A microscopic image showing several green, spherical algae cells. The background is a textured, light green color, likely representing the surrounding algal matrix or other cellular components.

Sergio Rosales-Mendoza

Algae-Based Biopharma- ceuticals

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Preface

Photosynthetic microorganisms have been used for the benefit of human beings since ancient times. For instance, the first known report on the use of cyanobacteria as food was published in 1520 by Hernán Cortés, commenting the fact that Aztecs ate tecuitlatl, a cake made from Spirulina that was an important part in their diet. In the case of eukaryotic algae, important biotechnology applications have been developed for many species. The invention of molecular cloning and genetic engineering tools allowed for the development of numerous products that tremendously favored the human and animal health worldwide. For instance, the recombinant biopharmaceuticals (BFs) such as insulin, cytokines, monoclonal antibodies, and subunit vaccines allowed for the treatment, cure, or prevention of many diseases saving millions of lives. Improvements on the platforms for producing these recombinant BFs are under development or still needed. During the last decade, algae species have been explored as a next-generation platform for BFs production with clear advantages in terms of efficacy, safety, and cost. The current developments comprise the production of several BFs in some algae species, which have been evaluated at the preclinical level with positive outcomes. Moreover, the ambitious objectives in this field consist in the use of whole algae cells for the development of photosynthetic biomaterials for regenerative medicine and for the oral delivery of BFs eliminating the need for purification and sterile injections. This book provides an updated outlook on the use of algae for the production and delivery of BFs. Although the case of *Chlamydomonas reinhardtii* is emphasized since the majority of the studies have been performed in this model microalga, the use of other algae species such as *Dunaliella* sp., *Phaeodactylum tricornutum*, and *Schizochytrium* sp. is also covered.

First, the features of algae as convenient hosts for the production of BFs are analyzed in terms of production costs, biosynthetic capacity, and safety (Chap. 1). Second, the genetic engineering tools for algae species are described. Nuclear- and chloroplast-based expression approaches are analyzed and compared in terms of biosynthetic advantages, gene expression complexity, and DNA transfer approaches (Chap. 2). In the following sections, Chaps. 3, 4, 5, 6, and 7, the state of the art on producing distinct types of BFs in algae species is presented. Although this book is

mainly focused on BFs, considering that the production of compounds with health-promoting properties are achieved using genetically engineered algae strains, Chap. 8 deals with nutraceuticals. In Chap. 9, the developments reported thus far are placed in perspective and challenges for the field are discussed. Critical future prospects comprise the following: optimizing large-scale production in bioreactors, implementing glycoengineering approaches, optimizing nuclear expression, exploring new approaches for oral delivery, and implementing regulatory frameworks to accomplish technology transfer and regulatory approval of algae-made BFs.

Consequently, this book constitutes a key reference on the use of algae in the BFs production field, providing an updated outlook on the achievements accomplished thus far and transmitting a prospective view for this biotechnological application.

I thank all my colleagues whose time and efforts constituted a relevant support in this project, especially to Ileana García-Silva and Omar González-Ortega.

San Luis Potosí, Mexico

Sergio Rosales-Mendoza

Contents

| | |
|--|----|
| 1 The Biopharmaceuticals Field and Algae as Expression Hosts | 1 |
| Introduction | 1 |
| Biopharmaceuticals Market and Current Limitations | 3 |
| Current Platforms for the Large-Scale Production of BFs | 5 |
| General Features of Microalgae | 6 |
| Features of Algae and Implications in BFs Production | 7 |
| Relevant Algae Species | 9 |
| <i>Phaeodactylum tricornutum</i> | 9 |
| <i>Dunaliella salina</i> | 10 |
| <i>Chlamydomonas reinhardtii</i> | 10 |
| <i>Schizochytrium sp.</i> | 10 |
| Prospective View | 11 |
| References | 11 |
| 2 Genetic Engineering Approaches for Algae | 15 |
| Introduction | 15 |
| Construction of Genes and Expression Vectors | 16 |
| Transformation Techniques | 20 |
| <i>Agrobacterium tumefaciens</i> | 20 |
| Biostatic | 22 |
| Glass Beads Treatment | 23 |
| Electroporation | 23 |
| Expression Modalities | 23 |
| Nuclear and Chloroplast-Based Expression | 23 |
| Inducible Expression | 24 |
| Overview of Algae Transformation Achievements | 26 |
| Advances for Transgene Expression in the Model Alga <i>C. reiinhardtii</i> | 28 |
| Multigene Expression and Organelle Targeting | 28 |
| Generation of Mutant Strains with High Productivity | 33 |
| Fusion to Protein Partners | 34 |

| | |
|--|----|
| The Transformosome Concept | 34 |
| Prospective View | 35 |
| References | 35 |
| 3 Algae-Made Vaccines Targeting Human Diseases | 41 |
| Introduction | 41 |
| Gut Associated Immune System and Oral Vaccination | 42 |
| Vaccines Targeting Infectious Agents | 46 |
| <i>Plasmodium falciparum</i> | 46 |
| <i>Staphylococcus aureus</i> | 49 |
| <i>Human Papillomavirus</i> | 49 |
| <i>Influenza Virus</i> | 50 |
| <i>Hepatitis B Virus</i> | 53 |
| <i>Human Immunodeficiency Virus</i> | 54 |
| Vaccines Targeting Non-communicable Diseases | 55 |
| Type I Diabetes | 55 |
| Atherosclerosis | 55 |
| Hypertension | 55 |
| Allergy | 56 |
| Prospective View | 56 |
| References | 59 |
| 4 Algae-Made Vaccines Targeting Animal Pathogens | 65 |
| Introduction | 65 |
| Algae-Based Vaccines | 66 |
| <i>Classical Swine Fever Virus</i> | 66 |
| <i>White Spot Syndrome Virus</i> | 67 |
| <i>Taenia Solium</i> | 68 |
| <i>Foot-and-Mouth Disease Virus</i> | 69 |
| <i>Porcine Circovirus</i> | 70 |
| Prospective View | 70 |
| References | 73 |
| 5 Algae-Made Antibodies and Immunotoxins | 77 |
| Introduction | 77 |
| Current Developments on Algae-Made Antibodies and Immunotoxins | 79 |
| Large Single-Chain (Isc) Antibody Against Herpes Simplex Virus (HSV) Glycoprotein D | 79 |
| Full-Length Antibodies Against the Anthrax Protective Antigen 83 | 82 |
| An Anti-hepatitis B Surface Protein Antibody Produced in <i>Phaeodactylum tricornutum</i> | 83 |

| | |
|---|------------|
| Camelid Antibodies Against Botulinum Neurotoxin Serotype A (BoNT/A) | 84 |
| Immunotoxins Targeting CD22+ Cells | 85 |
| Prospective View | 88 |
| References | 91 |
| 6 Algae-Made Cytokines and Growth Factors | 95 |
| Introduction | 95 |
| Section I | 96 |
| Human Interferon β 1 | 96 |
| Human Vascular Endothelial Growth Factor | 98 |
| High Mobility Group Protein B1 | 99 |
| Section II | 100 |
| Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand | 100 |
| Tumor Necrosis Factor Alpha Produced in <i>Dunaliella salina</i> | 101 |
| <i>C. reinhardtii</i> Secreting VEGF for the Development of Photosynthetic Biomaterials in Tissue Engineering | 102 |
| Prospective View | 102 |
| References | 105 |
| 7 Other Biopharmaceuticals Produced in Algae | 109 |
| Introduction | 109 |
| Antimicrobial Peptides | 109 |
| Rabbit Neutrophil Peptide-1 | 109 |
| Lactoferricin | 110 |
| Fibronectin Domains | 111 |
| Soybean Kunitz Trypsin Inhibitor | 113 |
| Hormones | 114 |
| Erythropoietin | 114 |
| Human Growth Hormone | 116 |
| Flounder Growth Hormone | 116 |
| Prospective View | 117 |
| References | 118 |
| 8 Algae-Made Nutraceuticals Produced Using Genetic Engineering Approaches | 121 |
| Introduction | 121 |
| Proteins | 122 |
| Bovine Milk Amyloid A Produced in <i>C. reinhardtii</i> | 122 |
| A Chimeric Protein Carrying Bioactive Peptides Produced in <i>C. reinhardtii</i> | 128 |
| Lipids | 129 |
| Carotenoids | 131 |
| Prospective View | 135 |
| References | 137 |

| | |
|---|-----|
| 9 Perspectives for the Algae-Made Biopharmaceuticals Field | 143 |
| Introduction | 143 |
| Key Perspectives for the Field of Producing BFs in Microalgae | 144 |
| Optimizing Nuclear Expression | 144 |
| Implementing Glycoengineering Approaches | 146 |
| Exploring New Approaches for Oral Delivery | 149 |
| Optimizing Large-Scale Production in Bioreactors | 150 |
| Expanding the Group of Species Used as Hosts | 155 |
| Technology Transfer and Regulatory Approval | 156 |
| References | 158 |
| Index | 165 |

Chapter 1

The Biopharmaceuticals Field and Algae as Expression Hosts

Introduction

A biopharmaceutical (BF) is defined as a complex molecule of biological origin that exerts a therapeutic action in humans. Among this type of compounds; vaccines, antibodies, hormones, clotting factors, growth factors, and cytokines are the most prominent agents. BF s are key components in addressing many life-threatening diseases, which are mainly infectious and non-communicable diseases that constitute a major challenge for public health systems in both developed and developing countries (Finckh et al. 2015; Rencz et al. 2015).

Biopharmaceuticals (BFs) contrast respect to traditional drugs in terms of manufacture processes, structure, and action. These are much more complex due to their high molecular weight, specific tridimensional or even quaternary structures, and heterogeneity given by the number of post-translational modifications that can be subjected to (Schellekens 2004). Recombinant DNA and hybridoma technologies made possible for large-scale production of BF s. Since BF s are of key importance in the fight of a myriad of both infectious and non-communicable diseases, these are produced at the industrial level typically in mammalian cells, yeast, or bacteria as the expression host. The election of the production platform mainly depends on the requirements determined by the BF complexity (e.g. required specific glycosylation patterns or oligomeric structure formation). BF s with high complexity are produced in appropriate eukaryotic platforms such as mammalian cells or yeast, while simpler molecules that do not require complex post-translational modifications; such as insulin and some interleukins, are typically produced in bacterial systems (Table 1.1).

Besides the cost of production, the proper administration of BF s is also an important part of the story. In poor countries, unsafe injection practices have led to the transmission of infections at substantial rates. Although progress has been recorded in this regard, much work is still needed (Pépin et al. 2014). In those cases in which the BF s are bioavailable and remain active following oral administration, the use of food-grade organisms as expression hosts opens the possibility of using them as the

Table 1.1 Comparison of hosts used in biopharmaceuticals production platforms

| Host | Cultivation cost | Diversity of genetic tools | Growth rate | Post-translational modification capacity | Glycoengineering tools | Endotoxins and possible presence of pathogens | Industrial production experience |
|-----------------|------------------|----------------------------|-------------|--|------------------------|---|----------------------------------|
| <i>E. coli</i> | ++ | ++++ | ++++ | + | + | Yes | ++++ |
| Yeast | ++ | ++++ | ++++ | +++ | ++ | No | ++++ |
| Mammalian cells | +++++ | ++++ | ++ | ++++ | +++ | Yes | ++++ |
| Algae | + | ++ | ++++ | +++ | + | No | ++ |

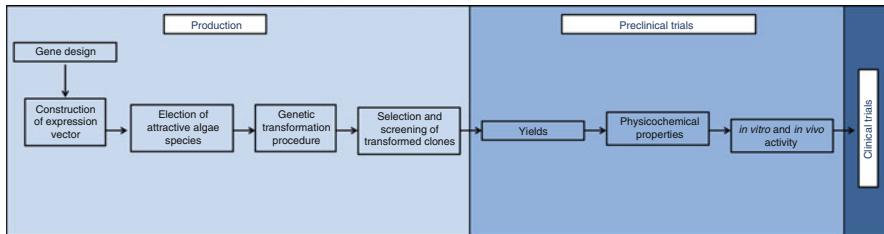


Fig. 1.1 General workflow for the development of microalgae-made biopharmaceuticals

oral delivery vector; thus alleviating the need for parenteral administration that is dependent on trained personnel and sterile devices (Rosales-Mendoza et al. 2015; Shaaltiel et al. 2015).

Algae species comprise a diverse group of organisms with attractive features for the production of BFs. The exploration of this topic has primarily focused on using the fresh water microalgae species *Chlamydomonas reinhardtii* as host, generating promising results in terms of yields and functionality. Therefore, the promising outlook generated in the pioneering studies led to efforts to explore this system for industrial implementation (Scaife et al. 2015; Fig. 1.1). Herein, the current outlook in the pharmaceutical field and the general aspects of algae as convenient platforms for the production of BFs are analyzed.

