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Microalgae, Functional Genomics and Biotechnology

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Abstract:

Microalgae have been studied for decades, but a new wave of research has recently begun as part of the search for renewable and sustainable energy sources. For economic optimization, microalgal biomass is being considered as a whole (main products and co-products) in an overall 'biorefinery' concept. Applications of microalgae cover a broad spectrum, including the food and (livestock) feed industries, bioenergy, cosmetics, healthcare and environmental restoration or protection. In the field of biotechnology, the access to genomic data is playing a growing role. As the cost of sequencing strategies has fallen, studies of gene function at the transcript, protein and biosynthesis pathway levels have multiplied. Notably, sequencing and mass spectrometry technologies are used to delineate the pathways of lipid synthesis, which will be valuable for the future application of microalgae in the biotechnology and biofuel industries. Another field making an applied use of genomics is the 'cell factory' approach, which uses the cell to manufacture (express) natural or recombinant proteins for diverse purposes. In this chapter, we present a vision of the potential future of genomics in the biotechnology of microalgae from several points of view.

Keywords: Microalgae ; Genomics ; Post-genomics ; Biotechnology ; Molecular farming ; Cell factory ; Domestication ; Biofuel ; Lipid ; Natural compound ; Biodiversity

1. Introduction

Microalgae in biotechnology are presently the focus of an unprecedented surge in interest and investment worldwide. Over recent decades, research predicted the explosion of attention this field would attact following the US Aquatic Species Program (Sheehan *et al.*, 1998), as microalgae can provide a new source of vegetal material. They offer complementary products to land plants and higher manipulability, but as the consequence of their large phylogenetic spread (reviewed in chapter I of this volume), they have vast unknown metabolic potential because most species are, as yet, unexamined.
Driven by the giants of the energy industry, the race to develop mass microalgal production capacity started about five years ago, fuelled by hundreds of millions of US dollars targeting the production of renewable biofuels. The challenges we face today are to adapt and improve existing methods, develop new processes, and achieve a drastic reduction in costs. The objective is to use this green biomass in its entirety and not only for energy production. The potential is huge and the fields of study numerous, offering very high added value in the areas of new energy (oil, hydrogen and fermentation), healthcare (pigments, enzymes and secondary metabolites), food (human or animal), environmental management (depuration and assimilation mechanisms) and industry (recovery of silica, enzymes or pigments). Here, we have chosen to focus our presentation on the world of microalgae, their broad fields of application, the advances in genomics for biotechnologies and some of the bottlenecks that need to be overcome.

1.1. Microalgae

We use the term *microalgae* to cover a heterogeneous group of single-celled photosynthetic organisms, including photosynthetic eukaryotes and photosynthetic prokaryotes like *Prochlorococcus* and *Synechococcus*, which are of major global importance and considered as key players among phytoplanktonic organisms in oligotrophic oceanic areas. It would be vastly overambitious to attempt to cover the biotechnological potential of the entire aquatic photosynthetic world in one book chapter, so this review will address only the genomics and biotechnology of eukaryotic microalgae.

Depending on environmental conditions such as salinity, light, temperature, pH and nutrient concentrations, the size and appearance of microalgae can change profoundly, making their identification difficult without molecular tools. The estimated number of described species ranges between 40 000 and 60 000, but estimations of the number of undescribed species range from hundreds of thousands to millions of species spread over the globe (Norton *et al.*, 1996, Sastre and Posten, 2010). In comparison, only 250 000 land plant species have been recorded. Half of the world‘s oxygen is produced via microalgal photosynthesis. Microalgae contribute up to 50% of all aquatic productivity and 25% of global productivity (Raven and Falkowski, 1999). They are the foundation of the aquatic food chain and have colonised nearly all biotopes, from the polar ice to deserts and hot springs. They have adapted to extreme environments, living in salt marshes, acidic environments or conditions with very low light. Through their presence on the surface of the oceans, which cover 70% of the earth, they play a major role in global climate regulation, as a machine that transforms CO_2 into organic matter (Raven and Falkowski, 1999).

Ancestors of the present day cyanobacteria invented photosynthesis as far back as 3.6 billion years ago (Gould *et al.*, 2008) and the primary endosymbiotic event at the origin of all photosynthetic eukaryotes can be traced to 1.8 billion years ago, (Finazzi *et al.*, 2010). The number and the diversity of algal species offer a whole new field of research when considering their potential commercial applications and biotechnology. Although progress still needs to be made on culture techniques, algal production systems on scales from a few litres up to cubic metre volumes, in photobioreactors or open ponds, are now a reality at the

industrial level. Microalgae have clear advantages over land plants. Their photosynthetic yields are slightly better than those of land plants (Wijffels *et al.*, 2010), and the fact that they live in an aqueous medium gives them direct access to their nutrients and explains why they display higher growth productivity. As an example, the productivity of classic crops in Europe is around 1 to 2 g/m^2/day (dry weight), whereas the microalgae in small and medium-sized enterprises on the Atlantic coast produce around 10 g/m^2/day. Additionally, aqueous cultures in marine water offer the advantage of using land unsuitable for food crops, avoiding the much-publicised dilemma between ‗food and fuel'. Other differences between land plants and microalgae that could give microalgae the advantage include the possibility of performing continuous cultures in photobioreactors with a high level of control, the potential to couple microalgal production with the disposal of effluents that provide nutritive components, the attractive idea of using industrial CO_2 sources, and the saving of freshwater by cultivation of microalgae in seawater. The opportunity to cultivate in photobioreactors offers the additional possibility of adjusting and adapting culture conditions in real time, allowing growers to react instantaneously to the culture situation. The biological diversity of microalgae provides an exceptional range of adaptability and represents a vast potential as a source of food and feed, biomaterial, original molecules and applications in the broad field of biotechnology. Gene transfer of the means to produce selected molecules by genetic engineering will provide a complementary production method for novel compounds.

1.2. Applications of microalgae

The current and forthcoming applications of microalgae are numerous and diverse, including food, feed, healthcare, industry and energy. Although the use of cyanobacteria in food dates back many hundreds of years, advances in this area were made in the 20th century (Habib *et al.*, 2008). The market for microalgae as food and food supplements is dominated by the Cyanobacteria *Spirulina platensis* (also called *Arthrospira platensis*), the Chlorophyta *Chlorella sp.*, and in France, the diatom *Odontella aurita*. In addition, the green microalga *Dunaliella salina* is used for its beta-carotene, *Haematococcus pluvialis* for astaxanthin and the Cyanobacteria *Aphanizomenon flos-aquae* as a dietary supplement. Investigation is still needed on the use of other microalgae as food, requiring effort to be made for the acceptance of these alternative sources. For example, cookies made from the Haptophyta *Isochrysis galbana*, rich in omega-3, have already been produced (Gouveia *et al.*, 2008).

The area in which microalgae were first mass produced was aquaculture. Phytoplanktonic organisms are an essential food for the rearing of molluscs and fish, especially to feed the early life stages of bivalves, for which microalgae must be provided as live food. A large production capacity is devoted to this activity worldwide. Although around 40 microalgal species are used in this way, the number routinely grown is closer to a dozen. The technology and skills developed as part of this culture are important for the future of microalgal biotechnology. Microalgae could become an important source of land animal feed. The most common species used for this are *Spirulina*, *Chlorella* and *Scenedesmus*. In chicken farming, it is reported that the incorporation of 5 to 10% microalgae in the diet has an effect on the colour of the meat and egg yolk (Becker, 2007). The potential substitution of fish oil with algae oil has also been discussed (AbuGhazaleh *et al.*, 2009).

Algae also offer several benefits in the field of human healthcare. Land plants and animals lack the enzymes to synthesise polyunsaturated fatty acids (PUFAs) longer than 18 carbon atoms. Long-chain PUFAs like gamma-linolenic (GLA), arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), produced by microalgae, accumulate in most marine animals. Sufficient consumption of such fatty acids could have beneficial effects on human health. The oil from the stramenopile *Schizochytrium sp*. (permitted as a food ingredient) contains 35–45% DHA. In comparison, most conventional oils rich in omega-3 (walnut oil, canola oil) contain about 10% alpha-linolenic acid, the precursor of omega-3. The production of these PUFAs will undoubtedly be a major challenge in the coming years.

Algal pigments, such as carotenoids, are already commercially exploited but are also the subject of intensive research. The most popular among these are beta-carotene, alpha-carotene, lutein, lycopene and zeaxanthin. Even though the main supply of astaxanthin to colour salmon is 95% of synthetic origin, natural sources such as the green microalga *Haematococcus pluvialis* are authorised in Japan and Canada (Lorenz and Cysewski, 2000). Among other marine pigments of interest, the phycobiliproteins are a very unusual class identified in microalgae. First commercialised in clinical and immunological analysis, broader uses in industry and therapy are envisioned (Sekar and Chandramohan, 2008).

The uptake of oxygen by organisms can cause the formation of dangerous derivatives, including singlet oxygen and free radicals. These forms of highly reactive oxygen species (ROS) play an important role in various chronic diseases (cancer, atherosclerosis, osteoarthritis, Parkinson‘s, etc.) or acute reactions (inflammation, septic shock, etc.). However, ROS production can also be used as a means of therapy in human health. Indeed, photo-dynamic therapy (PDT) is an innovative discipline calling for photosensitive molecules with a tumour tropism that react to light and destroy the surrounding tissues by ROS production. Only a few drugs are presently in use for PDT. Less than a dozen molecules have been identified so far and none are, as yet, considered very efficient. It is, however, a promising field as our laboratory was able to identify a group of molecules from microalgae that is 30 times more efficient than the best commercial gold standard (Unpublished work, Patrice, T., Cadoret, J. P., Picot, L., Kaas, R. and Berard, J. B.).

The polysaccharides extracted from the red microalga *Porphyridium purpureum* have been proven to have antiviral activity on cell lines, as well as *in vivo* in rabbits (Huheihel *et al.*, 2002). Indeed, red algae have been studied for their polysaccharide contents both for health (Matsui *et al.*, 2003) and industry applications (Gourdon *et al.*, 2008). Apart from structural polysaccharides, some microalgae synthesise exopolysaccharides. These polymeric compounds form a hydrophilic and polyanionic matrix, retain water and trap cations, allowing the microalgae to resist desiccation. These properties suggest that the algae could be useful for biotechnological applications in environmental fields, through the detoxification of biotopes polluted by heavy metals (Pb, As, Hg, Cd) and in the recovery of some metals such as gold and uranium. The physicochemical characteristics of polysaccharides—particularly their rheological, lubricant and flocculent properties—have been suggested for various applications.

A few hundred microalgae are classified as dangerous due to their toxin production. Among the 90 recorded species, 70 belong to the dinoflagellate group. The potential applications of these toxins in human healthcare have been reviewed by Camacho *et al.* (2006). Characteristics such as the antifouling properties of microalgae could be exploited produce a range of ‗biogenic‘ products (Bhadury and Wright, 2004).

