández E, García-Maroto F, Vigara J, León R. Validation of a New Multicistronic Plasmid for the Efficient and Stable Expression of Transgenes in Microalgae. *International Journal of Molecular Science.* 22;21(3):718. January 2020. Doi:10.3390/ijms21030718.

- **Ana Molina-Márquez**; Vila M, Vigara J, Borrero A, León. The Bacterial Phytoene Desaturase-Encoding Gene (CRTI) is an Efficient Selectable Marker for the Genetic Transformation of Eukaryotic Microalgae**.** *Metabolies.* 12;9 (3):49, March 2019. Doi: 10.3390/metabo9030049.

- Kathleen Saavedra; **Ana María Molina-Márquez**; Nicolás Saavedra; Tomás Zambrano and Luis A. Salazar. Epigenetic Modifications of Major Depressive Disorder. *International Journal of Molecular Sciences*. 17 – 8. August 2016. Doi: 10.3390/ijms17081279.

2. WORKS SUBMITTED TO NATIONAL/INTERNATIONAL CONFERENCES OR WORKSHOPS

- R. Rengel, **A. Molina,** T. García, J. Díaz, J. Vigara, R. León. Biotechnological application of nitrogen starvation for the production of chemical precursosrs from the microalga *Chlamydomonas reinhardtii.* XV National Meeting of the Nitrogen Metabolism group. 2022. Poster.

- **A.M. Molina-Márquez**, S. ketelborn, P. Hegemann, R. León. Manipulation of the carotenoid profile of phytoplankton by treatment with ribonucleoproteins-Cas9. III International Congress of Young Researchers of the Sea. Motril. Granada (Spain). 2021. Oral communication.
- M.R. Pereira Jaramillo, **A. Molina-Márquez**, M. López-López, F.J. Ostos, José A. Lebrón, M.R.E Idrissi Benhnia, P. López-Cornejo, R. León**. Estudio de la capacidad antimicrobiana de nanotubos de carbono frente a patógenos de interés acuícola.** International Congress of Young Researchers of the Sea. Motril. Granada (Spain). 2021. Poster.
- **Molina-Márquez A**., Hornero-Méndez D., Vigara J. and León R. **Characterization of a new pigment mutant of *Chlamydomonas reinhardtii*. Póster.** 19th International Conference on the Cell and Molecular Biology of Chlamydomonas (Chlamy 2020+1). 2021. Poster.
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- Vila M., Sendra M., Yeste P., **Molina A**. y León R. Study of the immune response in *Mitylus galloprovincialis* against ecotoxic particle of polystyrene nanoplastic**.** II International Congress of Young Researchers of the Sea. Málaga (Spain). 2019. Poster.
- Rengel R., **Molina AM**., García-Maroto F., López-Alonso D., Vigara J., León R. Synergistic effect of overexpression of genes involved in TAG biosynthesis and substrate supply on the lipid profile of *Chlamydomonas reinhardtii*. II International Congress of Young Researchers of the Sea. Málaga (Spain). 2019. Poster.
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- Vila, M.; **Molina**, **A.M**.; León, R. Strategies to enhance the carotenoid production of carotenoid in microalgae. I International Congress of Young Researchers of the Sea. Cadiz (Spain). 2018. Poster
- **Molina A.,** Rengel R., Vila M., Vigara J., León R. Optimization of the Microalgal Expression Vector Phyco69. 18 th International Conference on the Cell and Molecular Biology of *Chlamydomonas.* Whasington (EEUU). 2018. Poster
- Vila M., **Molina A.,** Rengel R., Vigara J., Díaz- Santos E., León R. Simultaneous Expression of Two Promotorless Genes in the Nucleus of *Chlamydomonas reinhardtii*. 18 th International Conference on the Cell and Molecular Biology of *Chlamydomonas.* Whasington (EEUU). 2018. Poster.
- **A. Molina**, R. Rengel, M. Vila, J. Vigara y R. León. Analysis of the heterologous expression genes through the multicistronic expression vector Phyco69. IX Conference on Aquaculture in the South Atlantic Coast. Cartaya (Huelva). 2018. Poster.
- M. Vila**, A. Márquez**, J. Vigara y R. León. Strategies to obtain microalgae rich in carotenoids for aquaculture. IX Conference on Aquaculture in the South Atlantic Coast. Cartaya (Spain) 2018. Poster.
- **A. Molina**, M. Vila, R. León. New strategies to genetic manipulation of the carotenoid synthesis pathway of eukaryotic microalgae. National Meeting of the carotenoid in microorganisms, plants, diet and health. Valencia (Spain) 2017. Poster.
- **A. Molina**, R. Rengel, M. Vila y R. León. Validation of a multicistronic expression vector for microalgae. National Biotechonology Congress. Murcia (Spain). 2017. Póster.

- **A. Molina-Márquez**, M. Vila, R. León. Enhancement of the carotenoid biosynthesis pathway in the unicelular microalga *Chlamydomonas reinhardtii.* VIII Southern Atlantic Coastal Aquaculture Workshop. Cartaya (Spain) 2016. Poster.

- R. León, M. Vila, **A. Molina**, A. León, P. Gómez, R. Rengel, C. J. Vigara. Microalgae: from auxiliary cultures to source of high added value products. VIII Southern Atlantic Coastal Aquaculture Workshop. Cartaya (Spain) 2016. Oral communication.