Biopharmaceuticals Market and Current Limitations

Two main biologic drug discovery phases can be identified: recombinant human proteins generated in the 1980s and even more complex products including monoclonal antibodies (mAbs) generated in the 1990s (Kellermann and Green 2002; Shellenkens 2004). The approvals for BFs by the Food and Drug Administration (FDA) have shown a sustained growth over the last two decades (Fig. 1.2). There are more than 180 therapeutic proteins and peptides approved by this regulatory agency thus far. Moreover the commercialization of biologics reached \$157 billion in 2011, which constitutes 19% of the global pharmaceutical market (EvaluatePharma 2012; IMS 2012). MAb are the largest group of BFs serving as therapeutic agents in a diverse group of conditions. In 2010 mAb sales were of \$18.5 billion, representing 36% of the total BFs sold (Aggarwal 2011). In 2011, a \$44.6 billion global market for mAb was calculated and with an estimated annual growth rate of 5.3% leads to \$58 billion in sales for 2016 (www.bccresearch.com/market-research/biotechnology) (Elvin et al. 2013).

Although patents allowed large profits for the developer companies, the expiration of the patents and/or data protection for the first major group of BFs such as insulin, human growth hormone, and erythropoietin; has opened the opportunity to start the era of biosimilars, which will allow for increasing pharmaceutical supply coverage. A biosimilar is defined as a biological product that is highly similar to a reference product, notwithstanding minor differences in clinically inactive

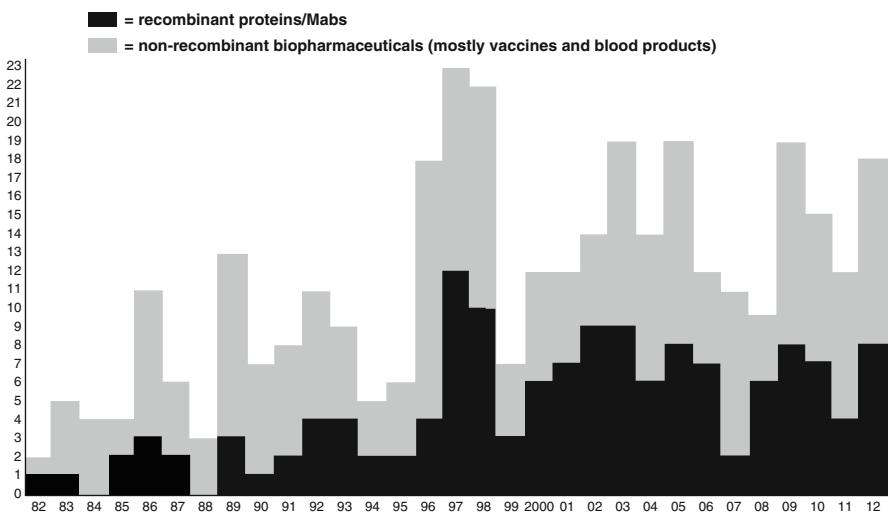


Fig. 1.2 Number of FDA new biopharmaceutical approvals. A sustained growth in the number of BFs and a US market of 157 billion USD in 2011 indicate the relevance of BFs in human health care (Graph taken from Rader (2013) and www.biopharma.com/)

components that can be produced in a different host. However, these products are similar enough to guarantee no significant clinical difference with respect to the reference product (Ornes 2015). It has been estimated that over the next decade, biosimilars will decrease overall direct spending on biologics in the United States by \$44 billion (Mulcahy et al. 2014).

It is considered that the safety profile and clinical experience on using the original products will serve as a valuable reference to promote a smoother path to the development and approval of biosimilars. However, BFs have 100–1000 times higher molecular weight than small chemical molecules and thus are inherently more variable and complex; which is given in part by the microheterogeneity of the protein structure and different glycosylation profiles. Therefore, it should be considered that BFs are complex molecules whose structure and clinical performance is critically influenced by the production process. Due to these aspects the regulatory framework for biosimilars is different to those for generics. Biosimilars must show no clinically meaningful differences in their safety, purity, and potency with respect to the reference product (Daller and Daller 2015). Both the EU and the US have prepared guidance documents for biosimilars that will result in biotherapeutics that are as safe and efficacious as the innovator product.

The first biosimilar approved by the US FDA consists of Zarxio (filgrastim-sndz), approved on March 6, 2015. This is a myeloid growth factor manufactured by Sandoz, a subsidiary of Novartis, and is biosimilar to Amgen's Neupogen (filgrastim). Estimations of a US pharmacy benefit management organization (Express Scripts) indicate that the adoption of this biosimilar has the potential to reduce US drugs costs by around \$5.7 billion in the following 10 years. Moreover, they

estimated that the adoption of other 11 biosimilar drugs will achieve a further \$250 billion in savings (Burki 2015).

Moreover, biobetters constitute an important concept in this field. A biobetter consists on the originator molecule that possesses improved parameters as a consequence of specific alterations, making it more efficacious, less frequently dosed, better targeted, and/or better tolerated (Beck 2011; Strohl 2015). Innovative expression hosts offer the opportunity to accomplish the development of biobetters. For instance glycoengineered BFs produced in moss have been proposed as a source of biobetters (Reski et al. 2015).

Sadly, the cost of innovative biopharmaceuticals produced under genetic engineered procedures has often been prohibitive, especially in poor countries. In developed countries the high cost of BFs causes cost-cutting measures in other therapeutic areas (Haustein et al. 2012). In 2012, the FDA granted 18 new biopharmaceutical product approvals covering a broad range of innovation and novelty that translate into a positive impact on healthcare and the market. This group of biopharmaceuticals comprises eight recombinant proteins, including two monoclonal antibodies (mAbs) and one engineered antibody-like “trap” molecule (Rader 2013).

Current Platforms for the Large-Scale Production of BFs

Mammalian Cell Lines The production of BFs in mammalian cells is characterized by the most appropriate post-translational processing, high yields of proteins with optimum functionality and half-life (Almo and Love 2014). Chinese hamster ovary (CHO) cells represent the most frequently host cell system used for industrial manufacturing of BFs. The advantages of this host comprise the capability to produce high quality BFs having human-like post-translational modifications in gram quantities (up to 1 g/L). Although some advances in bioprocess, media, and vector optimizations; as well as in host cell engineering technologies have been achieved, limitations of this platform include the following: high cost of the culture media, limited growth, low productivity, and stress resistance. In addition, there is an inherent risk of contamination with pathogenic virus or prions since hemoderivates and animal products are used during upstream processing (Fischer et al. 2015). This factor requires strict purification processes and proper control quality procedures leading to costly BFs.

Bacteria Bacterial systems represented by *Escherichia coli* as the typical host offer high recombinant protein yields (up to 3 g/L) and fast growth. However, *E. coli* is not a proper host in general for producing complex BFs requiring elaborated post-translational modifications (Terpe 2006). Although the production of BFs in bacteria is more economical than in mammalian cells, the presence of endotoxins in this host requires proper approaches for their removal. In addition the recombinant material is frequently produced in an insoluble form as protein bodies, which facilitate purification but a further expensive refolding process must be performed not always leading to a functional protein (Overton 2014; Singh and Panda 2005).

Yeast The yeast species *Saccharomyces cerevisiae* and *Pichia pastoris* are attractive hosts for the production of functional BFs. In fact, the first recombinant vaccine approved for human use in 1981 was produced in *S. cerevisiae* and this species is currently used in the manufacture of vaccines against hepatitis B virus and Human papillomavirus (Bill 2015). These organisms offer high yields (in the range of 10–30 g/L) and efficient protein secretion, however hyperglycosylate proteins and thus BFs may become immunogenic or allergenic (Celik and Calik 2012). Nonetheless, this aspect is evaluated case by case.

Given this outlook, the development of innovative BFs production platforms is a relevant aim in this area. Proper functionality aided by a high biosynthetic capacity, safety, low-cost for production, and easy delivery are the main desired attributes. New platforms meeting these requirements are needed, especially in low-income countries where there is an urgent need for making BFs more accessible. During the last decades progress in the genetic modification procedures led to the design of the next generation platforms for the production of BFs. Over the last 30 years, several efforts have been conducted to explore new platforms; including those based in plant cells (Streatfield et al. 2015), moss (Reski et al. 2015), silkworms (Tada et al. 2015), and filamentous fungi such as corn-smut (Juárez-Montiel et al. 2015). In fact, for the case of vaccines the term food-grade vaccines has been employed to refer orally-delivered vaccines produced in food-grade organisms (Rosales-Mendoza et al. 2015).

Outstandingly one of the BFs approved by the FDA in 2012 is Elelyso, a recombinant glucocerebrosidase from Protalix and Pfizer, which constitutes a milestone in the field since it is the first plant-made FDA-approved biopharmaceutical. Elelyso is manufactured through suspension carrot cell culture in disposable plastic bioreactors (Tekoah et al. 2015). The product and the manufacturing process were developed by Protalix BioTherapeutics and constitute a key example of innovation in bioprocessing. This example illustrates the potential of using low cost systems for producing BFs. Therefore the use of technologies based in photosynthetic organisms as a source of recombinant BFs, which has been researched over the last decades, has become a reality.

Among the next generation platforms for BFs production, algae species are gaining attention as a convenient platform for the production and in some cases as the delivery vehicle of BFs.

General Features of Microalgae

Microalgae are a polyphyletic group of unicellular eukaryotic organisms that can occupy both aquatic and terrestrial environments adopting photosynthetic, heterotrophic, or mixotrophic lifestyles. Algal lineages hold a long evolutionary history responsible for a high diversity (Dorrell and Smith 2011). Algae naturally occur in environments with wide range of temperatures, light intensities, pH, and salt concentration. In fact, some species grow in a symbiosis with animals (Barsanti et al.

2008; Varshney et al. 2015). Moreover, microalgae hold wide characteristics in terms of cellular architecture and biosynthetic capacity. Therefore several microalgae species possess attractive characteristics for industrial application because of their rapid cell division and photosynthetic growth, leading to a higher productivity per unit land area than any plant system.

The first report on the culture of microalgae for experimental purposes was authored by Warburg (1919), which consisted in the cultivation of *Chlorella* for the study of photosynthesis. In terms of cultivation for industrial purposes, *Chlorella* was cultured in the 1950s in pilot plants in Massachusetts and Tokyo (Richmond and Soeder 1986). The interest on developing biotechnological applications for microalgae has increased substantially during the last decades in several areas. Currently algae constitute the basis for formulation of dietary supplements and animal feed. Algae compounds are also part of the formulation of cosmetics and pharmaceutical products (Apt and Behrens 1999; Luiten et al. 2003; Yamaguchi 1997).

Features of Algae and Implications in BFs Production

Attractive Biosynthetic Capacity and Low Production Cost Many algae species can be grown phototropically, and thus sun light can drive biomass production using minimal, low cost media. Photosynthesis enables the production of biomolecules in a sustainable manner without the requirements of a fixed carbon source, which cannot be achieved in the conventional bacterial, yeast, or mammalian hosts. Eukaryotic microalgae possess a cellular complexity allowing for the compartmentalization of biochemical reactions, which is not possible in bacteria. Valuable compounds naturally produced by microalgae including pigments, such as β -carotene and astaxanthin, and polymers, such as alginate, carrageenan and agar; are currently exploited at a commercial level.

Technologies for culture and optimization are important achievements in this field. Early studies have proven the influence of different growth conditions on the chemical composition of microalgae (Spoehr and Milner 1949). It is now well recognized that some kind of stress promote the production of valuable compounds. With the coming of genetic engineering, algae became attractive hosts for recombinant protein production. Overall, algae possess the capacity to produce recombinant protein at convenient levels for the purification or direct delivery of BFs. Eukaryotic algae possess a chloroplast that produces disulfide isomerases, chaperones (Schroda 2004), and peptidyl propylisomerases (PPIases) (Breiman et al. 1992); which allow for the formation of disulfide bridges (Kim and Mayfield 1997). Therefore, this platform surpasses the biosynthetic capacity of the bacterial systems and offers relatively high yields at the chloroplast level (up to 10 % of total soluble protein, TSP). As eukaryotic organisms, microalgae possess the machinery for the efficient synthesis of nuclear-encoded proteins through complex post-translational modifications that mainly occur in the endomembrane system (Anyaogu and Mortensen 2015).

Current developments allow for production of competitive levels of recombinant proteins in green algae. The approach consisted in non-photosynthetic strains since the heterologous gene replaces an essential gene for photosynthesis. However, recent advances achieved the development of clones in which photosynthesis was reestablished with the concurrent production of highly valuable proteins with attractive yields under greenhouse conditions (Gimpel et al. 2015b). Another attractive feature explored in some algae species is the capability to secrete the BF into the culture media, which greatly facilitates purification (Lauersen et al. 2015). This approach has been successfully applied in the case of antibodies produced in *Phaeodactylum tricornutum* (Hempel and Maier 2012) and other recombinant proteins produced in *Chlamydomonas reinhardtii* (Lauersen et al. 2013; Rasala et al. 2012).

Genetic Engineering Feasibility Current technologies allow for the genetic modification of either nuclear or chloroplast genomes. Several tools such as promoters, untranslated regions (UTRs), reporter genes, selectable markers, recombination regions in the case of chloroplast transformation, and promoters have been evaluated in algae (Rosales-Mendoza et al. 2012). Several approaches for achieving genetic modifications are available for algae species and Chap. 2 of this book contains a detailed compilation of such technologies. With the recent advances in genetic engineering tools, remarkable developments on modifying/enhancing the production of valuable compounds in microalgae are expected. Intensive research on applying genetic engineering to enhance the production of valuable metabolites (nutraceuticals) is ongoing and an outlook on this topic is presented in Chap. 8.