Some algal extracts are considered emollients, and are incorporated into anti-aging creams to prevent wrinkles and stimulate collagen synthesis; their ultraviolet (UV) protection properties are also being researched. Although many of the marketing claims about algal bioproducts still need to be proven, business prospects justify the interest shown in this field. *Arthrospira* and *Chlorella*, are again those involved in the anti-aging and regenerative products (Spolaore *et al.*, 2006). However, while many applications of microalgae are already in existence, genomics is opening up still more opportunities.

1.3. Genomics and microalgae

The rise of next-generation sequencing (NGS) technologies, accompanied by a sharp fall in their cost, has led to the acquisition of important genomic data on microalgae since the 1990s. The pace of the availability of microbial genomes is obviously increasing with NGS technologies, and in addition to the 14 nuclear genomes available (see chapter II and III of

this volume for a review), the gene repertoire of many additional species is now available through transcriptomics, as discussed below. Due to its phylogenetic proximity to land plants and because many molecular tools are available, the Chlorophyta *Chlamydomonas reinhardtii* was chosen as a model among photosynthetic organisms and the sequencing of its entire nuclear genome completed in 2007 (Merchant *et al.*, 2007). Comparative phylogenomic analyses have provided insight into the evolution of plants and animals, allowing genes to be associated with photosynthesis and flagellar functions, and links established between ciliopathy and the composition and function of flagellae. Over the past decade, many post-genomics and genetic tools have been used on this species, including microarrays, antibodies, RNAi and genetic transformation. These approaches have enabled the exploration of metabolic pathways and biological processes such as responses to stress, the circadian clock (Matsuo and Ishiura, 2011), photosynthetic electron transport chains (Hermsmeier *et al.*, 1994), mechanisms of carbon concentration (Yamano and Fukuzawa, 2009) and flagellar assembly (Iomini *et al.*, 2009). In addition, proteomic studies have provided major research contributions in the areas of photosynthesis, molecular biology and evolution (Rolland *et al.*, 2009, Muhlhaus *et al.*, 2011). The other alga species sequenced were chosen due to their ecological role, phylogenetic distribution or harmful nature. Sequencing provided extensive information on the evolution of these species, helped to identify metabolic pathways and specific genes and clarified processes involved in the cycles of iron, calcium, silica, urea and nitrogen. In addition, sequence data provide essential references for matching with post-genomic investigations, including transcriptomic and proteomic analyses.

The gene content of microalgae is only beginning to be explored. Microalgal genomes can be structurally complex and sizes range from 12.6 Mbp for the Chlorophyta *Ostreococcus tauri* and 168 Mbp for the Haptophyta *Emiliania huxleyi* to an estimated 10,000 Mbp for the Dinophyta *Karenia brevis* (see chapter XI for a discussion of genome size variations in algae). These large genome sizes can preclude full-genome sequencing, thus enforcing the use of transcriptome sequencing to build gene catalogues. Many authors have made this choice, although aware of the risk of neglecting non-transcribed sequences. Among the species studied in this way, we can mention the Ochrophyta *Pseudochattonella farcimen*, which is associated with fish mortalities (Dittami *et al.*, 2011), green microalgae *Chlorella vulgaris* UTEX 395 (Guarnieri *et al.*, 2011)*, Dunaliella salina* (Zhao *et al.*, 2011) and *D. tertiolecta* (Rismani-Yazdi *et al.*, 2011) and the coccolithophore *Emiliania huxleyi* (Von Dassow *et al.*, 2009). Transcriptomic data have been used for phylogenomics and opened the way for functional post-genomics approaches to the study of physiology, environmental adaptation, life cycles, metabolism and signal transduction pathways. Several major projects for transcriptome sequencing are currently underway (Table 1). One example is the ‗Marine Microbial Eukaryotic Transcriptome Project', which aims to sequence the transcriptomes of approximately 750 samples expected to represent hundreds of species and strains with key ecological roles and evolutionary importance in the tree of microeukaryotes (http://marinemicroeukaryotes.org/). To date, 39 microbial algal transcriptomes have been sequenced (Table 1). In order to establish a reference database from ecologically and phylogenetically relevant photosynthetic protists for the ‗Tara Oceans expedition', the ‗Prometheus project' is proposing to sequence about 30 species of ecological or phylogenetic importance (http://oceans.taraexpeditions.org) (Karsenti *et al.*, 2011).

We can therefore hope, in a few months or years, to have a very large number of new transcriptomic and genomic data for algae. The development of genomics has already made a major contribution to fundamental research on photosynthetic eukaryotes in the fields of functional biology, global ecology and the evolution of organisms. These data will accelerate the commercialisation of alga-derived compounds by providing a framework for hypothesis-based strain improvement programs built on an improved fundamental understanding of the specific pathways and regulation of networks. These studies are also the source of new biotechnologies that will be presented in the following sections.

2. Biotechnology and microalgae

For 2011, a search using the two keywords ‗microalgae' and ‗biotechnology' returned 51 publications in Web of Science database. More than a third of these were on energy and biofuels. In second position, with 20% of the papers, came work on different cultivation and extraction techniques. Cell factories, i.e., the production of recombinant proteins, came in third position, with a number of technical advances in *Chlamydomonas sp.*

2.1. Microalgal Lipids as Biofuel and Food

2.1.1. Algal lipid synthesis: The contribution of genomic data

Compared with land plants, the lipid composition of algae shows great specificity, such as the presence of long-chain PUFAs or the species-specific absence of phosphatidylcholine and phosphatidylserine in the membranes, replaced by diacylglyceryltrimethylhomoserine (DGTS) (Guschina and Harwood, 2006). In addition, for many algal species, high-energy reserves of triacylglycerol (TAG) accumulate in large amounts in lipid droplets in response to different types of stress or nutrient deficiency. TAG represent > 50% of the algal dry weight and serve for membrane synthesis or carbon storage (Hu *et al.*, 2008), making it possible to obtain oil yields 10 times higher per hectare than with land plant species. Recent soaring oil prices, diminishing world reserves and the environmental damage associated with fossil fuel consumption have led to increased interest in using algae as an alternative and renewable feedstock for fuel production. The development of the microalgal biodiesel industry depends primarily on the reduction of production costs, and one strategy to achieve this is to increase lipid productivity. This explains the large investment being placed in such technology, and demonstrates why most genomics work on algae is aimed at describing and orienting their lipid metabolism (Norsker *et al.*, 2011).

Many studies have been conducted on land plants to understand their mechanisms of lipid synthesis and the development of reserves in their seeds. It was reported that environmental conditions (nutrients, salinity, light, etc.) affect microalgal fatty acid accumulation (for a review, see Hu *et al.*, 2008). However, molecular mechanisms that trigger and control the accumulation of storage lipids in microalgae are poorly understood. Genomic data have allowed the identification of new enzymes and helped to show how lipid pathways interrelate with energy and carbohydrate metabolism (Wallis and Browse, 2010). Until recently, the molecular mechanisms involved in regulatory pathways in algae were still poorly understood. With genomic data and genetic tools available for the green microalga *Chlamydomonas reinhardtii*, lipid metabolism has been mainly studied in this species and overviews of these findings can be found in several papers (Guschina and Harwood, 2006, Khozin-Goldberg and Cohen, 2011, Moellering and Benning, 2010). Many genes of *C. reinhardtii* involved in fatty acids and TAG metabolism have been identified based on their orthological relationships to fungi and land plants. In green microalgae, starch synthesis shares common carbon precursors with lipid synthesis. In *C. reinhardtii,* it has been shown that shunting of carbon precursors from the starch synthesis pathway may facilitate carbon partitioning into the fatty acid synthesis pathway resulting in enhanced production of TAG (Li *et al.*, 2010b). Identification of genes and biosynthetic pathways implicated in lipid biosynthesis is usually made using starchless mutants. With regard to the metabolism of TAGs, genomic data have shown conservation of the main biosynthetic pathways between microalgae and seed plants. Briefly, fatty acids are synthesised in the chloroplasts, in which acetyl-CoA carboxylase (ACCase) provides the malonyl-CoA substrate for the biosynthesis of fatty acids thanks to the fatty acid synthase, a multifunctional enzymatic complex (Guschina and Harwood, 2006). Free fatty acids are then either used for the synthesis of membrane lipids or exported to the endoplasmic reticulum for the biosynthesis of TAGs. This synthesis involves the sequential

transfer of acyl groups from acyl-CoA to different positions of glycerol-3-phosphate. Most acyltransferases and a phosphatases involved have been identified in the genome of *C. reinhardtii* (Merchant *et al.*, 2011). Nevertheless, significant differences from land plants were observed in the TAG pathways of *C. reinhardtii*, such as the absence of the extra-plastidic lysophosphatidyl acyltransferase in the genome and the presence of new enzymes that are, as yet, poorly characterised (Hu *et al.* 2008). Most recently, an alternative chloroplast pathway of TAG synthesis was identified in *C. reinhardtii* (Fan *et al.*, 2011). TAG accumulates in lipid droplets, in which proteomics techniques revealed the importance of a major lipid droplet protein (MLDP). Miller *et al.* (2010) used 454 and Illumina technologies for transcriptomic analysis, and showed how nitrogen deprivation redirects lipid metabolism. In brief, genomic and post-genomic data have allowed lipid metabolism pathways and regulation to be characterised in the Chlorophyta *C. reinhardtii.* However, this alga is not an oleaginous species. With the great diversity that exists among algae, specific studies are now being conducted on lipid-accumulating species in numerous laboratories around the world.

2.1.2. Algal lipids as biofuel

Very recently, several studies have used post-genomics to study the lipid metabolism of high oil-content algae. This illustrates a real drive in the exploration of the functional metabolism of oleaginous algae. In 2011, Rismani-Yazdi *et al.* (2011) published the NGS and transcriptome annotation of a non-model member of the Chlorophyta: *Dunaliella tertiolecta.* Genes encoding key enzymes were identified by homology and metabolic pathways involved in the biosynthesis and catabolism of fatty acids, TAG and starch were reconstructed (Rismani-Yazdi *et al.*, 2011). A few months later, similar work was reported in a strain of the oil-producing green alga *Botryococcus braunii* (Baba *et al.*, 2011). In parallel, proteomic approaches have identified new proteins involved in the storage of TAG in the lipid droplets of the Chlorophyta *Haematococcus pluvialis* (Peled *et al.*, 2011). Guarnieri *et al.* (2011) reported a comprehensive proteomic and transcriptomic investigation of lipid accumulation in the unsequenced green alga *Chlorella vulgaris* UTEX 395. The authors presented the first utilisation of a *de novo* assembled transcriptome as a search model for proteomic analysis. The regulation of fatty acid and TAG biosynthetic pathways was analysed under nitrogen limitation. This oleaginous species is extensively studied due to its relatively fast growth rate, its value as both a food supplement and a potential biofuel feedstock and its ability to produce high–economic value molecules and to remediate heavy metals from wastewater. For these reasons, the genome of the Chlorophyta *Chlorella variabilis* NC64A was previously sequenced by Blanc *et al.* (2010). However, difficulties were encountered in identifying proteins by comparing data with strains of species from the same phylum. The researchers pointed out the importance of having unique sequence data to study species and strains of interest (Guarnieri *et al.*, 2011).