- A. Léon-Vaz, **A. Molina-Márquez**, J. Vigara, R. León. Development of a procedure for the cryopreservation of chlorophyte microalgae at -80ºC. XIII National Meeting of the Nitrogen Metabolism Group. Villanueva de la Serena (Badajoz). 2016. Poster.

3. PARTICIPATION IN PROJECTS

- **Use of carbon nanotubes in agriculture for the inhibition of the bacterial nitrification process** (NANOCARB). Fertinagro-Biotech agri-food sustainability chair, 2022. PI: Manuel López López. 5 000 €.

- **Study and spreading of the biomedical applications of new strains of microorganisms isolated from the Andalusian Algarve coast**. Andalusian government, 2021. PI: Ana María Molina Márquez. 7 200 €.

- **Stereoselective antimicrobial peptides (D-AMP), CAS9-ribonucleoproteins and marine microorganism as sustainable biological weapons in the fight against pathogens in aquaculture** (FITORIBOPEP) PY20_00728. PI: Rosa León Bañares. I+D+i+JA-PAIDI-Retos, Andalusian gobernment, 2020. 110 288 €.

- **Adapting microalgae metabolism for the production of colourless bioactive and bioavailable carotenoids** (BESTALGAE4CAROT) PID2019-110438RB-C22. MICIIN, 2019-2020. PI: Rosa León Bañares. 121 000 €.
- **Cross-border network for the development of innovative products in microalgae** (0055_ALGARED_PLUS_5_E), VA INTERREG cross-border program, POPTEP-2014-2020. PI: Rosa León Bañares. 321 420 €.

- **Study of the immune response in *Mitylus galloprovincialis* against ecotoxic particle of polystyrene nanoplastics** (ERI-NP). the International Campus of Excellence of the Sea (CEIMAR). PI: Marta Vila Spínola. 4 000€.

- **Dissemination of the biotechnological poptential of the salt flats of the Algarve Andalusian coast, and study of the carotenoids present in the isolated exptremophilic microorganisms**. Andalusian government, 2019. PI: Marta Vila Spinola. 4 800 €.

- **Diffusion of the biotechnological potential of the salt flats of the Andalusian-Algarve coast: isolation of extremophilic microorganisms and identification of bioactive substance with biomedical applications**. Andalusian government, 2018. PI: Marta Vila Spinola. 4 740 €.

- **Recombinant microalgae as a platform for the production and supply of oral vaccines and antimicrobial peptides in aquaculture** (AGL2016-74866-C32-R) MINENNCO. 2016-2019. PI: Rosa León Bañares. 90 000 €.

4. PATENTS

- **Use of the *CRTI* gene in a selection method of herbicide resistant algae.** 201830544/2701381. July 2022.

5. GRANTS

- Predoctoral European mobility grant of the International Campus of Excellence of the Sea (CEIMAR). January-april 2020

- Predoctoral International mobility Erasmus+ of the Campus of International Agri-Food Excellence (ceiA3). February-may 2018.

6. INTERNATIONAL STAYS

- **Institute of Biology Experimental Biophysics, University of Humbolt, Berlin (Germany).** International predoctoral stay, january-april 2020. Genetic improvement of phytoplackton using CRISPR technology to obtain nutraceutical and pharmacology products.

- **Centre for Marine and Environmental Research (CIMA), University of Algarve, Faro (Portugal)**. International predoctoral stay, February-May 2018. Study of bioethanol production from algal biomass.

7. TEACHING

- **Practical teaching in the Biochemistry Molecular Biology subject of the Degree in Chemistry** at the University of Huelva. 3 Credicts. Course of 2021/2022.

- **Co-direction of the Final Degree Project in the Degree of Chemistry**: "Study of the antimicrobial capacity of the carbon nanotubes against to pathogenics with aquaculture interest". University of Huelva. Course 2020/2021.

- **Co-direction of the Final Degree Project in the Degree of Chemistry**: "Subcloning of the antisense sequence in a expression plasmid of microalgae". University of Huelva. Course 2018/2019.

8. LABOR CONTRACTS

- Labour contract for specific work and service to carry out a research project (402): **Stereoselective antimicrobial peptides (D-AMP), CAS9-ribonucleoproteins and marine microorganism as sustainable biological weapons in the fight against pathogens in aquaculture** (FITORIBOPEP) PY20_00728. From 01 may 2023 to 30 june 2023.

- Labour contract for specific work and service to carry out a research project (401): **Adapting microalgae metabolism for the production of colourless bioactive and bioavailable carotenoids** (BESTALGAE4CAROT) PID2019-110438RB-C22. Desde el 01 de marzo de 2021 hasta el 30 de abril de 2023.
- Labour contract for specific work and service to carry out a research project (401): **Red transfronteriza para el desarrollo de productos innovadores con Microalgas, (0055_ALGARED_PLUS_5_E).** Desde el 01 de agosto de 2020 hasta el 31 de diciembre de 2020.

- Contrato de trabajo por obra y servicio determinado para la realización de un proyecto de investigación (401): **Red transfronteriza para el desarrollo de productos innovadores con Microalgas, (0055_ALGARED_PLUS_5_E).** Desde el 19 de noviembre de 2018 hasta el 18 de noviembre de 2019.

- Contrato de trabajo por obra y servicio determinado para la realización de un proyecto de investigación (501): **Red transfronteriza para el desarrollo de productos innovadores con**

Microalgas, (0055_ALGARED_PLUS_5_E). Desde el 13 de noviembre de 2017 hasta el 12 de noviembre de 2018.