Large-Scale Production Under Full Containment Algae can be cultivated in bioreactors at a large scale. Some algae species are currently used in the industry for the large-scale production of food supplements and biofuels (Gangl et al. 2015). Thus, this aspect is already established and will greatly facilitate the adoption of specific algae species for the production of BFs. For instance, the non-photosynthetic microalgae *Schizochytrium spp* is currently used for the production of docosahexanoic acid (DHA) (Barclay et al. 1994).

At the industrial level, the goal for setting platforms for biofuel production have prompted the development of technologies to achieve effective and economical microalgae cultivation systems; as well as separation and harvesting of microalgal biomass (Chen et al. 2011; Show et al. 2015). Since algae can be grown under different microalgal metabolisms (i.e. phototrophic, heterotrophic, mixotrophic, and photoheterotrophic growth) and light sources, there is a wide spectrum of possibilities for industrial production in bioreactors. This outlook will greatly help in the establishment of efficient and commercially viable technologies for the exploitation of algae species in the production of BFs.

However, it should be recognized that some limitations in the large-scale cultivation of microalgae exist. Some of the aspects requiring optimization include light capture in dense cultures (Béchet et al. 2015), resource-use efficiency, harvesting, and expanding the metabolic engineering approaches making commercial exploitation economically viable (Gimpel et al. 2015a). One concern

Table 1.2 Compilation of the main algae species used in the BFs production field thus far

| Microalgae | Type | Phototrophic growth | Use in BFs production | Maximum production scale assessed | References |
|----------------------------------|---------------------------|---------------------|-----------------------|-----------------------------------|----------------------------|
| <i>C. reinhardtii</i> | Sweet water (chlorophyta) | + | ++++++ | 100 L bags (greenhouse) | Gimpel et al. (2015b) |
| <i>Phaeodactylum tricornutum</i> | Marine (diatom) | + | ++ | Flask | Hempel et al. (2011, 2012) |
| <i>Dunaliella salina</i> | Marine (chlorophyta) | + | ++ | Flask | Geng et al. (2003) |
| <i>Schizochytrium sp.</i> | Marine (diatom) | - | + | 10 L fermentors | Bayne et al. (2013) |

in the case of genetically engineered organisms is the undesired gen flow, which is of particular attention in the case of plants cultivated in open fields (Lucht 2015). In this regard, algae systems offer the alternative of having fully containment by using close fermenters or photobioreactors. Species in which these processes are well established include *C. reinhardtii*, *Phaeodactylum tricornutum*, *Schizochytrium* sp., and *Dunaliella salina*. Some perspectives in the field of algae production at the industrial level are presented in Chap. 9.

High Safety Due to the Absence of Human Pathogens Contamination with virus or prions is a risk when mammalian cells are used as host for BFs production. In some cases the production of BFs has been seriously hampered due to contamination with virus (Aggarwal 2009). Thus far no human pathogenic organisms are known to naturally occur in algae species, therefore there is no concern about the presence of human virus or prions in algae species used for BFs production. In fact, some algae species hold a GRAS (generally recognized as safe) status and thus can be safely administered to humans by the oral route (Rosales-Mendoza et al. 2015).

It is clear that algae species offer substantial advantages for the large-scale production of BFs since these organisms possess some of the strengths of the mammalian and bacterial systems. The following subheadings describe the main algae species explored in this field as convenient biofactories and possible delivery vehicles of BFs (Table 1.2).

Relevant Algae Species

Phaeodactylum tricornutum

Diatoms are a diverse and ubiquitous group of eukaryotic microalgae that occur in diverse environments (freshwater, marine, and terrestrial) (Round et al. 1990). These organisms are photosynthetic autotrophs and important contributors of CO₂

fixation and O₂ production in the oceans. Diatoms also possess commercial uses as feeds in aquaculture (Gladue and Maxey 1994) and lipid production (Barclay et al. 1994). *Phaeodactylum tricornutum* is a species of diatoms used as model in distinct disciplines. *P. tricornutum* has high growth rates and genetic transformation methodologies have been developed for this species (Apt et al. 1996; Hempel et al. 2011; Hempel and Maier 2012) in addition it has been successfully used for the production of BFs such as antibodies (See Chap. 5).

Dunaliella salina

Dunaliella salina is a unicellular, bi-flagellate, naked green alga lacking a rigid cell wall (Emeish 2013); which is able to grow under high salt concentration, high light intensity, high temperature and a wide pH range (Cifuentes et al. 1996). Several methods were developed to genetically modify *D. salina*, including electroporation, particle bombardment, *Agrobacterium tumefaciens*, glass beads treatment, and LiAc/PEG-mediated methods; being the last two the most efficient (Feng et al. 2009, 2014). Remarkably, *D. salina* is commercially used for pigment production at industrial levels (Ben-Amotz and Avron 1990; Ben-Amotz 1993; Olaizola 2003). This species has also been used for the production of some vaccines (see Chaps. 3 and 4).

Chlamydomonas reinhardtii

Chlamydomonas (including *Chloromonas*) is one of the largest green algal genera with more than 600 species described (Pröschold et al. 2001). *Chlamydomonas reinhardtii* is a fresh water alga used for decades as a model organism for molecular biology, including flagellar motility, chloroplast dynamics, biogenesis, and genetics (Gutman and Niyogi 2004; Rochaix 1995). *C. reinhardtii* was transformed at the chloroplast genome level more than 25 years ago (Boynton et al. 1988). In the early 2000s the use of *C. reinhardtii* in the BFs field begun with the expression of antibodies (See Chap. 5). In fact the large majority of the research on this topic is currently performed with *C. reinhardtii*, since it is the best characterized microalgal species and commercial applications of this development are in fact ongoing (Rosales-Mendoza et al. 2012). These cases are analyzed throughout this book.

Schizochytrium sp.

Schizochytrium sp. is a globose and pale yellow marine microalgae (thraustochytrid), closely related to heterokont algae, that possesses a thallus thin-walled. β-carotene is the only carotenoid pigment in this species and up to 40 % of its total

fatty acids is DHA (Yokoyama and Honda 2007). *Schizochytrium* can be propagated at the industrial scale in heterotrophic conditions in which low cost medium is used and no complex photobioreactors are required since the process does not depend of light irradiation. Since *Schizochytrium* is grown in marine water-based culture media, its industrial uses do not interfere with fresh water sources used for agriculture (Ren et al. 2010). This microalga is currently used as food supplement in mammals and poultry (Meale et al. 2014). A vaccine candidate against Influenza virus has been produced in this species (see Chap. 3).

Prospective View

The BFs market demands innovations in production and delivery. Most importantly, since costs are not affordable for developing countries, platforms requiring low infrastructure and having low production cost are needed. Algae constitute a robust platform for the production of biopharmaceuticals. Therefore, one alternative consists on the production of biosimilars, next generation molecules, or even biobetters in algae species since these hosts offer remarkable advantages over the conventional ones. Further chapters of this book will discuss the tools involved in this technology, also specific cases in which algae are a promising source of new products will be presented.

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Chapter 2

Genetic Engineering Approaches for Algae

Introduction

Genetic engineering constitutes the essence of the modern biotechnology that has allowed many strides in the medical biotechnology. The concept of modifying DNA with the aid of restriction enzymes was reported in 1971 using SV40 DNA (Danna and Nathans 1971). This study used the *EcoRI* restriction endonuclease, which was previously discovered by the groups headed by Herb Boyer (Yoshimori 1971). Further pioneering studies performed by Herb Boyer and Stan Cohen consisted in using DNA ligase to join a DNA molecule with “sticky” ends, produced by digestion with *EcoRI* (Cohen et al. 1973), to a plasmid DNA molecule previously digested with *EcoRI*; thus having complementary ends. The recombinant DNA was cloned into the easily grown *E. coli*. Since then, a multibillion-dollar biotechnology industry was developed based on this technology.

Superior organisms were subsequently subjected to genetic modification with success. For instance, plant transformation was achieved by using *Agrobacterium tumefaciens* as transformation vector (Herrera-Estrella et al. 1983). The first genetically modified alga was subsequently generated using the model microalgae *Chlamydomonas reinhardtii*, which was transformed at the nuclear level (Debuchy et al. 1989; Kindle 1990). To date, several other species have been successfully subjected to genetic engineering approaches. On the other hand, chloroplast transformation mediated by particle bombardment was achieved for *C. reinhardtii* almost at the same time than nuclear transformation (Blowers et al. 1989; Boynton et al. 1988). This chapter provides a guide on the genetic engineering procedures in algae, from gene design and vector construction to the transformation itself.

Construction of Genes and Expression Vectors

The first step on obtaining a transgenic organism consists in isolating the gene of interest and constructing an appropriate DNA vector that allows for the transfer, insertion, and expression of the transgene. The design and synthesis of the transgene encoding the biopharmaceutical (BF) of interest takes into consideration several aspects. For instance, a particular organelle can be selected for protein accumulation or the protein can be rather secreted into the culture medium. For protein secretion, specific signal peptides are genetically fused to the open reading frame (ORF) of interest; which is inserted in the nuclear genome. Specific tags can be also genetically fused to facilitate protein detection or protein purification (Terpe 2003; Fig. 2.1). Gene design also comprises the inclusion of flanking restriction sites to facilitate the expression vector construction through cloning steps. Once defined the above mentioned aspects, the full-length coding sequence can be obtained from cDNA libraries or genomic libraries and modified by molecular cloning steps to obtain the selected design. An alternative consists in obtaining the gene by chemical/enzymatic synthesis. A number of companies currently offer the gene synthesis service, which greatly facilitates this task. Gene synthesis is based on a combination of organic and molecular biology techniques that achieve the production of genes without the need of a precursor template DNA (Hoover and Lubkowski 2002).

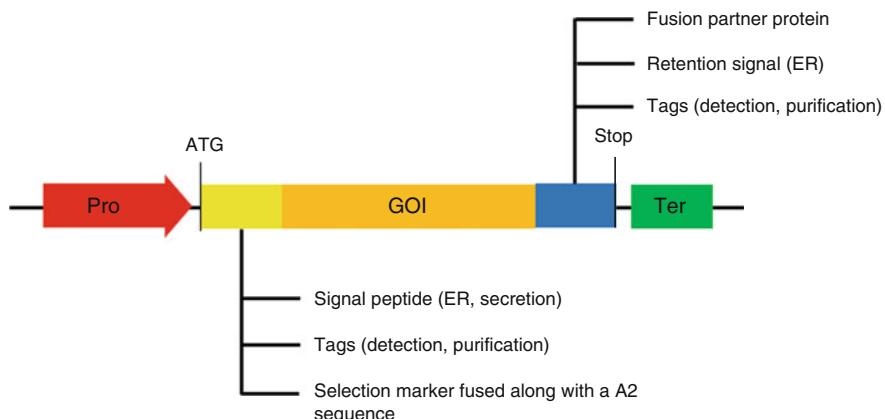


Fig. 2.1 Illustration of the elements useful in the design of transgenes. The open reading frame of interest is defined according to the specific target protein sequence. Several protein fusions can be produced for particular objectives: tags for easy detection or purification; signal peptides or retention signals for specific subcellular localization or secretion (e.g. endoplasmic reticulum, ER); to enhance expression the BF can be genetically fused to the selection gene marker along with the 2A picornaviral sequence that allows for the release at the translational level of the individual BF and the antibiotic resistance enzyme, or to a protein partner that enhances protein yields (e.g. large subunit of ribulose bisphosphate carboxylase) along with a processing sequence to recover the BFs

Gene synthesis has the advantage of allowing the optimization of codons according to codon usage in the target host, which maximizes the translation rate (Heitzer et al. 2007; Quax et al. 2015). Several groups have confirmed the influence of codon optimization on protein yields (Surzycki et al. 2009). In fact, codon optimization has been identified as a key factor not only for translational efficiency but also for transcript stability; while unfavorable GC content leads to poor gene expression due to induction of heterochromatinization (Barahimipour et al. 2015). In addition, the removal of undesired introns or restriction sites can also be conveniently performed during the design of synthetic genes. After isolation, the gene of interest is generally cloned into a standard cloning vector for confirmation purposes through sequencing. A subsequent subcloning step is required to assemble the expression vector.