Although lipid biosynthesis pathways have been studied in several species, very few studies focus on the regulation of these pathways. Given the induction of TAG biosynthesis by different stresses, it is likely that the mechanisms for the regulation of TAG synthesis differ between algae and seed plants, as the latter produce oil during a specific phase of their life cycle and in specialised tissues. The means of regulation are presently of great interest, as these are the key to engineering algal crop production without causing weakening through nutrient stress. Although transcriptomics offer a wealth of information on gene expression, the processes of mRNA splicing, ribosome recruitment and post-translational regulations of proteins are not well understood in algae and transcriptomic analysis does not adequately define the control points for metabolic regulation.

By providing insight into the mechanisms underpinning lipid metabolic processes, results can be of use for the genetic manipulation of organisms to enhance the production of feedstock

for commercial microalgal biofuels. By 1996, Dunahay and co-authors were able to overexpress ACCase in the diatom *Cyclotella cryptica*, which is a key enzyme in the biosynthesis of fatty acids (Dunahay *et al.*, 1996). However, no increase in the amount of lipid was observed. In expressing recombinant thioesterases to enhance the expression of shorter chain length fatty acids, Radakovits *et al.* (2010) were able to improve the level of lauric and myristic acids in the diatom *Phaeodactylum tricornutum*. This creates an advantage for biofuel feedstock because biodiesel made from saturated short or medium chain length fatty acids has a relatively low cloud point and is resistant to oxidation. In addition, several studies have shown metabolic shifts in starchless mutants of *Chlamydomonas reinhardtii* in favour of an overexpression of TAG (Moellering *et al.*, 2009, Wang *et al.*, 2009, Li *et al.*, 2010a). In a starchless mutant, Moellering *et al.* (2009) inhibited the expression of MLDPs by RNAi, which increased the size of the lipid globules (Moellering *et al.*, 2010) but also resulted in decreased growth. In contrast, the fatty acid content of a starchless selected mutant of *Chlorella pyrenoidosa* was doubled without detriment to its growth characteristics (Ramazanov and Ramazanov, 2006). This suggests that it is possible to improve the productivity of microalgae using lipid selection strategies. To date, the genomic data available on the selected species is still patchy, and reverse genetic tools are completely absent in these species. We also lack genetic information on the molecular mechanisms leading to these beneficial mutations. The exponential increase of genomic and post-genomic technology should enable biologists to acquire data, and reverse genetic tools should improve our understanding of the metabolism of these lipids and demonstrate ways in which these processes can be improved.

Recently, we put one of the first varietal selection strategies into action in our laboratory. We used successive rounds of UV mutation and cell sorting to improve the TAG production of the Haptophyta *Isochrysis galbana* affinis Tahiti (a strain related to the *Isochrysis galbana* strain), a species that offers numerous advantages for lipid production. This approach, which does not create genetically modified organisms (GMOs), allowed us to obtain a strain that accumulates twice the amount of neutral lipids as the original without affecting the growth rate (Rouxel *et al.*, 2011).This strategy quickly improved the performance of an unsequenced selected species, so similar strategies will now be tried on other species and other valuable molecules. From now on, the acquisition of transcriptomic and proteomic data will be used to identify genes and molecular processes involved in the increase of lipid accumulation.

2.1.3. Algal lipids as feed and food

Apart from the high importance of TAG from algae, the identification of enzymes involved in the synthesis of PUFAs, such as the long-chain PUFAs AA, EPA and DHA, is of great interest due to the health benefits they offer. Production of PUFAs involves a consecutive series of desaturations and elongations of the fatty acyl chain. Until recently, numerous authors isolated and characterised lipid metabolism and enzymes using biochemical technologies. These studies are reported in a review by Guschina and Harwood (Guschina and Harwood, 2006). Over the last few years, authors have used genomic data to understand the biosynthetic pathways of PUFAs. Because of the putative role of PUFAs in the virulence of the fish pathogen *Pseudochattonella farcimen*, Dittami and co-authors analysed the expressed sequence tags (ESTs) of this species. Focusing their attention on PUFA metabolic pathways, they identified new specific desaturases related to this virulence (Dittami *et al.*, 2011). In the same way, the ESTs of *Myrmecia incisa*, a green coccoid freshwater microalga rich in AA, were analysed and a putative new elongase was identified (Yu *et al.*, 2011). Pan *et al.* (2011) sequenced the genome of the high PUFA-content species of Heterokonta *Nannochloropsis oceanica* using next-generation Illumina sequencing technologies. Sequence similarity–based investigation identified new elongase- and desaturase-encoding genes involved in the biosynthesis of long-chain PUFAs, which provide the genetic basis of its rich EPA content.

To date, major lipid primary metabolism has been well studied in model species, but regulation pathways, catabolism and secondary metabolic pathways of lipids are complex and rarely studied. Many metabolites of lipids have high biotechnological potential. The control of lipid metabolism, which is highly regulated, is of great interest as a means of increasing the lipid yields in culture. Furthermore, strategies using random mutations and strain selection have succeeded in increasing the lipid content of selected strains, but without a clear understanding of the mechanisms involved. This demonstrates that there are still many gaps in the knowledge that would help us to optimise lipid production from algae. Genomic and post-genomic studies on a variety of microalgae will provide the basis for identifying metabolic and signalling pathways.

2.2. Bioactive Natural Products

Commercial applications of microalgae include their use as natural sources of valuable macromolecules, such as carotenoids and phycocolloids. Due to the exceptionally high diversity of the different groups and the low level of exploration carried out so far, algae are a burgeoning reservoir of high added-value compounds. During the last decade, full genome analysis unveiled numerous new natural products in bacteria and fungi. Indeed, it appears that many of their genomes contain more gene clusters coding for the biosynthesis of natural products than natural products isolated from these same species (Winter *et al.*, 2011). Similar results have been observed in microalgae. For example, *in silico* analysis of the Heterokonta *Aureococcus anophagefferens* genome revealed the presence of five berberine bridge enzymes involved in the synthesis of toxic isoquinoline alkaloids, although this type of alkaloid had never been previously identified in this harmful species (Gobler *et al.*, 2011). Genomic exploration of microalgae appears to be a promising way to discover new bioactive products. To date, the analysis of available genomes has aided the identification of pathways to known compounds, thereby greatly facilitating regulatory and functional investigations. The search for enzymes involved in the biosynthesis of polyketides, isoprenoids, non-ribosomal peptides, oxylipins and alkaloids was conducted *in silico* by looking for homologous genes of land plants in sequenced genomes of microalgae (for review, see Sasso *et al.*, 2011). Although some pathways have been elucidated, there are still many gaps in our knowledge of the metabolism of the secondary metabolites. For example, isoprenoids comprise numerous bioactive molecules such as sterols, phytohormones, phytol, prenylated quinones and carotenoids, which have numerous qualities of interest for biotechnology. While the common first steps of the synthesis of isoprenoid compounds have been well described (Lohr *et al.*, 2012), very little is known about the biosynthesis of secondary isoprenoids except for the carotenoids. The genetic basis of the biosynthetic pathways of sterols and carotenoids in algae has been examined in detail by phylogenomics across several phyla of algae in order to gain insight into the evolution and diversity of photosynthetic eukaryotes (Frommolt *et al.*, 2008, Cui *et al.*, 2011, Desmond and Gribaldo, 2009) (see chapter II, III and IV of this volume). This has led to the identification of genes in organisms where pathways had not been identified before and demonstrated the steps by which more new enzymes could be discovered. The induction and regulation of astaxanthin and carotenoid biosynthesis in Chlorophyta such as *Sphaerella lacustris* or *D. salina* has received considerable attention owing to the increasing use of secondary carotenoids as a source of pigmentation for fish in aquaculture, and their potential as free-radical quenching drugs in cancer prevention. In aiming to identify the proteins involved in the regulation and biosynthesis of astaxanthin, comparative proteomics and transcriptomics were applied to the chlorophytes *Haematococcus pluvialis and H. lacustris* (*re-named Sphaerella lacustris*) under nitrogen starvation and irradiance stress (Kim*et al.*, 2006, Eom *et al.*, 2005, Tran *et al.*, 2009a), and the regulated genes identified. These genes putatively play a role in signal transduction from stress to the cellular defence system and activate the biosynthesis of astaxanthin. Complementary in-depth analysis should confirm the significance of these results. These genes include potential targets to increase the expression of astaxanthin.

Overall, it is clear that our understanding of secondary metabolism and its regulation is still rudimentary. Secondary metabolites include a large number of natural bioactive products, many of which are unknown. *In silico* genome analyses are a key to the identification of new metabolic and signalling pathways. Post-genomics can be applied to identify physiological conditions that lead the expression of new pathways, and so identify hitherto undetected metabolites.

2.3. Molecular Farming

The extraction of natural substances remains the main source of supply for a large number of pharmaceutical molecules. However, since it is possible to identify the genes responsible for building a protein molecule, they can be introduced into cultured cells, which then become cell factories, making millions of copies of the desired product. This strategy—the expression of molecules with high added value in recombinant cell systems—offers extraordinary opportunities for the development of a very promising biotechnology market (estimated to be worth up to several tens of billions of dollars, depending on the information source) (Schmidt 2004, Gasdaska *et al.* 2003). The production systems available are bacteria, yeasts and animal or plant cells, which are genetically modified to produce insulin, growth hormones, monoclonal antibodies and other therapeutic proteins. Each system has advantages and disadvantages relating to factors such as cost, production safety, ease of extraction, purification and complexity of producing the molecules. Some solutions, however, combine a number of benefits, putting them in a strong position for the future of this industry.

Microalgae have several advantages over other expression systems for the production of recombinant proteins, such as: (1) a high growth rate (they commonly double their biomass within 24 h), (2) easy cultivation at a low production cost (they only require water and nutrients), (3) the possibility of performing post-transcriptional and post-translational modification as in other eukaryotic expression systems and (4) photobioreactor culture methodologies that prevent transgenes from escaping into the environment, which is a potential risk when using land plants (Janssen *et al.*, 2003).

Several interesting reviews on transgenic tools describe the use of microalgae as a platform for production of recombinant proteins (Walker *et al.*, 2005, Hallmann *et al.*, 2007, Bozarth *et al.*, 2009, Potvin and Zhang, 2010). Here, we focus on recent progress and results on transgenic microalgae technology for the production of therapeutic recombinant proteins, and discuss the contribution of genomic studies for the optimisation of genetic manipulation in microalgae.

2.3.1. Transgenic microalgae as a platform for biopharmaceutical proteins

In this section, we provide a review of biopharmaceutical proteins expressed in microalgae systems according to their intracellular cell localisation (chloroplastic or nuclear). The interest in the N-glycosylation of pharmaceutical proteins will also be discussed.