Basic and common elements in expression vectors include a selectable marker expression cassette (comprising promoter/selectable marker gene/terminator) and a Gene of Interest (GOI) expression cassette (comprising a promoter, the ORF of interest, and a terminator or 3'UTR) (Fig. 2.2i). In addition, some elements that are

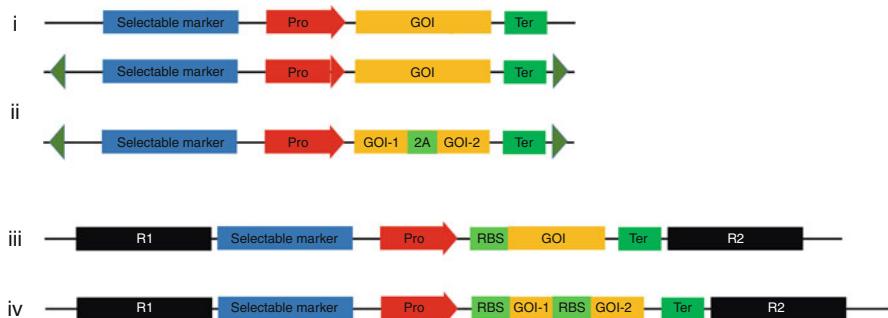


Fig. 2.2 Graphic description of algae expression vectors. Distinct strategies can be implemented for transgene installation in algae. Basic and common elements in such vectors include: a selectable marker expression cassette (in blue) comprising promoter/selectable marker gene/terminator 3'UTR, and a gene of interest (GOI) expression cassette comprising a promoter (*Pro*, in red), an open reading frame (GOI), and a terminator or 3'UTR (Ter). When nuclear transformation is intended, the expression vectors can consist of (i) a minimal vector containing the required elements for expression of the GOI and selection of transformants, which are used under physical transformation methods; or (ii) binary vectors, which are functional for *A. tumefaciens*-mediated transformation and contain the borders that mediate the transfer of the foreign DNA recognized in trans by the *A. tumefaciens* machinery (vir proteins). The 2A picornaviral sequence (in green) can be employed for the expression of two independent proteins through a single transformation event, where a ribosome skip mechanism allows splitting the polypeptides during translation. Chloroplast transformation vectors comprise, in addition to the regulatory and coding sequences, one of two regions that mediate homologous recombination with the plastome; such sequences are plastome sequences targeting homologous recombination to achieve the insertion at a specific point in the chloroplast genome. Chloroplast vector can be monocistronic (iii) or bi- poly-cistronic (iv). The latter are based on the use of distinct ORFs, each of them containing a ribosome binding site (RBS, in green) but transcribed under the same promoter

specific to the transformation method may be included. When performing nuclear transformation mediated by a physical method (such as electroporation, glass bead treatment, or particle bombardment), the expression vector consists of a minimal arrangement comprising the required elements for expression of the GOI and selection of transformants. In this case it occurs a random integration of the heterologous DNA. On the other hand, binary vectors are used when nuclear transformation mediated by the bacteria *Agrobacterium tumefaciens* is pursued. These vectors possess two replication origins: one functional in *E. coli*, where the plasmid is propagated in the subcloning steps required during vector construction and confirmation; and a second replication origin that is functional in *A. tumefaciens*, which is the host that mediates the transfer of the heterologous DNA in the form of a T-DNA/vir proteins complex. The particular elements in binary vectors are the borders (left and right, named LB and RB; respectively), which mediate the transfer of the foreign DNA as an artificial T-DNA that is recognized in *trans* by the *A. tumefaciens* machinery (vir proteins) (Fig. 2.2ii).

A recently developed technology comprises transcriptional fusions that allow for the nuclear expression of more than one independent protein from a single expression cassette. This approach is based on the 2A picornaviral sequence that activates a ribosome skip mechanism during translation (Fig. 2.2ii). This approach has allowed for the construction of functional bi or multicistronic expression vectors (Rasala et al. 2012, 2014), which is explained in detail in the section -Recent advances for transgene expression in the model alga *C. reinhardtii*.

Regarding chloroplast transformation vectors, these comprise in addition to the basic regulatory elements and the ORF of interest, one or two flanking regions that mediate homologous recombination with the plastome; such sequences come from chloroplast genome regions, they are isolated from chloroplast DNA and subsequently cloned into the transformation vector in a manner that flank the expression cassettes to be transferred to the target host at an specific insertion site (Figs. 2.2iii and 2.3). Vectors having only one recombination region induce a single recombination event with the limitation that the insertion of the whole plasmid vector occurs as a reversible integration (Goldschmidt-Clermont 1998). Therefore, transgene stability depends on maintaining selective pressure (Fig. 2.4; Purton 2007).

Interestingly, the expression in chloroplasts offers the possibility of polycistronic arrangements. This is due to the prokaryotic-like machinery of plastids, where ribosome binding sites (RBS) can mediate the translation of different ORFs present in a transcript generated in a process driven by a single promoter (Fig. 2.2iv).

In terms of tools for molecular cloning techniques that allow for vector construction, the conventional cloning mediated by restriction enzymes/ligase provides a reliable approach but demands time and a detailed design of the cloning strategies. However new technologies are facilitating the assembly of vectors. For instance, Gateway technology relies on performing molecular cloning procedures through the recombination machinery from the phage λ . Interestingly, this technology has been applied to the construction of chloroplast transformation vectors and has the advantage of providing faster and highly efficient cloning procedures that are not limited to specific restriction sites (Oey et al. 2014).

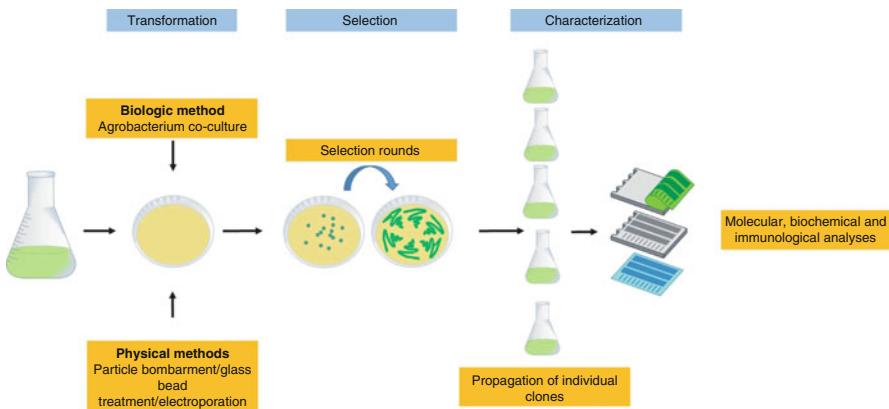


Fig. 2.3 A general workflow for algae transformation. (i) Axenic cultures of the target species are established. (ii) Cells are subjected to DNA transfer through the chosen technique (Agrobacterium infection or physical methods). (iii) Cultures are further subjected to selection (also Agrobacterium killing step, when required). For chloroplast transformation several rounds of selection are required to achieve homoplasmy and ensure stable transformation events. (iv) Individual clones are analyzed to determine the transgene presence and recombinant BFs properties (yields, in vitro and in vivo activity, etc.)

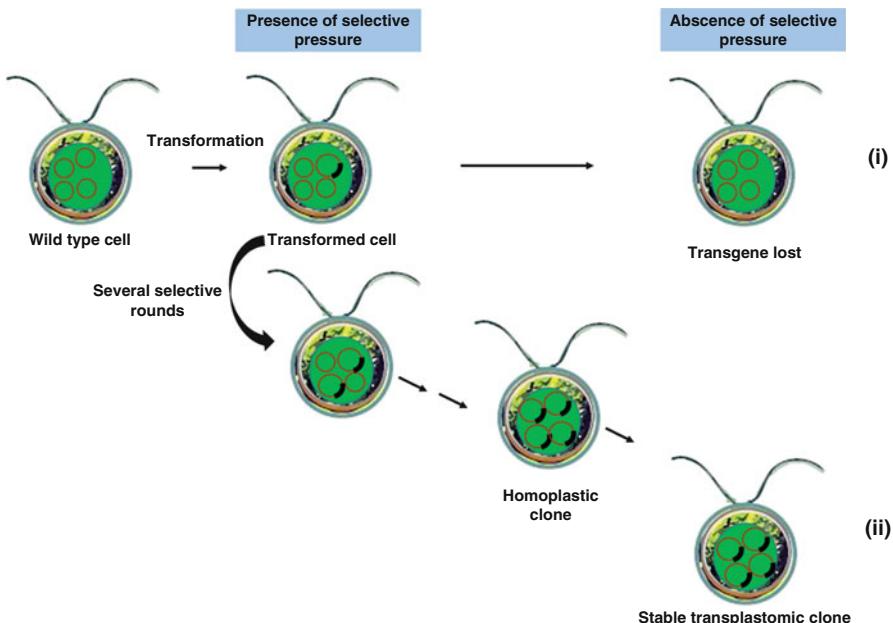


Fig. 2.4 Development of homoplasmic strains. When chloroplast transformation is pursued, a critical aspect is the generation of homoplasmic clones. Since the chloroplast genome is polyploid (up to 100 genome copies per chloroplast), initial transformation events possess only few transformed plastomes. Therefore, selective pressure is required during a long period of time (several culture rounds) to allow for the accumulation of transformed plastomes eliminating the wild type ones (i). This procedure ensures the stability of the transplastomic state; otherwise, once selective pressure is eliminated, wild type genomes will predominate and transgene will be eventually lost (ii)

Transformation Techniques

The development of genetic engineered algal strains shares the following common steps: (i) establishment of axenic cultures of the target species; (ii) conducting the transformation procedure by the proper methodology; (iii) selecting transformed clones, which is typically performed by adding antibiotics to the culture medium; (iv) propagating the selected clones; and (v) characterizing the candidate clones at the molecular and functional levels by detecting the transgene, expression patterns, and activity of the target BF (Fig. 2.5). Several technologies to develop genetic engineered algae strains have been developed thus far being described in the present section (Table 2.1).

Agrobacterium tumefaciens

The transformation mediated by *A. tumefaciens* relies on its ability to serve as biological vehicle to transfer heterologous DNA into the target cells. A number of proteins derived from both the target host and the bacterial pathogen are involved in this complex process (Gelvin 2012). In the natural infection *A. tumefaciens* causes crown gall tumors on various plant species, including many important crops; which is consequence of the replication of a single-stranded copy of the bacterial transferred (T)-DNA located on the tumor-inducing (Ti) plasmid that is transferred and

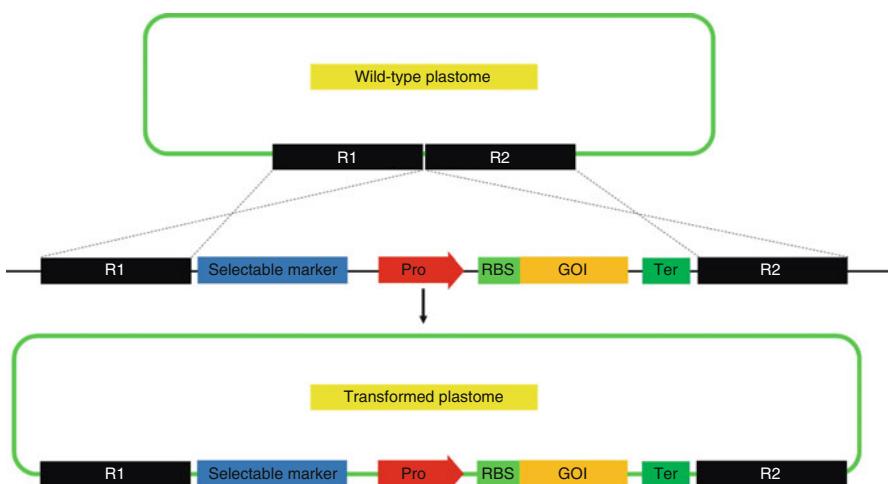


Fig. 2.5 Schematic representation of a typical vector for site specific insertion of expression cassettes into algae chloroplast genome. The foreign DNA (the gene of interest and regulatory sequences) is flanked by homologous regions from the target plastome, which mediate a double crossover leading to the insertion of the heterologous DNA into the target insertion site at the plastome. This approach allows for the development of stable transplastomic clones that are selected with the corresponding antibiotic according to the selectable marker included in the vector

Table 2.1 Features of the transformation methods available for algae species

| Method | Cost | Integration pattern | Special requirements |
|----------------------------------|-----------|---|-----------------------------|
| Glass bead method | Low | Low heterologous DNA definition High copy number | Cell wall deficient strains |
| <i>Agrobacterium tumefaciens</i> | Low | High heterologous DNA definition Low copy number | Biological compatibility |
| Electroporation | High | Low heterologous DNA definition | Specialized equipment |
| Particle bombardment | Very high | Low heterologous DNA definition | Specialized equipment |

integrated into the genome of the target cell. The T-DNA encodes several genes involved in auxin and cytokinin biosynthesis, whose expression alters cell cycle having as consequence the formation of tumors. On the other hand a set of T-DNA genes are involved in the biosynthesis of opines, which are metabolized mainly by Agrobacterium (Bourras et al. 2015). These sophisticated mechanisms allow Agrobacterium to be an efficient phytopathogen that controls the plant cell, which serves as its source of valuable metabolites. Although Agrobacterium mainly infects dicotyledonous plants in nature, it has been applied in the algae genetic engineering arena providing an efficient tool to genetically modify a number of species (Tzfira and Citovsky 2006).