Although no recombinant protein produced by transgenic algae is yet available on the market, some therapeutic proteins have been successfully produced using microalgae, mainly the Chlorophyta *Chlamydomonas reinhardtii,* for which suitable transgenic tools and genomic data are available (for all three genomes: nuclear, chloroplastic and mitochondrial). Mayfield‘s group has done considerable work on the chloroplastic expression of recombinant protein in *C. reinhardtii* (Rasala and Mayfield, 2011a). Indeed, the majority of microalgal therapeutic proteins have been produced by chloroplasts (Table 2). Transgenic protein can accumulate to much higher levels in the chloroplast than when expressed by the nuclear genome because plastids lack disadvantages such as gene-silencing mechanisms (Bock,

2007). Indeed, expression of foreign proteins remains very low for reasons that are not yet fully understood (Potvin and Zhang, 2010). The chloroplast of *C. reinhardtii* has been used to produce a range of recombinant proteins, including reporters such as glucuronidase (GUS), luciferase (LUC), green fluorescent protein (GFP), industrial enzymes, vaccines and therapeutic enzymes (Rasala and Mayfield, 2011a).

The first therapeutic protein expressed by transgenic microalgae was produced at Mayfield's laboratory using chloroplast transformation in the green microalga *C. reinhardtii*. In the study of Mayfield *et al.* (2003), the entire IgA (Imunnoglobulin A) heavy chain protein (HSV8-lcs) fused to the variable region of the light chain was expressed and accumulated as a soluble protein able to bind to the herpes virus protein. Nevertheless, the expression yield was too low (detectable only) for commercial use, even though several regulation sequences (promoters) were tested. Regulation sequence aspects will be examined in the next section. This previous study was completed by the chloroplastic expression of a single chain fragment variable antibody (HSV8-scFv) that accumulated to 0.25% of total soluble protein (TSP) (Mayfield and Franklin, 2005). In their next study, the same team successfully increased the accumulation of a bioactive mammalian protein, bovine mammary-associated serum amyloid A (M-SAA), to 5% of TSP with by chloroplasts, using different promoter sequences and an interesting strategy consisting of replacing an endogenous gene by the expression cassette (Manuell *et al.*, 2007). Recently, a full-length human monoclonal antibody was expressed in the chloroplast of *C. reinhardtii*, proving that this eukaryotic green alga is capable of synthesising and assembling a full-length antibody in transgenic chloroplasts (Tran *et al.*, 2009b). More recently, a study was conducted to examine the versatility of algal chloroplasts for the expression of seven different therapeutic proteins: human erythropoietin (EPO), the 10th and 14th human fibronectin type III domains (14FN3 and 10FN3), human interferon β1, the human vascular endothelial growth factor (VEGF) isoform, the high mobility group protein (HMGB1) and the human proinsulin. Of the seven proteins tested, four were successfully expressed in transgenic chloroplasts to above 2% of TSP (Rasala *et al.*, 2010). However, no detectable expression was shown for EPO or interferon β1. Like Mayfield's group, other research groups have successfully shown that the chloroplast of *C. reinhardtii* is a perfect platform to produce recombinant proteins at an economically viable cost (Zhang *et al.*, 2006, Yang *et al.*, 2006, Wang *et al.*, 2008). In addition to therapeutic proteins, some vaccines have been successfully produced in algal chloroplasts. Indeed, a fusion protein between the foot and mouth disease virus VP1 and the cholera toxin B subunit (as mucosal adjuvant) was reported to accumulate to 3% of TSP in transgenic algal chloroplasts (Sun *et al.*, 2003). This fusion protein retained both specific ganglioside-binding affinity and antigenic function. A classical swine fever virus (CSFV) E2 recombinant protein was also successfully expressed in chloroplast to around 2% of TSP and observed to have immunological activity (He *et al.*, 2007). Surzycki *et al.* (2009) reported a strong expression of the white spot syndrome VP28 protein by chloroplasts to around 10.5% of TSP. Moreover, in this study, the authors attempted to determine factors affecting the level of recombinant protein expression, which will be covered in the next section. Recently, Dreesen *et al.* (2010) reported the oral immunisation of mice by transgenic algae expressing (to 0.7% of TSP) the *Staphylococcus aureus* fibronectin-binding domain D2 fused to the cholera toxin B subunit.

It is important to reiterate that all these studies were carried out using transgenic chloroplasts of the green algae *C. reinhardtii*. To our knowledge, there are no reports of biopharmaceutical protein expression by transgenic chloroplasts in other microalgae.

Although it is estimated that most of the therapeutic human antibodies used in therapy do not require glycosylation, other therapeutic proteins require the correct glycosylation pattern to function properly (Dove, 2002). Nevertheless, nuclear expression of therapeutic proteins remains limited because of some problems in reducing yield expression (Potvin and Zhang,

2010). Transgenic microalgal technologies are still in their infancy and the therapeutic proteins expressed by the nuclear genome are still rare in microalgae.

Initial work has been done by Hawkins and Nakamura (1999) to produce human growth hormone in the extracellular medium of *Chlorella sorokiniana* and *C. vulgaris C-27*. In a subsequent study, growth hormone of sole was produced and expressed as a stable product in *C. ellipsoidea* (since renamed *Chloroidium ellipsoideum*). Soles fed with these transgenic microalgae increased in size by 25% (Kim *et al.*, 2002). Another research team has shown the efficient expression and biological activity of rabbit neutrophile peptide-1 in *C. ellipsoideum* cells (Chen *et al.*, 2001).

Recently, Dauvillée *et al.* (2010) expressed a nuclear protein corresponding to the *Plasmodium* antigens that fuse to granule-bound starch synthase (GBSS), a protein involved in the starch matrix of plants and algae. The C-terminal domains from apical major antigen (AMA1) or major surface protein (MSP1) fused to GBSS were both efficiently expressed in nuclear cells and targeted starch particles in the chloroplasts, taking advantage of the transit peptide on the GBSS protein. Although expressed in the nucleus, these fusion proteins directly targeted starch granules, avoiding post-translation modification such as N-glycosylation. Immunogenicity tests for both fusion proteins were successfully performed in mice (Dauvillee *et al.*, 2010).

More recently, diatoms have also been used as cell factories to produce recombinant proteins. Diatoms are an algal group of great ecological importance. Their contribution to global CO_2 fixation represents around 40% of marine carbon production. Diatoms like *Phaeodactylum tricornutum* represent an interesting subject for a variety of biotechnological applications, and this species has become a model organism for the diatoms (Bowler *et al.*, 2008, Siaut *et al.*, 2007, Hempel *et al.*, 2011a and b). Indeed, its whole genome has been sequenced and molecular tools for functional genomics are available (Maheswari *et al.*, 2009, Siaut *et al.*, 2011). To date, diatoms have not been employed for expression of any biopharmaceutical proteins, but a research team has recently reported the first stable expression of a full-length human antibody and the respective antigen in *P. tricornutum* (Hempel *et al.*, 2011b). In this study, the antibody and respective antigen were both expressed and accumulated within the endoplasmic reticulum (ER) using the ER retention signal. Interestingly, while the same expression vector and molecular tools were used for the expression of both these recombinant proteins, different expression levels were observed for the antibody (7.8% of TSP) and antigen (0.7% of TSP). This result confirms that not all foreign proteins are equally expressed (Potvin and Zhang, 2010).

At our laboratory, we became interested by the potential of microalgae as a means to produce therapeutic proteins (Cadoret *et al.*, 2008). This interest led to the creation of a private company by our laboratory: Algenics. Algenics is the first privately owned European biotechnology company focusing on innovative uses of microalgae to produce recombinant biotherapeutics. Using microalgae as a platform for recombinant proteins, our laboratory filed a patent on the production of glycosylated proteins in microalgae (Cadoret *et al.*, 2009). Recently, as proof of the concept, we successfully produced another therapeutic protein, murine erythropoietin (mEPO), in the diatom *P. tricornutum* (Unpublished work, Carlier, A., Bardor, M., Lerouge, P., Delavault, P., Saint-Jean, B., Gerard, A., and Cadoret, J.P.). The data show that recombinant mEPO accumulates to around of 0.05% of TSP (or 300 µg/L). This recombinant EPO is glycosylated and able to bind the human EPO receptor *in vitro* with the same affinity. These results, combined with Hempel's data, confirm the high potential of diatoms to express biopharmaceutical proteins.

This last result corroborates the expression specificity of some foreign proteins according to cell localisation and/or algal taxon. Indeed, no detection of recombinant EPO has been reported in Chlorophyta *C. reinhardtii* transgenic chloroplasts (Rasala *et al.*, 2010). In

contrast, Eichler-Stahlberg *et al.* (2009) observed a minor accumulation of recombinant EPO up to around 100 µg/L in nuclear expression by *C. reinhardtii* cells. Thus, EPO protein accumulates differently and at different expression levels according to cell localisation or species.

To conclude, many efforts have been made to produce biopharmaceutical proteins at a level sufficient to be economically viable, but extensive research to optimise microalgae as cell factories still needs to be done. Recent success in microalgal transgenesis and input from genomic data will allow a response to the growing demand for biopharmaceutical molecules. However, microalgae can also provide compounds other than pharmaceutical proteins. Indeed, an interesting study has recently been reported that used microalgae to produce industrial products such as bioplastic: Hempel and co-workers (2011) expressed three prokaryotic enzyme genes in the diatom *P. tricornutum* to produce poly-3-hydroxybutyrate (PHB). These genes (i.e., a ketolase, an acetoacetyl-CoA reductase and a PHB synthase) are able to synthesise PHB from acetyl-CoA in diatom cells up to a level of 10.6% of algal dry weight.

Of the post-translational modifications encountered in eukaryotic proteins, N-glycosylation is the most prevalent of those that appear essential for biological functions (biological activity, short half-life). Moreover, glycosylation is of particular interest for biopharmaceutical proteins, since more than 70% of biopharmaceuticals are glycoproteins. Glycosylation capability is an advantage for any system used to produce biopharmaceuticals. This pathway is currently well understood among the different production systems available today, such as cultured mammalian, yeast and plant cells. Plants have N-glycosylation capability similar to mammalian cells. However, N-glycosylation patterns processed in plant cells differ from those of humans and other mammals. In plants, N-linked glycans contain β(1,2)-xylose and α(1,3)-fucose instead of the α(1,6)-fucose found in mammals. These plant-specific glycans are considered to be potentially antigenic and/or allergenic epitopes (Bakker *et al.*, 2001). Several strategies have been studied to remove the antigenic potential of plant-specific glycans. One simple approach is aglycosylation to obtain recombinant protein with no N-glycosylation by mutating the N-glycosylation sites of expressed genes (Conley *et al.*, 2009). This approach is effective if the biological activity is not affected by aglycosylation.

Another approach consists of retaining the foreign protein in the ER using KDEL/HDEL retention signals to avoid plant-specific glycan residues such as β-(1,2)-xylose and α-(1,3)-fucose (Ko *et al.*, 2003, Gomord *et al.*, 2004, Petruccelli *et al.*, 2006). Indeed, glycosylation processing in the ER is conserved between the plant and animal kingdoms and restricted to high mannose-type N-glycans, whereas the further glycosylation process in the Golgi apparatus, where additional glycans are added for glycan maturation, is highly diverse. Another approach to eliminating plant-specific glycan residues is to knock out the gene expression of glycosyltransferases involving β-(1,2)-xylosylation and α-(1,3)-fucosylation (Gomord *et al.* 2004). However, in addition to eliminating plant-specific sugar, humanisation of N-glycosylation is also essential for the production of authentic glycosylated recombinant proteins in plants. The strategy to humanise plant N-glycans consists of expressing mammalian glycosyltransferases, which would complete N-glycan maturation, in plants (Bakker *et al.*, 2001).

So far, little information regarding the glycosylation of microalgae is available and it is interesting, both from a purely scientific point of view and for biotechnological applications, to determine their capacity for this process. Our laboratory published the first *in silico* N-glycosylation study in microalgae. Using the genomic data available for *P. tricornutum*, we identified specific genes coding enzymes involved in the N-glycosylation pathway in diatoms (Baiet *et al.*, 2011). Moreover, by structural analyses of N-linked glycans, this study also demonstrated that *P. tricornutum* proteins carry mainly high mannose-type N-glycans. Interestingly, other recent biochemical studies have reported the existence of special

glycosyltransferase and glycosylation pathways, unique to the red alga *Porphyridium sp.* (Levy-Ontman *et al.*, 2011).

The emergence of genomic data in microalgae will provide the opportunity to perform comparative genomic studies and to dissect biosynthetic pathways such as N-glycosylation. Recently, we initiated new studies to evaluate the N-glycosylation pathway of microalgae representing different phyla: green and red microalgae, glaucophytes, alveolates, stramenopile and haptophytes. This study will help us to determine how this specific process evolved within the eukaryotes. Moreover, demonstrating that microalgae are a suitable alternative system for the production of biopharmaceuticals requires the demonstration of their N-glycosylation capability.

2.3.2. Genomic strategies for optimising recombinant protein expression

In this section, we report three strategies commonly used to optimise recombinant protein accumulation in microalgae.

2.3.2.1. Translation optimisation by codon usage bias

Specific variations in codon usage are often cited as one of the major factors impacting protein expression level. The presence of rare codons that are correlated with low levels of their endogenous tRNA species in the cell can reduce the translation rate of target mRNA. The classical strategy to bypass this problem is to redesign genes to increase their expression level. For this, two approaches have been attempted, both of which require choosing from a vast number of possible DNA sequences. The first approach consists of assigning the most abundant codon of the host of a given amino acid in the target sequence. The second uses translation tables based on the frequency distribution of the codons in an entire genome or for a range of highly expressed genes. This approach was successfully used in *Chlamydomonas reinhardtii* to improve the expression level of foreign proteins such as GFP in the nucleus (a 5-fold increase) (Fuhrmann *et al.*, 1999) and chloroplasts (increased up to 80-fold) (Franklin *et al.*, 2002). Similar studies using a codon-optimised human antibody gene or luciferase reporter gene confirmed that codon bias plays an important role in protein accumulation in chloroplasts of *C. reinhardtii* (Mayfield *et al.*, 2003, Mayfield and Schultz, 2004).

The nuclear and chloroplastic genome of *C. reinhardtii* may exhibit different codon bias and thus, adjustment of codons in foreign gene sequences is necessary to obtain a high rate of protein production. To overcome this issue, the codon adaptation index (CAI) is used as a quantitative tool to predict the expression level of transgenes based on their codon usage. Several molecular software programs are available to determine and optimise codon usage. A list of these programs is given in Villalobos *et al.* (2006).

This approach, which consists of optimising the codon usage of transgenes, was successfully used in the green alga *C. reinhardtii* and diatom *Phaeodactylum tricornutum*. Specific codon usage is a field that will benefit from the contribution of future microalgal genomic and transcriptomic sequences.

2.3.2.2. Identification of promoter sequences

Genome data is also necessary to identify functional sequences such as promoter, 5‘ and 3‘-untranslated region (UTR) sequences that regulate the gene expression rate. These sequences are specific for each gene and microalgal strain. Due to the presence of plastid and nuclear genomes in microalgae, there are different types of promoter sequences according to cell localisation. Plastid transgenes are expressed under the control of an

endogenous promoter and 5‘ and 3‘-UTR. Overall, promoter sequence control transcription and 5‘-UTR mediate mRNA stability, and translation initiation and 3‘-UTR regulate stability and act in the termination of transcription. The same sequences were found for nuclear promoters, but other regulated sequences such as intron sequences are also involved in the regulation of nuclear gene expression. Previous studies identified sequences within the 5‘-UTR that were involved in RNA stability and used as a means to increase recombinant protein synthesis. For a comprehensive review of chloroplast translation regulation, see Marin-Navarro *et al.* (2007).

Concerning chloroplastic transformation in microalgae, the green alga *C. reinhardtii* has been intensively studied. Among chloroplastic promoters for the expression of foreign proteins (Table 3), the endogenous *atpA*, *psbD*, *rbcL* and *psbA* promoters are generally used (Hallmann *et al.*, 2007, Specht *et al.*, 2010). An excellent study performed by Barnes *et al.* (2005) reported the effect of various promoters and UTRs on recombinant proteins in the chloroplast of *C. reinhardtii*. Using different combinations of chimeric proteins corresponding to the promoters and 5‘-UTRs of chloroplast genes, *atpA*, *rbcL*, *psbA*, *psbD* and *16S* rRNA, fused to the GFP reporter and followed by 3‘-UTR of either gene, they observed different protein accumulation levels. Moreover, they showed that mRNA accumulation is, in general, proportional to protein accumulation. Also, according to chimeric construction, they observed that the 5‘-UTR sequence had a significant impact on recombinant protein production while 3‘-UTR had little effect. The highest level of reporter protein was found using the *atpA* or *psbD* promoter and 5‘-UTR, while a minor protein accumulation level was observed under control of *rbcL* and *psbA* and no expression was seen using the *16S* rRNA promoter and 5‘-UTR (Barnes *et al.*, 2005).

Interestingly, the *psbA* promoter fused with its 5‘-UTR was actually the most used (Manuell *et al.*, 2007, Surzycki *et al.*, 2009). Recently, Rasala *et al.* (2011b) reported a high recombinant protein expression level with the *psbA* promoter in comparison to the levels reached with the *atpA* promoter. It remains unclear why certain regulatory elements induce a high expression level in some genes but not in others (Marin-Navarro *et al.*, 2007). The *psbA* promoter and 5‘UTR are the most studied, but essentially require a *psbA*-deficient genetic background for high foreign protein accumulation (Rasala and Mayfield, 2011a).

Other exogenous promoter sequences have been used in *C. reinhardtii* chloroplasts. Kato *et al.* (2007) showed the functionality of the inducible system of the *lac* operon of *Escherichia coli* in *C. reinhardtii* chloroplasts. At the same time, a riboswitch was reported to act as a translational regulatory factor in *C. reinhardtii* (Croft *et al.*, 2007). Finally, all the data suggest that the translation mechanism and mRNA accumulation are primarily controlled by the promoter and 5‘-UTR, and that the choice of these sequences is a critical factor to consider for each protein of interest in order to achieve high yields of recombinant proteins. On the other hand, to our knowledge, no chloroplast transformation has been reported for microalgae other than *C. reinhardtii* and the unicellular flagellate protist *Euglena gracilis*, leaving the way open for research to study this mechanism of expression in other microalgae.

Concerning nuclear promoters, several studies have been performed in different taxa of microalgae using endogenous, exogenous and synthetic promoters (Table 3). The most widely used constitutive promoter in the chlorophyte group is *RbcS* (RuBisCO small subunit). Interestingly, some endogenous promoters of *C. reinhardtii* can be used in other Chlorophyta algae. Indeed, the *C. reinhardtii RbcS* promoter has been successfully used in the green alga *Dunaliella salina* (Sun *et al.*, 2005), *Chloroidium ellipsoideum* formerly *Chlorella ellipsoidea* (Kim *et al.*, 2002), the Heterokonta *Nannochloropsis oculata* (Chen *et al.*, 2008, Li *et al.*, 2008a) and the Chlorarachniophyta *Lotharella amoebiformis* (Hirakawa *et al.*, 2008). A chimeric promoter using heat shock protein A (*HSP70A*) fused to *psaD* was also successfully used in *C. reinhardtii* (Fischer and Rochaix, 2001, Schroda *et al.*, 2000), and more recently in

the multicellular alga *Gonium pectorale* (Lerche and Hallmann, 2009). The same strategy, using *HSP70* fused to *CAb* (chlorophyll-binding protein), was reported in the charophyte *Closterium peracerosum* (Abe *et al.*, 2011). Usual plant promoters, such as the cauliflower mosaic virus *35S* with *Ubiquitin-Ω*, have been also tested in some microalgae (Kumar *et al.*, 2004, Jarvis and Brown, 1991, Chen *et al.*, 2001, Wang *et al.*, 2007), and recently the *35S* promoter demonstrated efficiency in the diatom *P. tricornutum* (Sakaue *et al.*, 2008), Chlorophytes *Haematococcus sp. (Kathiresan et al., 2009)* and *Dunaliella bardawil (Anila et al., 2011)* and the Heterokonta *Nannochloropsis sp. (Cha et al., 2011a)*. Moreover, inducible promoters have been chosen for some algae. Indeed, the gene expression under the control of the nitrate reductase promoter is switched off when cells are grown in the presence of ammonium, and becomes switched on when cells are transferred to a medium containing nitrate. This approach was reported for the diatom *Cylindrotheca fusiformis* (Poulsen and Kroger, 2005) and recently in *Chlorella vulgaris* (strain not reported) using the *NR* cassette (promoter and 5‘ and 3‘-UTR of nitrate reductase) of the diatom *Phaeodactylum tricornutum (Niu et al., 2011)*. A similar strategy was applied in *P. tricornutum* using the endogenous *NR* cassette (Hempel *et al.*, 2011a, Hempel *et al.*, 2011b) and exogenous *NR* cassette from the diatom *Cylindrotheca fusiformis* (Miyagawa *et al.*, 2009). Miyagawa-Yamaguchi *et al.* (2011) reported the same approach in another diatom, *Chaetoceros sp.,* using the *NR* cassette of the diatom *Thalassiosira pseudonana*. The study performed by Niu *et al.* (2011) is particularly interesting as the diatom *NR* cassette was shown to be functional in green algae, suggesting that this type of inducible promoter could be universally employed across diverse species of algae.

In contrast to plastid promoters, several studies have been performed on nuclear promoters in diatoms. So far, unlike in green algae, the RuBisCO small subunit gene of diatoms is encoded by the chloroplast genome, and its promoter is not adapted for nuclear transformation. Other promoters were identified from genomic and transcriptomic data from diatoms. Early studies reported protein expression using the acetyCoA carboxylase (*Acc1*) promoter in diatoms *Cyclotella cryptica* and *Navicula saprophila* (Dunahay *et al.*, 1995). Members of the family of light-inducible fucoxanthin chlorophyll (*Fcp*) promoters have also been used to produce foreign protein in diatoms *P. tricornutum* (Apt *et al.*, 1996, Falciatore *et al.*, 1999, Zaslavskaia and Lippmeier, 2000) and *Thalassiosira sp.* (Falciatore *et al.*, 1999, Poulsen *et al.*, 2006). In contrast to the *NR* promoter, the *Fcp* promoter appears to be more specific to the host as, for example, the *Fcp* promoter of *P. tricornutum* is not functional in *Cylindrotheca fusiformis* (Poulsen and Kroger, 2005).