The native T-DNA has been adapted for genetic engineering purposes by removing the hormone and opine biosynthesis genes. The most used methodology relies on the use of a binary vector that contains an artificial T-DNA comprising expression cassettes for a selectable marker gene and one or more genes of interest, which are flanked by the T-DNA left and right border sequences. The T-DNAs are engineered in *Escherichia coli* and subsequently transferred into the Agrobacterium strain containing a T-DNA-less Ti plasmid (“helper” Ti plasmid), which provides the virulence (vir) functions required for the processes of T-DNA transfer and integration into the plant genome (Fig. 2.2ii). Algae transformation comprises a co-culture step of the Agrobacteria containing the engineered vectors with alga cells. The selection process is performed subsequently by transferring the cells to antibiotic-containing medium, which is selected according to the marker gene used.

Remarkably, the Agrobacterium-mediated insertion leads to the stable integration of intact heterologous DNA in one or a reduced number of loci with minimal genomic rearrangements (Gheysen et al. 1991). Based on these characteristics, the Agrobacterium-mediated transformation is the method of choice for over-expression or down-regulation of genes of interest in functional basic research or for the generation and commercialization of superior crops. This last application is based on the precise replication through the T-DNA border sequences upon infection and the low-copy T-DNA insertion into the genome. It should be considered that chloroplasts transformation by means of *A. tumefaciens* is not successfully accomplished, thus a number of alternative transformation approaches have been developed including physical methods for the DNA transfer.

Biolistic

Biolistic constitute the most efficient procedure to transfer DNA into organelles, in particular chloroplasts. The technique consists in bombarding, with microparticles, the target cells placed on a cultivation agar plate under vacuum. Gold or tungsten particles of 0.5–1 µm in diameter are used. These particles are previously coated with the expression vector DNA with the aid of calcium chloride and spermidine (Sanford 1990). The accelerated particles cross the cell wall, the plasma and chloroplast membranes allowing for the vector DNA delivery into the organelle (Taylor and Fauquet 2002). Although less used, this method can also achieve nuclear integration of the transgene constituting an alternative for that purpose.

In particular, when transplastomic approaches are pursued, several rounds of selection must be performed to eliminate the untransformed genomes and achieve both homogeneity in the transgenic state of the organism and maximum productivity. This stage is called homoplasmy and represents an important aspect that ensures transformation stability since, once the selection pressure is removed, no WT genomes exist and therefore there is no risk of reversion to the WT state. In contrast, chimeric organisms (heteroplasmy state) are prone to transgene lost once the selection pressure is removed (Purton 2007; Fig. 2.6). The main limitation of this methodology is the requirement of specialized instrumentation. Also, when

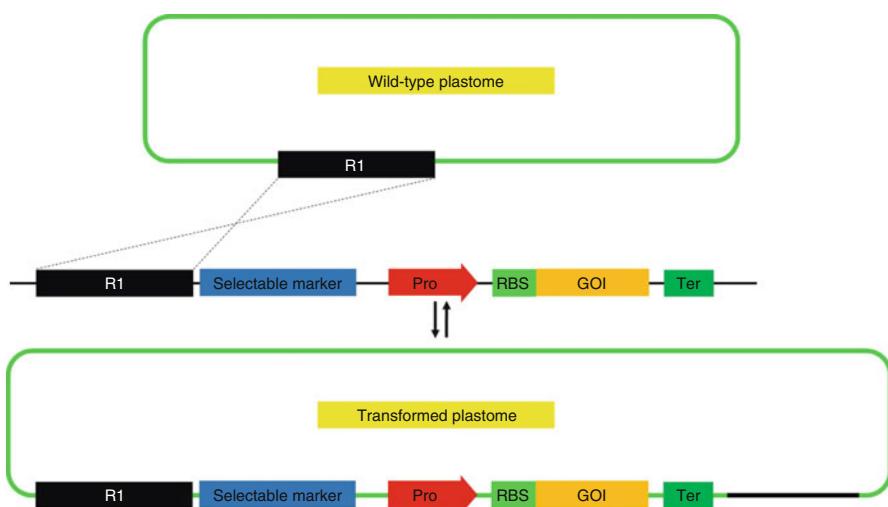


Fig. 2.6 Schematic representation of a single recombination chloroplast transformation vector for algae. The foreign DNA (the gene of interest and regulatory sequences) is placed next to a homologous region that mediates a single crossover leading to the insertion of the full-length plasmid vector into the target site at the plastome. This approach allows for the development of reversible transplastomic clones that are selected with an antibiotic according to the selectable marker included in the vector

nuclear transformation is pursued, complex integration patterns and multiple copy insertions are frequently observed, which are associated to gene silencing.

Glass Beads Treatment

Alternative physical methods for algae transformation include the treatment of the target cells, in the presence of the vector DNA and polyethylene glycol, with glass beads under shaking with the aid of a vortex (Kindle 1990). This procedure can yield up to 1000 transformants per µg of DNA. Nevertheless this protocol is essentially restricted to strains lacking of cell walls (induced by mutations or enzymatic treatment), which imposes some limitations since they are more sensitive to shearing stress. The advantages of this method over the particle bombardment include its simplicity, low cost, (does not require access to specialized equipment) and a general low copy number of the integrated transgene.

Electroporation

Electroporation is a genetic transformation technique that consists in applying an electrical field to cells in order to increase the permeability of the cell membrane, which allows introducing heterologous DNA into the cell (Neumann et al. 1982). The electroporation method for Chlamydomonas was established by Brown et al. (1991). Initially, cell wall deficient strains were transformed using this method, in which important variables include in this method include osmolarity, temperature, exogenous DNA concentration, voltage, and capacitance. The optimized method allows for an efficiency of up to 2×10^5 transformants per µg of DNA (Shimogawara et al. 1998), which is two orders of magnitude higher than the efficiency of the glass beads method. The advantages of electroporation are its application to species having cell wall and its high efficiency. The main disadvantages comprise random integration and the requirement of specialized equipment. However, it should be considered that the transgene copy number is lesser in electroporation when compared to the particle bombardment method (Kindle et al. 1989).

Expression Modalities

Nuclear and Chloroplast-Based Expression

Nuclear and chloroplast-based expression can be applied for the production of bio-pharmaceuticals (BFs) and each alternative has singular features and limitations that should be considered case by case based on the characteristics of the BF of

interest. It should be consider that chloroplast and nuclear expression lead to differential contexts in terms of protein processing.

Chloroplast is an organelle present in photosynthetic eukaryotic algae where several biosynthetic pathways occur. For instance the synthesis of fatty acids, amino acids, and isoprenoids occur in this organelle. Chloroplast possesses a circular genome (plastome) and the transcriptional and translational machinery to synthesize proteins. In fact, unlike bacterial hosts, the *C. reinhardtii* chloroplast, which occupies up to 40% of the cellular volume, possesses chaperones (Schroda 2004), peptidyl propylisomerases (PPIases) (Breiman et al. 1992), and protein disulfide isomerases (PDIs) (Levitin et al. 2005); which enable the proper synthesis of complex proteins requiring disulfide bonds for correct folding (Tran et al. 2013).

Another interesting feature is given by the chloroplast capability to express genes arranged in co-transcribed operons. For the production of BFs at large scale, attaining high protein yields is a priority. Chloroplast-based expression offers high protein productivity due to high expression derived from the high copy number of transformed genomes per chloroplast and a general absence of gene silencing with no position effects since the integration is site-directed and mediated by homologous recombination (Bock and Warzecha 2010; Maliga 2002).

Limitations to consider for the chloroplasts-expressed BFs consist in the lack of complex post-translational modifications, such as glycosylation, that is of relevance for some BFs. In addition, it should be considered that protein secretion into the culture media is not possible under chloroplast-based expression.

In contrast, the expression of nuclear-encoded proteins is a different scenario since proteins can be subjected to glycosylation when directed to the secretion route by the inclusion of specific leader peptides in the BFs sequence (Rasala et al. 2014). One alternative consists in inducing the secretion of the recombinant protein into the culture medium, which allows for an easy recovery and purification of the BFs (Rasala et al. 2012). On the other hand, retention signals can allow for accumulating the BF in the endoplasmic reticulum (ER) lumen.

Inducible Expression

Inducible expression systems have also been reported for algae species, generating important perspectives for the BFs production field since these approaches could make viable the expression of proteins with toxic effects in the algae; or allow optimizing the productivity.

One inducible system reported by Surzycki et al. (2007) consists in placing, at the nuclear level, the *Nac2* gene under the control of the copper-repressible nuclear *Cyc6* promoter (Merchant and Bogorad 1987). Since the nucleus-encoded chloroplast *Nac2* protein is required for the expression of plastid genes that are under the control of the *psbD* 5'UTR (Nickelsen et al. 1994), the chloroplast *psbD* mRNA of the PSII D2 reaction center protein was controlled under this approach.

The usefulness of inducible expression has been proven in the case of the expression of the *DILP-2* gene, which codes for a growth promoter. While no transformed clones were recovered when the gene was driven by a constitutive promoter, the inducible genetic system based in the *Nac2* protein allowed rescuing transformed clones expressing the target protein; which strongly suggests that the target protein is toxic and only the inducible expression allows for its production in alga (Surzycki et al. 2009).

A subsequent system was developed relying on the thiamine pyrophosphate (TPP)-responsive riboswitch from *C. reinhardtii*, where the addition of thiamine (vitamin B1) to the culture prevents expression of *Thi4* and *ThiC* due to an alternative splicing of their transcripts (Croft et al. 2007). Combining the use of the *Nac2* gene and the *Thi4* 5'UTR (TPP riboswitch), the control of *psbD* expression was achieved in a reversible way. The vitamin-repressible riboswitch was initially used to get insights on the role of two essential chloroplast genes (ribosomal protein S12, *rps12*, encoding for a plastid ribosomal protein; and *rpoA*, encoding for the α -subunit of chloroplast bacterial-like RNA polymerase), observing a successful repression of the target genes and relevant changes in terms of cell growth and gene expression profiles (Ramundo et al. 2013).

In the case of the species *Volvox carteri*, the promoter of the nitrate reductase gene (*nitA*) has shown to serve as a proper element for inducible transgene expression. The induction is given by the change of ammonium by nitrate as the nitrogen source (von der Heyde et al. 2015). An element that will also have implications in this area is a 44-base-pair region from the *C. reinhardtii* LHCBM9 promoter, which is essential for sulfur responsiveness. The photosynthetic light-harvesting complex (LHC) proteins play essential roles both in light capture, the first step of photosynthesis, and in photoprotective mechanisms. In contrast to other LHC proteins and the majority of photosynthesis proteins, LHCBM9 is a photosystem II-associated LHC protein that is up-regulated under sulfur deprivation conditions. Promoter deletion analysis revealed that the minimal region responsible for the responsive to sulfur deprivation is -136 and -180 relative to the translation start (Sawyer et al. 2015). These findings will be useful for the development of inducible expression systems based on sulfur deprivation.

Park et al. (2013) have characterized the promoter of the light-inducible protein gene (LIP) of *Dunaliella sp.*, which contains several light-responsive motifs. The light-responsive region was identified through evaluation of a truncated version of the promoter fused to a reporter gene. The promoter responded to high light (HL) in a light intensity-dependent manner. The fact that the truncation to a 100 base pair (bp) region abolished light induction suggests the presence of a negative cis-regulatory element upstream of the 100 bp fragment. This promoter is thus proposed as a tool to generate transgenic algae under high-light conditions.

These representative examples indicate that the characterization of inducible promoters is an active research field that is generating new tools to sophisticate the expression systems for recombinant proteins in microalgae, thus advances in this field are expected in the following years.

Overview of Algae Transformation Achievements

Since the end of the 80s several algae species have been successfully genetically modified (Table 2.3), most of these at the nuclear level. Ten species of green algae have been transformed. Stable transformation has been achieved for seven of them, including the unicellular model organism *Chlamydomonas reinhardtii* (Debuchy et al. 1989; Kindle et al. 1989); while only transient transformation was demonstrated in the other three. This group of species includes: two multicellular species, *Volvox carteri* (Schiedlmeier et al. 1994) and *Ulva lactuca* (Huang et al. 1996); and six species of red algae (two unicellular and one is the ultrasmall unicell *Cyanidioschyzon merolae*) (Minoda et al. 2004). Two multicellular species come from the genus *Porphyra* (Cheney et al. 2001) and *Kappaphycus* (Kurtzman and Cheney 1991). The group also includes *Gracilaria* (Gan et al. 2003). Stable transformation has also been shown for two brown macroalgae from the order Laminariales, *Laminaria japonica* (Qin et al. 1999) and *Undaria pinnatifida* (Qin et al. 2003). Laminaria is extensively farmed in China, Korea, and Japan and it is harvested from wild stands in several other countries. In the case of diatoms, stable transformation of four diatom species has been reported. The transformed dinoflagellates comprise *Amphidinium* and *Symbiodinium* (ten Lohuis and Miller 1998), and one euglenid; *Euglena gracilis* (chloroplast transformation).

Several promoters, terminators, and UTRs to efficiently drive the expression of transgenes at the nuclear and chloroplast level are available. A recent compilation of the regulatory elements and reporter genes is presented in Table 2.2 (nucleus) and Table 2.3 (chloroplast). The development of genetic engineering tools for algae is also an active research area. In the recent years several protocols have been implemented for several species including *Nannochloropsis*, *Volvox carteri*, *Penium margaritaceum*, *Parachlorella kessleri*, *Eudorina elegans*, among others. In addition, new protocols for some species and new selection markers have been reported (Table 2.4).