Some use of virus promoters other than *35S* has also been reported in algae, such as mammalian cytomegalovirus *CMV* in *P. tricornutum* (Sakaue *et al.*, 2008), and recently in the Chlorophyta *Platymonas subcordiformis* (Cui *et al.*, 2010), as well as the Rous sarcoma virus in the diatom *P. tricornutum* (Sakaue *et al.*, 2008).

Surprisingly, the use of microalgal virus sequences in algal expression constructs to enhance gene expression has still not been explored. To date, several algal viruses have been identified and their full genomes sequenced in some microalgal taxa, specifically chlorophytes, dinoflagellates, diatoms and haptophytes (for reports and reviews on this topic see, Nagasaki, 2008; Nissimov *et al.*, 2011; Schroeder, Oke, Malin, & Wilson, 2002; Van Etten & Dunigan, 2012; Wilson, Van Etten, & Allen 2009). To date, algal viruses represent a largely unexplored source of genetic elements for engineering algae and land plants. This approach has previously been used in both monocotyledonous and dicotyledonous land plants, as well as in bacteria (Mitra *et al.*, 1994). Another study reported the functionality of a translation enhancer element from the *Chlorella* virus in the plant *Arabidopsis thaliana* (Nguyen *et al.*, 2009).

Another strategy to increase the yield of recombinant proteins consists of adding intronic sequences to the expression vector to act as an endogenous enhancer. Indeed, while

regulation of gene expression occurs at the post-transcriptional level in the plastid, it appears that most regulation occurs both at the transcriptional and translational levels in the nucleus (Marin-Navarro *et al.*, 2007). The introns are non-encoding sequences but can affect the expression of genes by alternative splicing or through the regulation of transcription. In *C. reinhardtii*, Lumbreras *et al.* (1998) reported that the insertion of endogenous introns from heterologous genes increases the expression level. Recently, a similar approach has been used to increase the expression level of the *Renilla*-luciferase gene reporter in *C. reinhardtii* (Eichler-Stahlberg *et al.*, 2009). However, the way in which the introns affect the expression level is still unclear.

2.3.2.3. The challenges of transgene silencing and proteolysis

Transgene silencing is another problem for high-yield recombinant protein expression in plants and algae, but different strategies exist to overcome this obstacle. Indeed, gene silencing can function as a protective system against pathogens or viruses (Specht *et al.*, 2010). Plant virus–encoded suppressors of RNA silencing are useful tools for counteracting silencing, but their wide application in transgenic plants is limited because their expression often causes harmful developmental effects. To our knowledge, this approach has not yet been attempted in microalgae. Recently, another strategy to prevent transgene silencing was reported in *Chlamydomonas reinhardtii* using a process of UV mutation and selection by antibiotic resistance on a selective medium (Neupert *et al.*, 2009).

To date, most efforts to improve recombinant protein accumulation in plants or algae have focused on increasing protein expression. Moreover, proteolysis is also one of the factors that can affect the yield of recombinant protein accumulated and also lead to difficulties in purification due to degraded forms or non-functional protein (Doran, 2006, Surzycki *et al.*, 2009). However, proteolytic enzymes are essential for the degradation of misfolding or incorrectly processed endogenous proteins. Some strategies have been attempted to minimise foreign protein degradation in plants and microalgae, like producing recombinant proteins in other cell compartments that have an environment with less proteolytic activity. Indeed, for nuclear-expressed protein, the targeting of the ER using the HDEL or KDEL retention signal prevents the degradation of the foreign protein. A similar approach has been successfully used by our laboratory to express recombinant EPO in diatoms. Another approach used in plants consists of concomitantly producing protease inhibitor to neutralise endogenous protease (Doran, 2006).

3. Future Outlook

In this chapter, we tried to provide an overview of the principal applications of microalgae and show how genomics and post-genomics can improve their uses in biotechnology. Here, we mainly focused on some of the most popular applications. Without wishing to suggest that they are less important, we chose not to make a detailed review of other applications such as environmental biomarkers, silica synthesis from diatoms or hydrogen and methane production for energy. In any case, the future of microalgal biotechnology will depend on several steps, including domestication and a search for new intrinsic species characteristics, steps for which the contributions of ‗omics' technologies will be invaluable.

3.1. Domestication

A strategy comparable to that used for the domestication of crops is now making its way into the world of microalgae. This is a matter of selecting favourable mutations and finding markers that will help select the desired traits. In agriculture, the cross-breeding of species and selection of strains was conducted empirically for thousands of years before Mendel's

laws provided a scientific basis for species improvement. For sexual reproduction, considerable work remains to be done in microalgae. Knowledge of reproductive strategies is of major importance for maintaining strains, cultivating them on a long term-basis in continuous culture or envisaging selection strategies. Some algal groups have become the subject of increased attention concerning their reproduction strategies and sexual behaviour, including the diatoms (Chepurnov *et al.*, 2011). In the Coccolithophore *Emiliania huxleyi,* different morphotypes associated with different forms of ploidy have been observed and studied by transcriptomic analysis. This work revealed mechanisms involved in functional differentiation without proving that sexual reproduction occurs (Von Dassow, 2009). To date, there is too little knowledge to envisage the improvement of strains by cross-breeding and selection through sexual reproduction, so future studies in this direction will be of great interest.

Mutation followed by selection for favourable phenotypes has been used for crop plants, and some promising strategies are now beginning to emerge for algae. This domestication route calls for induced mutations and subsequent selection. Bonente *et al.* (2011) identified the major relevant points for the selection of H_2-producing *Chlamydomonas sp.*, namely a reduction of photosynthetic antenna size, an alteration of photosystem II to manipulate the oxygen concentration and a maximised electron flow towards hydrogenase. This strategy was thought to enhance carotenoid levels. Early studies involved *Dunaliella salina* and the selection of beta-carotene-rich strains (Shaish *et al.*, 1991). These were followed by a search for hyperproductive variants sorted by flow cytometry (Mendoza *et al.*, 2008), and recently there have been improvements in lutein production in the microalga *Chlorella sorokiniana* (Cordero *et al.*, 2011). This strategy was implemented to enhance overall lipid contents or EPA and DHA, in particular with the Haptophytes *Isochrysis galbana* (Molina Grima *et al.*, 1995) and *Pavlova lutheri* (Meireles *et al.*, 2003), the Heterokonta *Nannochloropsis oculata* (Chaturvedi and Fujita, 2006) or the chlorophyte *Dunaliella salina* (Mendoza *et al.*, 2008). In this context, the availability of a reliable marker, such as Nile Red or BODIPY for staining lipid bodies, greatly helps in the selection process. In the Haptophyte *Isochrysis galbana* affinis Tahiti*,* this strategy allowed our laboratory to select an improved strain that could stably produce twice the amount of TAG compared to its wild-type counterpart (Rouxel *et al.*, 2011).

We have reported that the improvement of microalgae for biotechnology uses will come through the domestication of strains, and this approach has already been initiated. Far from being in conflict, the different approaches (‗natural' *versus* ‗GMO') are complementary. Synthetic biology, synthetic genomics and genome engineering are disruptive technologies. Indeed, the development of ‗GMO' strategies is very promising for applications with very high added value such as the production of drugs or antibodies. However, taking into account the environmental risks arising with such transgenic species and societal pressure against their use, their culture will have to be performed in confined and controlled conditions. Their use for energy and food (large outdoor cultures) therefore seems somewhat inappropriate. The completely opposite point of view is that, given the immeasurable biodiversity of algae, the ideal alga for a given application is probably available in nature. Although this perspective is somewhat optimistic, the exploration of biodiversity was the source of the algae presently in use and will doubtless continue to be in the future. Screening this diversity will enable us to identify new, more efficient strains with new features, some of which may have uses that have not yet been imagined. This does not preclude subsequent domestication to improve these species for use in biotechnology.

3.2. Working towards a new algal metabolism, enzymes and compounds

As seen earlier in this chapter, the implementation of genomic and post-genomic approaches is now largely underway in the world of microalgae. In parallel, ecological approaches in metagenomics have only been seen very recently. These will hopefully lead to the

identification of a large number of presently unknown microalgae and, consequently, to new gene networks, enzymes and metabolic pathways. Due to the wide variety of microalgae and difficulties in cultivating certain of them, many metabolic pathways have remained out of our reach until the present. Metagenomics aims to analyse all of the genomic data in a given ecosystem without a strain isolation and cultivation step. This allows access to unknown mechanisms of potential biotechnological interest. In this situation, the ‗sequencing campaigns' on research cruises (Karsenti *et al.*, 2011) will offer new and valuable insight in the field of microalgal genomics. Chapter XI of this volume provides a review on the power and challenges of metagenomics for microbial algae. Conversely, metagenomics will be greatly aided by new methods like single cell genome analysis (Ebenezer *et al.*, 2011), which can improve methods of isolation and cultivation of new algae.

Among the wide variety of metabolic pathways conceivable across the diversity of microalgae, particular attention should be paid to metabolism from extremes environments. Like bacteria, although to a lesser extent, some microalgal species live under severe physico-chemical pressures such as high salinity, extreme temperatures from below zero to over 50°C, alkaline or acidic waters or very high irradiance. Additionally, some strains have been isolated downstream from industrial sites such as acid mine drainage, or in waters rich in contaminants such as metals (for review see Das *et al.*, 2009). Extremophiles offer numerous advantages, including: (1) the absence of contaminants in open door cultures subjected to physicochemical pressure; (2) their potential adaptation to industrial environments such as presence of toxins, radioactive elements or extreme pH, and consequently their potential use for the biocatalysis of effluents; and (3) their ability to produce enzymes with biotechnological applications. Proteomics have been carried out to highlight the adaptation mechanisms in the halophilic species *Dunaliella salina* (Liska, 2004, Katz *et al.*, 2007), for which the genome sequence will soon be available. Transcriptomics and comparative genomics have dealt with the very high biochemical versatility of thermo-acidophilic *Galdieria sulphuraria* {Weber *et al.*, 2004, Barbier *et al.*, 2005). Psychrophilic species have been identified, such as *Fragilariopsis cylindrus*, *Xanthonema sp.*, *Koliella antarctica* and *Chlamydomonas sp*. ICE-L. However, the culture of psychrophiles is far from being technically mastered, making post-genomic approaches difficult. In such cases, metagenomics would be an appropriate solution. Overall, efforts are still needed to isolate and cultivate extremophiles, and genomics will provide a source of new applications.