An interesting achievement in the last years consisted in advances in the genetic modification of the mitochondria genome through the expression of marker and reporter genes (Hu et al. 2011, 2012). Although a straightforward implication of this technology in the molecular pharming field has not been defined, these approaches may have positive impact in understanding the biology of algae. It is envisioned that genetic engineering at the mitochondrion level may lead to strains with improved metabolism/growth capacity.

Of particular interest is the report of efficient homologous recombination at the nuclear level for the species *Nannochloropsis sp*. This kind of report opens key perspectives for the implementation of gene targeting approaches in the benefit of the molecular farming field, such as developing glycoengineering strategies as in the case of other organisms (e.g. *Physcomitrella patens*; Rosales-Mendoza et al. 2014; see Chap. 9).

Table 2.2 Regulatory elements available for the expression of transgene in *C. reinhardtii* at the nuclear level

| Functional element | Name | Phytozyme gene ID | Property | Reference |
|---------------------|-------------------------|--|---|---|
| Promoters | HSP70A | Cre08.g372100 (196 bp upstream to ATG) | Typically used as constitutive; expression can be enhanced by high light and high temperature | Schroda et al. (2000) |
| | RBCS2 or HSP70A/RBCS2 | Cre02.g120150 (180 bp upstream to ATG) | Strong constitutive (refer to HSP70A) | Lumbrieras et al. (1998) |
| | PSAD | Cre05.g238332 (822 bp upstream to ATG) | Typically employed as strong constitutive; expression may be enhanced by high light | Fischer and Rochaix (2001) |
| | CYC6 | Cre16.g651050 (127 bp upstream to ATG) | Metal (Cu) responsive | Quinn and Merchant (1995) |
| | NIT1 | Cre09.g410950 (282 bp upstream to ATG) | Ammonium responsive | Ohresser et al. (1997) |
| | ATX1 | Cre09.g392467 (532 bp upstream to ATG) | Iron (Fe) responsive | Fei and Deng (2007) |
| | CA1 | Cre05.g248400 (194 bp upstream to ATG) | CO ₂ responsive | Villand et al. (1997) |
| | SQD2 | Cre01.g038550 (75 bp upstream to ATG) | Phosphate (P) responsive | Iwai et al. (2014) |
| | CAMV 35S | – | Enhancer and minimal promoter only | Ruecker et al. (2008) |
| Regulatory elements | METE | Cre03.g180750 (–574 to –89 bp from ATG) | Cobalamin (B12) suppression | Helliwell et al. (2014) |
| | RBCS2 intron 1, 2 and 3 | Cre02.g120150 | Enhance gene expression as intron in coding region or 5'UTR | Eichler-Stahlberg et al. (2009), Lumbrieras et al. (1998) |
| | THI4 | Cre04.g214150 (1414 bp 50UTR) | 5'UTR, thiamine (B) suppression | Croft et al. (2007), Moulin et al. (2013) |
| | RBCS2 | 32 aa | Rubisco small subunit 2 chloroplast transit peptide | Leon et al. (2007) |

(continued)

Table 2.2 (continued)

| Functional element | Name | Phytozyme gene ID | Property | Reference |
|-------------------------------|--|---------------------------------------|--|---------------------------------|
| Functional peptides | FD | 32 aa | Ferredoxin chloroplast targeting transit peptide | Leon et al. (2007) |
| | ARS2 | Cre16.g671350 (63 bp 50 end from ATG) | N-terminal secretion | Eichler-Stahlberg et al. (2009) |
| | 2 × simian virus 40 (SV40) | 20 aa | N-terminal nuclear target peptide | Rasala and Mayfield (2014) |
| | Foot-and-mouth disease virus (FMDV) 2A | 20 aa | Translational cleavage peptide | Rasala et al. (2012) |
| | Gaussia luciferase (gLuc) | 555 bp | Luciferase assay | Ruecker et al. (2008) |
| Reporter genes | GFP, mCHERRY, EYFP, DsRED, tdTOMATO, VENUS | 708–1437 bp | Fluorescent protein | Rasala et al. (2013) |
| | ARS | Cre16.g671400 (3012 bp cDNA) | Chromogenic assay | Davies et al. (1992) |
| Highly expressed host strains | UVM4 and UVM11 | – | High expression host, transgene silencing suppressed | Neupert et al. (2009) |

Adapted from Scaife et al. (2015)

Advances for Transgene Expression in the Model Alga *C. reinhardtii*

Multigene Expression and Organelle Targeting

Since *C. reinhardtii* is the species used as model in the field of algae biotechnology, most of the innovations in terms of genetic engineering have been achieved with it (Table 2.5). One notable challenge when nuclear expression is pursued in *C. reinhardtii* is transgene silencing (Cerutti et al. 1997; Fuhrmann et al. 1999; Neupert et al. 2009). An innovation in this field to overcome this limitation has been recently developed, consisting in a transcriptional linkage between the gene of interest and the gene coding for the marker gene *Ble*; through the 2A self-cleaving sequence (Rasala et al. 2012). Under this configuration, both proteins are produced separately at the translational level but transcribed from a single expression cassette.

Another key improvement has consisted in exploring specific protein localizations. For example, secretion approaches have been reported relying on the use of a secretion signal from the native extracellular *C. reinhardtii* carbonic anhydrase.

Table 2.3 Regulatory elements available for the *C. reinhardtii* chloroplast

| Functional element | Name | Properties | Source |
|--|-------------------------------|--|---|
| Transcriptional leaders | AtpA, psbD | Constitutive expression | Barnes et al. (2005) |
| | psaA-exon1 | Strong expression | Michelet et al. (2011) |
| Chloroplast-targeted peptides | psaD | 35 aa N-terminal | Fischer and Rochaix (2001) |
| | rbcS2, FD | 32 aa N-terminal | Leon et al. (2007) |
| Selectable marker | rrnS and rrnL point mutations | Spectomycin and erythromycin resistance | Kindle et al. (1991) |
| | arg9 | Arginine complementation | Remacle et al. (2009) |
| | aadA | Spectinomycin resistance | Goldschmidt-Clermont (1991) |
| | aphA6 | Resistance kanamycin | Bateman and Purton (2000) |
| | GFP | Fluorescent reporter | Franklin et al. (2002) |
| | gusA | b-Glucuronidase activity | Sakamoto et al. (1993) |
| | atpB | 5-Fluorodeoxyuridine resistance | Kindle et al. (1991) |
| | coda | Cytosine deaminase sensitivity to 5-fluorocytosine | Young and Purton (2014) |
| | p322 | psbA and 16S rRNA | Manuell et al. (2007) Barnes et al. (2005) |
| Destination sequences for homologous recombination | patpint-cg11 (atpB-int) | Inverted repeat and atpB 30UTR | Nickelsen et al. (1994) |
| | pLM7 (IR-int) | psbA and 5S/23S | Michelet et al. (2011) |
| | p72B | psbH and psbN | Bateman and Purton (2000) |
| | p71 | tscA and inverted repeat | Kindle et al. (1991) |

Adapted from Scaife et al. (2015)

Chloroplast functional parts are classified as transcriptional leaders (promoter and 5'UTR), chloroplast-targeted peptides, selectable markers, and destination sequences for homologous recombination

This approach allowed for an efficient secretion of the *Gaussia luciferase* marker into the culture medium of the *Chlamydomonas* wild type CC-1883 (WT) and in the mutant UVM4. The productivity reached up to 10 mg of recombinant protein per liter of culture. Secretion approaches are particularly useful since purification of the protein is greatly facilitated by processing the culture media, which is easily separated from the biomass containing a reduced number of non-target molecules (Lauersen et al. 2013).

In a similar approach Rasala et al. (2012) achieved the expression of the xylanase 1 (Xyn1) from *Trichoderma reesei*, which is an endo-β-1,4-xylanase (EC 3.2.1.8) that functions in the hemicellulose breakdown by hydrolyzing xylan and xylo-oligosaccharides. Such type of enzymes is of industrial relevance since they

Table 2.4 Recent advances on algae genetic engineering protocols in terms of new species transformed, new selection markers, and DNA transfer approaches

| Species | Transformation method | Gene marker | Transformation level | References |
|---|--|---|----------------------|--|
| <i>Nannochloropsis gaditana</i> , <i>N. oceanica</i> , <i>N. granulata</i> , <i>N. salina</i> , and <i>N. oculata</i> | Electroporation | <i>sh ble</i> | Nuclear | Radakovits et al. (2012), Kilian et al. (2011), Vieler et al. (2012), Li et al. (2014) |
| <i>Volvox carteri</i> | Particle bombardment | aminoglycoside 3'-phosphotransferase VIII (aphVIII) | Nuclear | Lerche and Hallmann (2014) |
| <i>Chlamydomonas reinhardtii</i> | Electroporation | Synthetic glyphosate acetyltransferase (GAT) gene for glyphosate resistance; Protoporphyrinogen oxidase (protox, PPO) for oxyfluorfen resistance; Phytoene desaturase (PDS) gene for norflurazon resistance | Nuclear | Brueggeman et al. (2014) |
| <i>C. reinhardtii</i> | Glass bead | tetX | Nuclear | Garcia-Echauri and Cardineau (2015) |
| <i>Penium margaritaceum</i> | Agrobacterium tumefaciens | hptII | Nuclear | Sørensen et al. (2014) |
| <i>Parachlorella kessleri</i> | <i>A. tumefaciens</i> | hptII | Nuclear | Rathod et al. (2013) |
| <i>Chlorella vulgaris</i> | Particle bombardment | nptII | Nuclear | Talebi et al. (2013) |
| <i>Eudorina elegans</i> | Particle bombardment | aminoglycoside 3'-phosphotransferase VIII (aphVIII) | Nuclear | Lerche and Hallmann (2013) |
| <i>Chlamydomonas reinhardtii cell-walled</i> | Square electric pulses-generating electroporator | hygromycin-resistant gene aph7 | Nuclear | Yamano et al. (2013) |
| <i>Chlamydomonas reinhardtii</i> | Particle bombardment | Sh ble | Mitochondrial | Hu et al. (2011, 2012) |
| <i>Nannochloropsis gaditana</i> | Electroporation | Sh ble | Nuclear | Radakovits et al. (2012) |
| <i>Nannochloropsis sp.</i> | Electroporation | Sh ble | Nuclear | Kilian et al. (2011) |

Table 2.4 (continued)

| Species | Transformation method | Gene marker | Transformation level | References |
|--|-----------------------|--|----------------------|----------------------------|
| <i>Closterium peracerosum-strigosum-littorale complex (C. psl complex)</i> | Particle bombardment | ble (phleomycin-resistant) gene | Nuclear | Abe et al. (2011) |
| <i>Gonium pectorale</i> | Particle bombardment | aminoglycoside 3'-phosphotransferase VIII gene (aphVIII) | Nuclear | Lerche and Hallmann (2009) |

A compilation of the reports from the last 5 years is presented

Table 2.5 Recent advances in the expression of recombinant proteins in *C. reinhardtii*

| Type of expression | Key elements | Transformation method | Main outcomes | Yields | Reference |
|--------------------|--|-----------------------|---|----------------------------|------------------------|
| Nuclear | Secretion signal of the native extracellular <i>C. reinhardtii</i> carbonic anhydrase | Glass bead | Compared to the native secretion signal of the <i>Gaussia</i> luciferase, up to 84 % higher recombinant protein production could be achieved. | 10 mg per liter of culture | Lauersen et al. (2013) |
| Nuclear | Targeting protein localization by using nucleus, mitochondria, chloroplast, and endoplasmic reticulum | Electroporation | The peptide signals allow for targeting the protein into distinct organelles | Not reported | Rasala et al. (2014) |
| Nuclear | Multicistron vector comprised of the <i>Ble</i> marker gene arranged as multicistron along with reporter genes having distinct localization signals (nucleus endoplasmic reticulum, mitochondria, and chloroplast. | Electroporation | Targeting the independent reporter proteins in two distinct organelles independently (ER and nucleus) | Not reported | Rasala et al. (2014) |

(continued)

Table 2.5 (continued)

| Type of expression | Key elements | Transformation method | Main outcomes | Yields | Reference |
|--------------------|---|-----------------------|--|---------------------------------------|-----------------------|
| Nuclear | UV light induced mutagenesis and screening for clones resistance to high concentration of selective agent | Glass bead method | Silencing mechanism were knocked out | 0.2 % TSP for GFP | Neupert et al. (2009) |
| Chloroplasts | Genetic fusion of the protein of interest to the carboxy-terminal end of an abundant endogenous protein (large subunit of ribulose bisphosphate carboxylase), including the native protein-processing site from preferredoxin (preFd) | Particle bombardment | A notable increase on productivity was achieved obtaining a protein lacking fusion partner | Increase of 33 times on protein yield | Muto et al. (2009) |

are applied in baking, textiles, pulp, and paper manufacturing; animal feed, and also have implications in cellulosic biofuel production (Polizeli et al. 2005). In *C. reinhardtii* strains, xyn1 accumulation was enhanced by approximately 100-fold when co-expressed with the *ble* marker and splitted by the 2A self-cleaving sequence; in comparison to the individual expression of xyn1. Moreover, the use of the ars1 secretion signal peptide allowed for high expression and efficient secretion of the recombinant protein into the culture broth. These studies represent a breakthrough for the field since they offer very high yields and easy purification of the product of interest.