3.3. Algal pathogens: Looking towards the future

Like land plants, phytoplankton are susceptible to diseases and parasitism, which impact their population dynamics and use in commercial industry. Interactions between bacteria and microalgae in the environment and in cultures are numerous, and bacteria can have beneficial or negative effects on the growth of microalgae. For a review, see Fukami *et al.* (1997). Numerous algicidal bacteria have been identified in the ocean, and their influence on algal bloom dynamics has been demonstrated (Mayali and Azam, 2004). Although they have not yet been associated with real economic losses in cultures, the experience in production of other marine species suggests that diseases will likely appear in parallel with the expansion of the industry. Viruses are extremely abundant in seawater and are believed to be significant pathogens to photosynthetic protists. They are known to affect the regulation of eukaryotic phytoplankton population densities. Since the discovery of the very high abundance of viruses in the marine environment, researchers have highlighted their possible ecological significance. To date, more than 40 viruses infecting marine microalgae have been isolated and characterised to different extents (Nagasaki, 2008). Several studies have focused on the relationship between eukaryotic microalgae and their viruses (for review see Nagasaki, 2008). Without going into great detail, it is interesting to note that most algae can use various strategies of resistance to their viruses, but the mechanisms involved are not yet clearly understood (Morin, 2008, Thomas *et al.*, 2011). The next chapter of this volume, ‗Genomics of Algal Host-Virus Interactions', reviews algal host-virus interactions. Finally, it

will be interesting to compare the emergence of pathogens such as plant viruses during this new agricultural revolution and increase in demand for algal culture by industry. Microalgal cultivation remains a niche market in almost all countries, but the increasing interest in sustainable biofuel sources has triggered a high investment in culture facilities all over the world. Consequently, intensive algal aquaculture using open pond systems for the mass culture of microalgae might favour disease outbreaks. Gachon *et al.* (2010) suggest that development towards intensive macroalgal production correlates with more damaging disease outbreaks. The best example is the case of edible red macroalgae *Porphyra sp.,* which represent a very valuable industry in Asia. The market is estimated to be worth about $1.5 billion worldwide, and has reported losses of about 10% of annual production due to oomycete pathogens, although these outbreaks can even lead to losses of 25 to 40% in some cases. To overcome this problem, sea farmers can use chemical treatments, but their use in large doses could have a real impact on ecosystems, as well as on production costs. A better understanding of the relationships between pathogens and microalgae would be useful to identify causes and possible solutions to overcome disease epidemics. Prophylactic and microbial flora management of cultures will probably be a key to the durability of production in the coming years. Varietal selection of microalgal strains for resistance to a large range of pathogens is one strategy that could increase the resistance of microalgae cultures. In any case, in the context of intensive microalgal production, we must anticipate future epidemics that will affect algal culture yields.

4. Conclusion

Due to the huge amount of diversity among microalgae, their applications have a very bright future. As seen in this chapter, the uses of microalgae are numerous and there is work for many research teams in many fields of specialisation.

Genomics and post-genomics have led to new areas of research and development and to the modernisation of our view of biology. The increase in sequencing capacities will soon face a data tsunami, a fantastic amount of data that will soon be generated by fast, low-cost sequencing methods. However, storage, calculation power, annotations and access to this information now pose a limit to its optimal exploration. Data mining and conversion of data into biological knowledge will be an important challenge in coming years. The confirmation of all the *in silico* analyses and discoveries will require a return to experimental testing, and the association of molecular data with biological functions will become vital work in the future (Lopez *et al.*, 2011).

The culture of microalgae for biomass production dates back to the 1940s, when it started in the USA before spreading to Europe, Japan and Israel (Grobbelaar, 2010). Since then, work has continued all over the world at different speeds, with irregular publication rates. Some of the early work still forms the basis for the today's revival of the microalgal trend (Sheehan *et al.*, 1998). The dramatic increase in the world population, concerns about the ecological equilibrium, pollution, the world energy demand and failing supplies of oil and coal have all led to a more ‗bio'-orientated attitude, meaning a general increase in the attention paid to ‗renewable' resources. From a global perspective, in the context of a demographic crisis, the major issues in the coming years will be to provide everyone with access to water, food, education and healthcare. In a world of limited resources (energy, clean water, arable land) and increasing anthropogenic pressure on the environment, the development of biotechnological processes to provide renewable energy, new molecules and molecular farming and cleaner industrial processes is one of the key challenges. Marine microalgae possess assets that make them suitable for some of these applications. While land plants are the subject of numerous programs aiming to use vegetal organisms for the so-called ‗green chemistry', the algae, particularly microalgae, are expected to participate in this race in a

complementary way. Although far less studied than their terrestrial counterparts, microalgae offer an, as yet, untapped diversity and manipulability that explain the enthusiasm and investment from around the world. Phytoplankton research is being revisited and enriched by modern techniques like molecular biology and biocomputing, and the ‗omics' technologies offer new insights into their biology. The young generation of students will have the chance, at the strictly scientific level, to be present when the majority of the genomes are still to be sequenced, the transcriptome is unknown and even the reproductive strategies or size of the genomes are undefined. The earth still hides a tremendous amount of original biology, including much that concerns microalgae. Their discovery, study, analysis and use will serve applications in all imaginable fields.

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Tables

Tables 1 : Ongoing microalgae transcriptomic projects

Phylum	Species	Strain	Status
Bacillariophyta	*Asterionellopsis glacialis*	1712	Assembly & Annotation
Bacillariophyta	*Chaetoceros sp.*		Assembly & Annotation
Bacillariophyta	*Corethron hystrix*	308	Assembly & Annotation
Bacillariophyta	*Cylindrotheca closterium*		Assembly & Annotation
Bacillariophyta	*Grammatophora oceanica*	410	Assembly & Annotation
Bacillariophyta	*Melosira sp.*	CCMP 2643	Sequencing
Bacillariophyta	*Navicula transitans*	80	Assembly & Annotation
Bacillariophyta	*Odontella sp.*		Assembly & Annotation
Bacillariophyta	*Odontella sinensis*	Grunow 1884	Sequencing
Bacillariophyta	*Skeletonema costatum*	1716	Sequencing
Bacillariophyta	*Stephanopyxis turris*	CCMP 815	Sequencing
Chlorarachniophyta	*Lotharella oceanica*	CCMP622	Assembly & Annotation
Chlorarachniophyta	*Lotharella globosa*	LEX01	Assembly & Annotation
Chlorarachniophyta	*Lotharella amoebiformis*	CCMP2058	Assembly & Annotation
Chlorarachniophyta	*Bigelowiella natans*	CCMP 2755	Assembly & Annotation
Chlorophyta	*Crustomastix stigmata*	CCMP3273	Sequencing
Chlorophyta	*Dolichomastix tenuilepis*	CCMP3274	Sequencing
Chlorophyta	*Micromonas sp.*	CCMP2099	Assembly & Annotation
Chlorophyta	*Nephroselmis pyriformis*	CCMP717	Assembly & Annotation
Chlorophyta	*Pyramimonas parkeae*	CCMP725	Assembly & Annotation
Chlorophyta	*Tetraselmis sp.*	GSL018	Sequencing
Cryptophyta	*Chroomonas mesostigmatica*	CCMP1168	Assembly & Annotation
Cryptophyta	*Cryptomonas paramecium*	CCAP977/2a	Assembly & Annotation
Cryptophyta	*Goniomonas pacifica*	CCMP1869	Sequencing
Cryptophyta	*Guillardia theta*	CCMP2712	Assembly & Annotation
Cryptophyta	*Hemiselmis andersenii*	CCMP644	Sequencing
Dinophyta	*Alexandrium minutum*	CCMP113	Sequencing
Dinophyta	*Crypthecodinium cohnii*	Seligo	Sequencing
Dinophyta	*Karenia brevis*	SP3	Sequencing
Dinophyta	*Oxyrrhis marina*	CCMP788	Assembly & Annotation
Dinophyta	*Oxyrrhis marina*	CCMP1795	Sequencing
Dinophyta	*Symbiodinium kawagutii*	CCMP2468	Sequencing
Euglenophyta	*Eutreptiella gymnastica*	NIES-381	Assembly & Annotation
Haptophyta	*Hyalolithus neolepis*	TMR5	Sequencing
Ochrophyta	*Dinobryon sp.*	UTEXLB2267	Assembly & Annotation
Ochrophyta	*Ochromonas sp.*	CCMP 1393	Assembly & Annotation
Rhodophyta	*Rhodosorus marinus*	769	Assembly & Annotation

Source : from http://marinemicroeukaryotes.org/project_organisms

Table 2: Biopharmaceutical Proteins Expressed In Microalgae

Gene expressed	Function	Host species and cell localization	Expression level achieved	Application	Source
HSV8-lsc	Mammalian antibody	*Chlamydomonas reinhardtii*, Chloroplast	Detectable	Pharmaceutical	(Mayfield *et al.* 2003)
CTB-VP1	Cholera toxin B subunit fused to foot and mouth disease VP1	*Chlamydomonas reinhardtii*, Chloroplast	3% TSP	Vaccine	(Sun *et al.* 2003)
HSV8-scFv	Classic single-chain antibody	*Chlamydomonas reinhardtii*, Chloroplast	0.5% TSP	Pharmaceutical	(Mayfield *et al.* 2005)
hMT-2	Human metallothionine-2	*Chlamydomonas reinhardtii*, Chloroplast	Detectable	Pharmaceutical, UV-protection	(Zhang *et al.* 2006)
hTRAIL	Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	*Chlamydomonas reinhardtii*, Chloroplast	~0.67% TSP	Pharmaceutical	(Yang *et al.* 2006)
M-SAA	Bovine mammary-associated serum amyloid	*Chlamydomonas reinhardtii*, Chloroplast	~5% TSP	Therapeutics, oral delivery	(Manuell *et al.* 2007)
CSFV-E2	Swine fever virus E2 viral protein	*Chlamydomonas reinhardtii*, Chloroplast	~2% TSP	Vaccine	(He *et al.* 2007)
hGAD65	Diabetes-associated anutoantigen human glutamic acid decarboxylase 65	*Chlamydomonas reinhardtii*, Chloroplast	~0.3% TSP	Diagnostics and therapeutics	(Wang *et al.* 2008)
83K7C	Full-length IgG1 human monoclonal antibody against anthrax protective antigen 83	*Chlamydomonas reinhardtii*, Chloroplast	0.01% dry algal biomass	Therapeutics	(Tran *et al.* 2009b)
IgG1	Murine and human antibodies (LC and HC)	*Chlamydomonas reinhardtii*, Chloroplast	Detectable	Therapeutics	(Tran *et al.* 2009b)
VP28	White spot syndrome virus protein 28	*Chlamydomonas reinhardtii*, Chloroplast	~10.5% TSP	Vaccine	(Surzycki *et al.* 2009)
CTB-D2	D2 fibronectin-binding domain of Staphylococcus aureus fused with the cholera toxin B subunit	*Chlamydomonas reinhardtii*, Chloroplast	0.7% TSP	Oral vaccine	(Dreesen *et al.* 2010)
10NF3, 14FN3	Domains 10 and 14 of human fibronectin	*Chlamydomonas reinhardtii*, Chloroplast	14FN3: 3% TSP 10FN3: detectable	Therapeutics	(Rasala *et al.* 2010)
M-SAA-Interferon β1	Multiple sclerosis treatment fused to M-SAA	*Chlamydomonas reinhardtii*, Chloroplast	Detectable	Therapeutics	(Rasala *et al.* 2010)
Proinsulin	Blood sugar level-regulating hormone, type I diabetes treatment	*Chlamydomonas reinhardtii*, Chloroplast	Detectable	Therapeutics	(Rasala *et al.* 2010)
VEGF	Human vascular endothelial growth factor isoform 121	*Chlamydomonas reinhardtii*, Chloroplast	2% TSP	Therapeutics	(Rasala *et al.* 2010)
HMGB1	High mobility group protein B1	*Chlamydomonas reinhardtii*, Chloroplast	2.5% TSP	Therapeutics	(Rasala *et al.* 2010)
NP-1	Rabbit neutrophil peptide-1	*Chlorella ellipsoidea*, nuclear	Detectable	Antimicrobial	(Chen *et al.* 2001)
ARS2-crEpo-his6	Human erythropoietin fused to ARS2 export sequence w/6xhis tag	*Chlamydomonas reinhardtii*, Nuclear	100 µg/L culture	Pharmaceutical, protein export	(Eichler-Stahlberg *et al.* 2009)
CL4mAb and HBsAg	Human antibody CL4mAB and the Hepatitis B surface antigen (HBsAg)	*Phaeodactylun tricornutum*, Nuclear	CL4mAb: 8.7% TSP HBsAg: 0.7% TSP	Vaccine	(Hempel *et al.* 2011b)
mEPO	Murine Erythropoietin	*Phaeodactylun tricornutum*, Nuclear	300 µg/L culture	Therapeutics	(Carlier A, unpublished work)

Source: modified from Spetch *et al.* (2011). Recent successes in therapeutic protein production in algae.