The Mayfield's group have also contributed with other advanced studies, considering that multi-gene engineering is required for therapeutics production (Keasling 2012) and metabolic engineering (Zhang et al. 2011); efforts to implement multi-gene expression and targeting proteins to distinct compartments in algae cells are required to expand the perspectives of the algae biotechnology.

Remarkably, further evaluations of the 2A-based bicistronic expression have been reported for distinct gene markers and proteins targeted to distinct cell compartments. The evidence indicate that the system is versatile since the gene markers provided the expected resistance phenotype and the native and foreign proteins of

interest bearing post-translational remnants of the extended FMDV 2A peptide retained the expected localization (Plucinak et al. 2015). Therefore, the 2A-based multicistronic approaches offer a reproducible and robust procedure for the production of BFs in algae species.

Another approach that was proposed to achieve multigene presence in a single strain was based in mating transgenic strains carrying a single transgene. This approach allows for the development of strains expressing four different reporter proteins, which were also properly targeted into distinct subcellular locations (the nucleus, ER, mitochondria, and flagella). Remarkably the transgene expression showed no major variation throughout the mating process, which suggests that gene silencing does not take place during gametogenesis or meiosis (Rasala et al. 2014).

Generation of Mutant Strains with High Productivity

Another important approach to achieve an efficient expression of BFs in algae consists of mutant strains developed as an attempt to improve expression of nuclear installed transgenes (Neupert et al. 2009). Under the hypothesis that low transgene expression is caused by an unusual tight chromatin structure or epigenetic processes, the group headed by Bock aimed at obtaining mutant clones through UV light exposure in which the transgene suppression mechanism is impaired. For measuring the efficiency of transgene expression the *CRY1-1* gene was used, which codes for a selectable marker whose expression level is in proportion with resistance to emetine (Nelson et al. 1994). The *CRY1-1* gene was co-transferred along with the *ARG7* selectable marker gene in an arginine-auxotrophic Chlamydomonas strain, expecting restoration of arginine prototrophy. Co-transformants were rescued and showed a phenotype of resistance to emetine at low concentrations (5–25 µg/mL), which represents a poor transgene expression. After UV mutagenesis, resistance to higher concentrations of emetine was observed suggesting that the genetic inactivation of the transgene suppression mechanism operating in Chlamydomonas occurred.

Ideally, this approach would represent the generation of strains able to express any transgene at high yields as a consequence of the elimination of the suppression mechanism. To test this hypothesis, the mutants were transformed with an the control of expression signals that were different from those driving the *CRY1-1* selectable marker gene and containing the paromomycin resistance gene APHVIII (Sizova et al. 2001). Three of the tested strains did not yield clones expressing GFP, while two of them resulted in GFP-expressing transformants at high frequency. About half of the obtained clones expressed GFP at high levels, suggesting that these two strains are mutants in which the epigenetic transgene suppression mechanism has been knocked out. Remarkably, the expression levels of GFP reached up to 0.2 % of total soluble protein (TSP) and the strains show no modification in the growth kinetics. An interesting phenomenon was the higher transformation efficiency observed

when another selectable marker gene was used. Therefore, these clones constitute a promising tool to develop clones expressing BFs at attractive levels.

Fusion to Protein Partners

A concept to enhance expression of recombinant proteins in the algal chloroplast has consisted in the genetic fusion of the protein of interest to the carboxy-terminal end of an abundant endogenous protein, such as the large subunit of ribulose bisphosphate carboxylase (Rubisco LSU). A further cleavable from the endogenous protein in vivo can be accomplished by including the native protein-processing site from preferredoxin (preFd) and thus the protein of interest is highly expressed and does not contain accessory unrelated sequences that are undesired for further applications. An increase of up to 33 times in protein yield has been reported under this approach (Muto et al. 2009).

The Transformosome Concept

An outstanding report stating the highest protein productivity in *C. reinhardtii* transplastomic clones (20 % of TSP) was reported by Surzycki et al. (2009). The authors presented an innovative concept that, rather than highlighting the relevance of the regulatory sequences used for expression, attributed the successful expression to other factors generating the concept of the transformosome; which supposes that individual transformed lines possess unique characteristics or transformation-associated genotypic modifications. This concept could explain the frequent cases of recalcitrant or low yielding clones in which some genetic events in transgenic strains may occur. The authors stated that “it is possible that in addition to inserting a transgenic gene into the chloroplast genome target site, additional insertions of the vector or DNA fragments also occur within the nuclear genome; this could result in a change in the function of nuclear gene (s) that regulate protein yield (such as proteases) or gene (s) that increase recombinant protein expression”. This is an innovative concept that may explain the lack of reproducibility commented by various research groups for obtaining clones expressing the expected protein. Therefore, the analysis of a very large group of candidate clones is recommended to increase the probability of rescuing high expresser clones.

These relatively recent advances add a new toolbox for algae biotechnology and are expected to accelerate the exploitation of *C. reinhardtii* for the production of high valuable proteins for various sectors, including the Pharma industry. In addition, all these developments achieved with the model microalga *C. reinhardtii* will be of key importance for translating the concepts to other species paving the way for the algae biotechnology field.

Prospective View

Significant advances in the field of genetic engineering approaches for algae species have been achieved over the last decade, consisting in new expression cassette configurations and high expresser mutant strains. In the model microalga *C. reinhardtii*, the chloroplast expression is almost considered as an optimized system with acceptable productivity, whereas nuclear-based expression has been improved but still requires further optimization and expansion of the knowledge on the gene expression regulation responsible for the generalized poor expression of transgenes. Thus, it is envisioned that new knowledge at the basic science level will allow for further systematic improvements in the productivity of nuclear-encoded BFs. An overall evaluation of the advances achieved for *C. reinhardtii* genetic engineering approaches indicates a promising potential and at the same time constitute a reference to accelerate the development of expression systems based on other algae species.

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Chapter 3

Algae-Made Vaccines Targeting Human Diseases

Introduction

Vaccination is a key intervention for the prevention or cure of several diseases and has saved millions of lives (Halloran and Longini 2014; Han 2015). However, the benefits of this tool should be expanded by improving vaccination coverage. The Expanded Programme on Immunization (EPI) was established by the WHO in 1974 to ensure full coverage of infants for vaccines recommended for routine use. The EPI includes Bacille Calmette-Guérin (BCG), diphtheria-tetanus-pertussis (DTP), polio, and measles vaccines (Bland and Clements 1998). According to World Health Organization (WHO) records, global coverage with these core vaccines increased from <5 % to ≥84 % and even additional vaccines have been added to the recommended schedule. In fact, coverage with the third dose of diphtheria-tetanus-pertussis (DTP) vaccine (DTP3) by 12 months of age is considered a relevant indicator of the immunization programme performance. Estimated global DTP3 coverage is in the range of 83–84 % since 2009 with projections of 84 % in 2013 (WER 2014). Although tremendous advances are made in these health interventions, it is necessary to enhance global immunization coverage in order to promote equity in the access to immunization services. This objective is complex to achieve and therefore requires the strengthening of policy processes, monitoring and evaluation, human resources, regular delivery and supply systems, local political commitment and ownership, involvement of civil society and communities, sustainable financing, and development of new vaccine technologies to decrease vaccination costs (WER 2014). In addition, the development of efficacious vaccines constitutes in parallel an urgent need to fight against high impact diseases for which no effective vaccines exist, such as tuberculosis, HIV/AIDS, malaria, among others (Hawn et al. 2014; Tanuma 2014).

High vaccination costs are due to complex downstream processing procedures during production, and the need for cold chain, specialized materials and personnel for the administration of the vaccines through injections (Branco et al. 2014;

Halloran and Longini 2014). As analyzed in Chap. 1, algae posses attractive features that can contribute to addressing current vaccinology challenges, including the following: they can be grown at low costs, offer attractive biosynthetic capacity, and can serve as oral delivery vehicles as some species lack toxic compounds and thus can be directly delivered orally without purification. In particular, an ambitious goal consists of developing oral vaccines, which are ideal since they involve a simplified production system where algae biomass can be freeze-dried and encapsulated for a straight forward oral administration with the subsequent induction of adaptive immune responses that can cover local or distant mucosal tissues as well as the systemic compartment. This immune profile is relevant when we consider the fact that mucosal compartments constitute the main port of entry of pathogens (Dlugońska and Grzybowski 2012; Owen et al. 2013). In addition, pharmaceuticals forms consisting of freeze-dried biomass could be easily distributed and administered.

However, oral vaccines often exhibit poor immunogenic potential for the reason that antigen uptake is seriously hampered by substantial antigen degradation and dilution in the gastrointestinal tract, poor antigen transport into the submucosa, and the tolerogenic nature of the gut associated lymphoid tissues (Mestecky et al. 2008). Algae-based vaccines are intended to surpass these challenges and render oral vaccines efficient. After the pioneering development of an algae-based vaccine was reported by Sun et al. in 2003, a number of candidates have been reported and are currently under preclinical investigation (Sun et al. 2003). This chapter is focused on the state of the art in developing algae-based vaccines for human use and highlights the potential of rendering new, convenient and low cost vaccines to fight global diseases (Table 3.1).

Gut Associated Immune System and Oral Vaccination

An effective oral vaccine can achieve the first line of immunological defense in the intestinal tract, which is the major site of pathogen entry. In addition, oral vaccines offer the possibility to induce immune responses at other mucosal compartments and at the systemic level. A typical effector mechanism at the mucosal level is the production of pathogen or toxin-specific secretory Immunoglobulin A (SIgA). The induction of such humoral responses is challenging since the vaccine formulation deals with the hostile environment of the gastrointestinal tract, which comprises low pH, protease activity, and the detergent activity of bile salts. Another factor that disfavors vaccine efficacy is a poor bioavailability of the antigen given by the antigen dilution and dispersion, and the presence of physical barriers, mainly by the mucus and the epithelium (Kunisawa et al. 2012). Thus the design of oral vaccines should include proper delivery vehicles as well as transmucosal carriers and adjuvants to optimize antigen bioavailability and processing, rendering high immunogenic activity.

Table 3.1 Compilation of antigens expressed in algae species as expression hosts

| Description of vaccine candidate | Route of administration | Adjuvant | Findings | Reference |
|---|-------------------------|--|---|-------------------------|
| Chimeric protein comprising the C-terminal domains from the Apical Major Antigen AMA1, or Major Surface Protein MSP1 and the granule bound starch synthase (GBSS), expressed in <i>C. reinhardtii</i> (chloroplast) | Oral | LTB | Immunogenic in mice and induced systemic protective immune responses. | Dauvillée et al. (2010) |
| | Intraperitoneal | Freund's | | |
| <i>Plasmodium falciparum</i> surface proteins 25 (Pfs25) and 28 (Pfs28) expressed in <i>C. reinhardtii</i> (chloroplast) | Intraperitoneal | Freund's | Both candidates are immunogenic in mice and induced antibodies able to recognize the target pathogen form. Pfs25 elicits transmission blocking antibodies. | Gregory et al. (2012) |
| <i>Plasmodium falciparum</i> surface protein 25 (Pfs25) expressed in <i>C. reinhardtii</i> (chloroplast) | Intraperitoneal | Alum, Toll-like receptor 4 [TLR-4] agonist glucopyranosyl lipid A [GLA] plus alum, squalene-oil-in-water emulsion, and GLA plus squalene-oil-in-water emulsion | Formulation with TLR-4 agonist in a squalene-oil-in-water emulsion showed higher immunogenic activity inducing parasite-blocking antibodies. | Patra et al. (2015) |
| Chimeric protein comprising the D2 fibronectin-binding domain of <i>Staphylococcus aureus</i> and cholera toxin B subunit, expressed in <i>C. reinhardtii</i> (chloroplast) | Oral | – | Immunogenic in mice, induced mucosal and systemic immune responses. Reduced bacteria load and protects against lethal challenge with <i>S. aureus</i> at 80 % | Dreesen et al. (2010) |

(continued)