TSP: Total Soluble Proteins

Table 3: Promoter Used For Microalgae Genetic Transformation

Host species of Microalgae	Promoter of gene and its product	Cell expression localization	Source of promoter	Source
Chlamydomonas reinhardtii	*arg7*, arginosuccinate lyase	Nuclear	*Chlamydomonas reinhardtii*	(Debuchy, 1989)
	35S, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	{Brown, 1991) (Tang *et al.*, 1995) (Kumar *et al.*, 2004)
	RbcS2, rubisco small subunit 2	Nuclear	*Chlamydomonas reinhardtii*	(Auchincloss *et al.*, 1999) (Fuhrmann *et al.*, 1999) (Sizova *et al.*, 2001) (Stevens *et al.*, 1996) (Nelson and Lefebvre, 1995) (Kovar *et al.*, 2002) (Cerutti *et al.*, 1997) (Cordero, 2011)
	HSP70, heat shock protein 70 (fused to other promoter)	Nuclear	*Chlamydomonas reinhardtii*	(Schroda *et al.*, 2000) (Eichler-Stahlberg *et al.*, 2009)
	Nos, nopaline synthase	Nuclear	*Agrobacterium tumefaciens*	(Hall *et al.*, 1993)
	Nit1, nitrate assimilation 1	Nuclear	*Chlamydomonas reinhardtii*	(Ohresser *et al.*, 1997) (Llamas *et al.*, 2002)
	Cop, chlamyopsin	Nuclear	*Chlamydomonas reinhardtii*	(Fuhrmann *et al.*, 1999)
	TubA1, alpha-tubulin	Nuclear	*Chlamydomonas reinhardtii*	(Kozminski *et al.*, 1993)
	β2-tubulin	Nuclear	*Chlamydomonas reinhardtii*	(Blankenship and Kindle, 1992) (Berthold *et al.*, 2002)
	CabII-1, chlorophyl-ab binding	Nuclear	*Chlamydomonas reinhardtii*	(Blankenship and Kindle, 1992)
	pcy1, plastocyanin	Nuclear	*Chlamydomonas reinhardtii*	(Quinn and Merchant, 1995)
	atpC, gamma-subunit of chloroplast ATPase	Nuclear	*Chlamydomonas reinhardtii*	(Quinn and Merchant, 1995)
	psaD, photosystem I complex protein	Nuclear	*Chlamydomonas reinhardtii*	(Fischer and Rochaix, 2001)
	atpA, alpha subunit of adenosine triphosphate	Chloroplast	*Chlamydomonas reinhardtii*	(Sun *et al.*, 2003)
	psbD, photosystem II D1	Chloroplast	*Chlamydomonas reinhardtii*	(Manuell *et al.*, 2007)
	RbcL, ribulose bisphosphate carboxylase large subunit	Chloroplast	*Chlamydomonas reinhardtii*	(Dreesen *et al.*, 2010)
	psbA, photosystem II psbA	Chloroplast	*Chlamydomonas reinhardtii*	(Rasala *et al.*, 2011b)
Dunaliella salina	*Ubi1- Ω*, ubiquitin-Ω	Nuclear	*Zea mais*	(Geng *et al.*, 2003)
	35S, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(Tan *et al.*, 2005) (Sun *et al.*, 2005) (Feng *et al.*, 2009) (Wang *et al.*, 2007b)
	NR, Nitrate reductase	Nuclear	*Dunaliella salina*	(Li *et al.*, 2007) (Li *et al.*, 2008a)
	RbcS2, rubisco small subunit	Nuclear	*Dunaliella salina*	(Sun *et al.*, 2005)
Dunaliella bardawil	*35S*, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(Anila *et al.*, 2011)
Chlorella ellipsoida	*35S*, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(Jarvis and Brown, 1991)
	Ubi1- Ω, ubiquitin-Ω	Nuclear	*Zea mais*	(Chen *et al.*, 2001)
	RbcS2, rubisco small subunit 2	Nuclear	*Chlamydomonas reinhardtii*	(Kim *et al.*, 2002)
Chlorella sorokiniana	*NR*, nitrate reductase	Nuclear	*Chlorella sp.*	(Dawson, 1997)
Chlorella vulgaris	*35S*, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(Cha *et al.*, 2011b) (Chow and Tung, 1997) (Chow and Tung, 1999) (Wang *et al.*, 2007a)
	NR, nitrate reductase	Nuclear	*Phaeodactylum tricornutum*	(Niu *et al.*, 2011)
Platymonas subcodiformis (Tetraselmis)	*CMV*, cytomegalovirus	Nuclear	Cytomegalovirus	(Cui *et al.*, 2010)

Nannochloropsis sp	*HSP70*, heat shock protein 70 / *RbcS2*, rubisco small subunit 2	Nuclear	*Chlamydomonas reinhardtii*	(Chen *et al.*, 2008) (Li and Tsai, 2008b)
	35S, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(Cha *et al.*, 2011a)
	VCP, violaxanthin/chlorophyl binding protein	Nuclear	*Nannochloropsis sp*	(Kilian *et al.*, 2011)
Haematococcus pluvialis	*SV40*, simian virus	Nuclear	simian virus	(Teng *et al.*, 2002)
	pds, phytoene desaturase		*Haematococcus pluvialis*	(Steinbrenner and Sandmann, 2006)
	35S, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(Kathiresan *et al.*, 2009)
Volvox carteri	*NR*, nitrate reductase	Nuclear	*Volvox carteri*	(Schiedlmeier *et al.*, 1994)
Gonium pectorale	*psD*, photosystem I complex protein / *HSP70*, heat shock protein 70		*Chlamydomonas reinhardtii*	(Lerche and hallmann, 2009)
Closterium peracerosum-strigosum litorrale	*HSP70* heat shock protein 70 / *Cab*, chlorophyl-ab binding Ch a/b-binding protein		*Closterium peracerosum-strigosum litorrale*	(Abe *et al.*, 2008) (Abe *et al.*, 2011)
Lotharella amoebiformis	*RbcS2*, rubisco small subunit 2	Nuclear	*Lotharella amoebiformis*	(Hirakawa *et al.*, 2008)
Cyclotella criptyca	*Acc1*, acetylCoA carboxylase	Nuclear	*Cyclotella criptyca*	(Dunahay *et al.*, 1995)
Navicula saprophila	*Acc1*, acetylCoA carboxylase	Nuclear	*Cyclotella criptyca*	(Dunahay *et al.*, 1995)
Phaeodactylum tricornutum	*fcpA*/*B*/*C*/*E*, fucoxanthin chlorophyll	Nuclear	*Phaeodactylum tricornutum*	(Apt *et al.*, 1996)
	fcpF, fucoxanthin chlorophyll	Nuclear	*Phaeodactylum tricornutum*	(Falciatore *et al.*, 1999)
	fcpA, fucoxanthin chlorophyll	Nuclear	*Phaeodactylum tricornutum*	(Zaslavskaia *et al.*, 2000)
	cah, carbonic anyhdrase	Nuclear	*Phaeodactylum tricornutum*	(Harada and Matsuda, 2005)
	CMV, cytomegalovirus; *PRSV-LTR*, rous sarcoma virus; *35S*, cauliflower mosaic virus 35S	Nuclear	Cytomegalovirus; Rous sarcoma virus; Cauliflower mosaic virus	(Sakaue *et al.*, 2008)
	fcpA, fucoxanthin chlorophyll	Nuclear	*Phaeodactylum tricornutum*	(Coesel *et al.*, 2009)
	fcp, fucoxanthin chlorophyll and *NR*, nitrate reductase	Nuclear	*Cylindrotheca fusiformis*	(Miyagawa *et al.*, 2009)
Cylindrotheca fusiformis	Pδ, frustulin α3	Nuclear	*Cylindrotheca fusiformis*	(Fischer *et al.*, 1999)
	NR, nitrate reductase	Nuclear	*Cylindrotheca fusiformis*	(Poulsen and Kroger, 2005)
Thalassiosira pseudonana	*fcp*, fucoxanthin chlorophyll	Nuclear	*Thalassiosira pseudonana*	(Poulsen *et al.*, 2006)
Thalassiosira weissflogii	*fcpB*, fucoxanthin chlorophyll	Nuclear	*Thalassiosira pseudonana*	(Falciatore *et al.*, 1999)
Chaetoceros sp.	*pTpNR* (nitrate reductase de *Thallassiosira psudomana*)	Nuclear	*Thalassiosira pseudonana*	(Miyagawa-Yamaguchi *et al.*, 2011)
Amphidinium spp. *Symbiodinium microadriaticum*	*35S*, cauliflower mosaic virus 35S	Nuclear	Cauliflower mosaic virus	(ten Lohuis and Miller, 1998)
Cyanidioschyzon merolae	UMP synthase	Nuclear	*Cyanidioschyzon merolae*	(Minoda *et al.*, 2004)
	β-tubulin	Nuclear	*Cyanidioschyzon merolae*	(Ohnuma *et al.*, 2008)
	cat, catalase	Nuclear	*Cyanidioschyzon merolae*	(Ohnuma *et al.*, 2009)
	apcC, phycocyanin-associated protein	Nuclear	*Cyanidioschyzon merolae*	(Watanabe *et al.*, 2011)
Porphyidium sp.	*AHAS*, acetohydroxyacid synthase	Nuclear	*Porphyidium sp.*	(Lapidot *et al.*, 2002)
Euglena gracilis	*psbA*, photosystem II complex protein	Chloroplast	*Euglena gracilis*	(Doetsch *et al.*, 2001)