Table 3.1 (continued)

| Description of vaccine candidate | Route of administration | Adjuvant | Findings | Reference |
|--|-------------------------|-----------------------------------|--|-----------------------------|
| Human papillomavirus A mutated version of the E7 oncoprotein from the Human papillomavirus, expressed in <i>C. reinhardtii</i> (chloroplast) | Subcutaneous | QuilA | Immunogenic in mice, induced systemic IgG responses and tumor protection | Demurtas et al. (2013) |
| Hemagglutinins (H5 or influenza B) expressed in <i>Schizochytrium</i> sp. (nuclear) | Parenteral | Alone or Addavax (squalene-based) | <i>Schizochytrium</i> sp. | Bayne et al. (2013) |
| Hepatitis B virus antigen (HBsAg) expressed in <i>D. salina</i> (nuclear) | - | | Yields reached 3.11 ng HBsAg/mg of soluble protein | Geng et al. (2003) |
| p24 HIV antigen expressed, expressed in <i>C. reinhardtii</i> (nuclear) | - | | Yields reached 0.25 % of the total cellular protein | Barahimipour et al. (2016) |
| Human glutamic acid decarboxylase 65 expressed in chloroplast as vaccine against type I diabetes, expressed in <i>C. reinhardtii</i> (chloroplast) | - | | Reacted with sera from diabetic patients and is immunogenic in NOD mice | Wang et al. (2008) |
| Chimeric protein comprising the p210 epitope from human ApoB100 and cholera toxin B subunit, expressed in <i>C. reinhardtii</i> (chloroplast) | Oral | None | Immunogenic in mice and induced long lasting systemic IgG responses | Beltrán-López et al. (2016) |

Table 3.1 (continued)

| Description of vaccine candidate | Route of administration | Adjuvant | Findings | Reference |
|---|-------------------------|----------|---|----------------------------|
| Nucleocapsid antigen from Hepatitis B virus (HBcAg) fused to angiotensin II, expressed in <i>C. reinhardtii</i> (nuclear) | — | | Yields reached 0.05 % of TSP | (Soria-Guerra et al. 2014) |
| CrAra h 1 and CrAra h 2, respectively, and a truncated CrAra h 1 consisting of amino acids 171–586 (CrAra h 1171–586) CrAra h 1 and CrAra h 2, respectively, and a truncated CrAra h 1 consisting of amino acids 171–586 (CrAra h 1171–586) Allergens from peanut (Codon-optimized Ara h 1 and Ara h 2 genes, and a truncated CrAra h 1 (amino acids 171–586), expressed in <i>C. reinhardtii</i> (chloroplast) | Intraperitoneal | None | CrAra h 1-core-treated mice showed low levels of mouse mast cell protease 7 (mMCP-7), slight drop in temperature and reduced basophil activation, indicating protective effect. | Gregory et al. (2016) |

The gut associated lymphoid tissues (GALT) are the primary target for oral vaccines. GALT comprises several different organized lymphoid structures, from which Peyer's patches (PPs) and isolated lymphoid tissue (ILF) are recognized as the sites for the induction of IgA responses (Fagarasan et al. 2010). PP comprise several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as interfollicular region (IFR) (Kunisawa et al. 2008). PP are covered with a specialized epithelial region, termed follicle-associated epithelium (FAE), containing specialized antigen-sampling microfold or membranous cells (M cells), which are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles,

and efficient transcytosis activity, allowing the selective and efficient transfer of antigens from the intestinal lumen into PPs (Foussat et al. 2001). Thus, M cells exert a critical role in antigen sampling and serve as gateway cells for the mucosal immune system. The next essential element are Dendritic cells (DCs), which are abundant in the subepithelial dome region (SED) under the FAE. DCs are specialized in taking up and processing the antigen to achieve the presentation of its epitopes to mucosal T and B cells, with the subsequent induction of adaptive immune responses (Milling et al. 2010). B cells are abundant in PPs and are mainly located in the follicle region, where germinal centers (GC) are formed in an environment that favors the class switching of B cells from IgM to IgA. Another component are T cells, some of which are found in the IFRs of the PPs, and comprise naive T cells or those with active phenotype, including IFN- γ -producing (Th1), IL-4-producing (Th2), and IL-10-producing Foxp3+ regulatory (Treg) cells (McGhee et al. 1989). A recent study demonstrated that at least some portions of Foxp3+ regulatory T cells differentiate into follicular helper T cells, which facilitate the B cell class switching to IgA+ B cells in the GC (Tsuji et al. 2009).

Once T cells have been antigen-primed, they support IgA class switching and somatic hyper mutation of B cells in the GC through antigen-specific interactions, CD40/CD40 ligand interaction, and cytokine expression (e.g., TGF- β , IL-4, and IL-21) (Fagarasan et al. 2010). In parallel, retinoic acid derived from PPs and DCs induces the expression on primed T and B cells of the gut imprinting molecules $\alpha 4\beta 7$ integrin and CCR9 (Mora et al. 2006). Therefore, oral immunization can achieve the induction of adaptive immune responses that have as effector mechanisms the secretion of IgA at the mucosal level, the production of systemic IgG, and expansion of Treg cells (Fig. 3.1). The latter have implications in the therapy against autoimmune and inflammatory diseases, which is discussed below. Herein, current developments on algae-made vaccines targeting human pathologies are analyzed.

Vaccines Targeting Infectious Agents

Plasmodium falciparum

Malaria is a parasitic disease, which is killing more children below 5 years of age than any other disease, especially in tropical regions (WHO 2015a). Dauphinais et al. (2010) reported the development of a *Chlamydomonas reinhardtii*-made vaccine based on the expression of Plasmodium antigens fused with the granule bound starch synthase (GBSS), which is the major protein associated with the starch matrix in plants and algae. Target Plasmodium antigens comprised the C-terminal domains from the Apical Major Antigen AMA1 and the Major Surface Protein MSP1, which were fused to a deleted version of GBSS with the aim to form antigen-starch complexes called ‘amylosomes’. Expression studies in *C. reinhardtii* revealed that the chimeric proteins bind to the polysaccharide matrix. Furthermore, immunogenicity

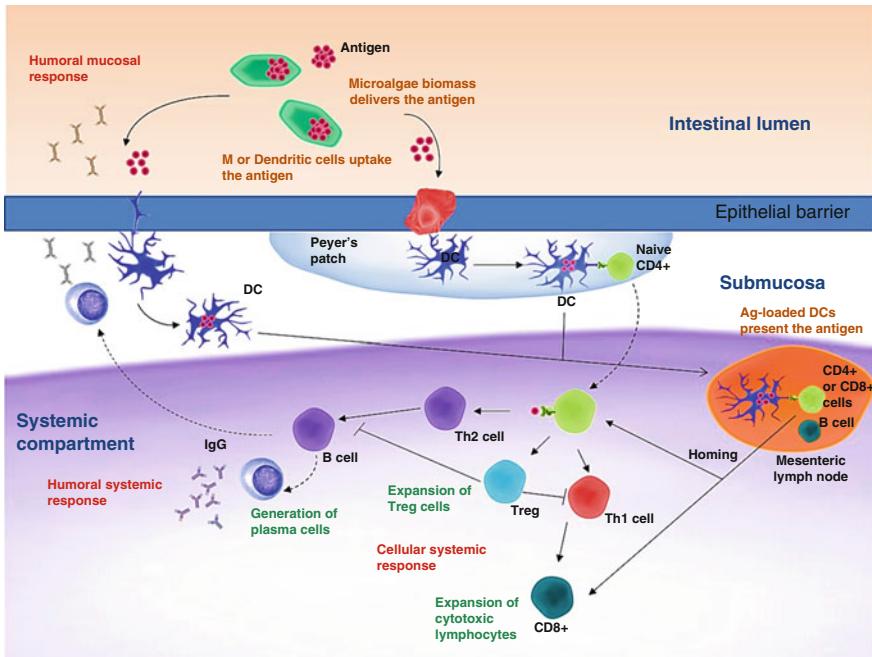


Fig. 3.1 Schematic description of the mechanisms behind the induction of adaptive immune responses following oral immunization with edible genetically engineered organisms expressing antigens. This type of vaccines, called food-grade vaccines, constitutes low-cost, easy to administer, and potentially efficacious vaccine. Mucosal and systemic adaptive immune responses can be induced following antigen (*Ag*) translocation by M (microfold) cells or dendritic cells (*DC*) into the submucosa, where DCs present the antigen to lymphocytes for the induction of humoral or cellular responses at the local and systemic levels. IgA and IgG responses as well as expansion of Treg are responsible for the vaccine effector mechanisms

of the purified ‘amylosomes’ was evaluated in mice subjected to intraperitoneal immunization using Freund’s adjuvant, or oral immunization using the B-subunit of the heat-labile enterotoxin (LTB) from *E. coli* as adjuvant. Animals were challenged with a lethal inoculum of *Plasmodium berghei*, and the remarkable finding was the fact that algae-immunized groups showed decreased degree of parasitemia and a higher survival rate and even infection recovery (Fig. 3.2).

In vitro studies also showed promising results since sera from mice immunized with the algae-made antigen inhibited the intra-erythrocytic asexual development of *P. falcifarum*, the most virulent plasmodial species. This amyloosome-based approach possesses a high immunogenicity due to the high molecular weight of the complexes, which enhance antigenicity and may confer enhanced stability in the gastrointestinal tract. However, this aspect is yet to be studied in detail comparing these aspects in amylosomes versus the soluble antigenic proteins.

Another malaria vaccine prototype was developed by Gregory et al. (2012). *Plasmodium falciparum* surface protein 25 (Pfs25) and 28 (Pfs28) antigens were

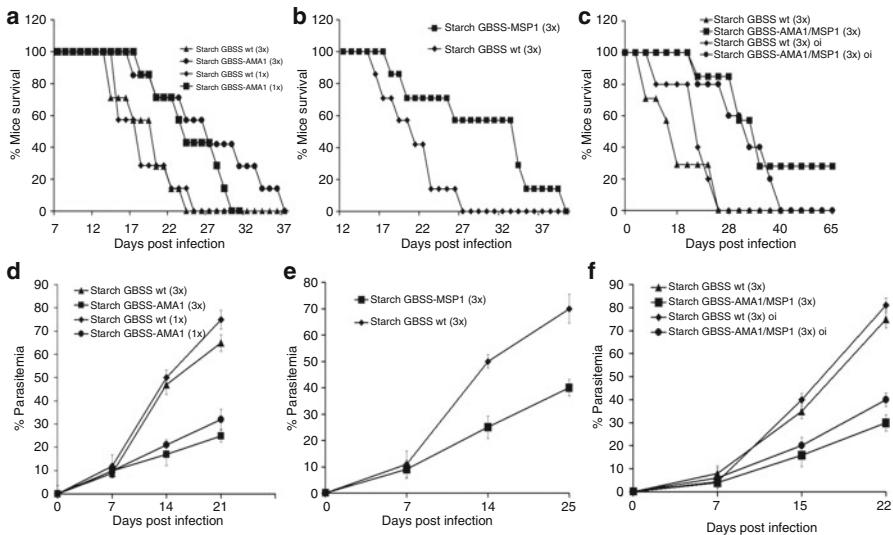


Fig. 3.2 An algae-made vaccine consisting of starch-bound antigens elicits protection in mice against a challenge with *P. berghei*. **(a)** Survival rates of mice vaccinated with one or three doses of starch containing GBSS-PbAMA_{1-C} or WT-starch co-administered with Freund's adjuvant. **(b)** Survival rates of mice vaccinated with three doses of starch containing GBSS-PbMSP₁₋₁₉ or WT-starch co-administered with Freund's adjuvant. **(c)** Survival rates of mice vaccinated with three doses of starch containing the mixture of 5 mg of GBSS-PbAMA_{1-C} and 5 mg of GBSS-PbMSP₁₋₁₉ or WT-starch with Freund's adjuvant. In all cases mice were subsequently challenged with a lethal dose of *P. berghei* ANKA strain. A group of mice were also fed three times with GBSS-PbAMA_{1-C}, GBSS-PbMSP₁₋₁₉ or WT-starch along with the B-subunit enterotoxin mucosal adjuvant. The panels **(d–f)** represent parasitemia profiles corresponding to mice vaccinated and challenged in experiments shown in panels **(a–c)**, respectively (Figure taken from Dauvillée et al. (2010))

expressed in *C. reinhardtii* through a transplastomic method. The algae-made Pfs25 and Pfs28 accumulated at levels 0.5 and 0.2 % of total soluble protein (TSP), respectively. After conducting animal immunization test with pure algae-made antigens, antibodies capable to recognize native proteins from *P. falciparum* sexual stage lysates were induced. However, according to experiments evaluating the reduction of oocysts in mosquito midguts, only Pfs25 was capable of eliciting antibodies with transmission blocking activity.

In a further study, the immunogenicity of the pure algae-made Pfs25 was assessed using the following human-compatible adjuvants: alum, Toll-like receptor 4 [TLR-4] agonist glucopyranosyl lipid A [GLA] plus alum, squalene-oil-in-water emulsion, and GLA plus squalene-oil-in-water emulsion. The immunization scheme comprised four doses administered by the i.p. route in mice. The formulation containing GLA plus squalene-oil-in-water adjuvant induced the strongest humoral response. Sera from mice immunized with this effective formulation showed a positive reaction with *P. falciparum* macrogametes and zygotes. Moreover, these samples blocked the development of parasites within the vector. Therefore, the authors

propose the algae-made Pfs25 co-administered with a TLR-4 agonist in a squalene-oil-in-water emulsion as an effective malaria candidate vaccine (Patra et al. 2015).

The Pfs48/45 antigens have also been explored for the development of algae-based candidate vaccines. Under a chloroplast expression approach, the C-terminal antigenic region of the Pfs48/45 was produced and evidence on expression and retention of antigenicity was generated and thus it is expected that this protein could retain the ability of inducing transmission blocking antibodies (Jones et al. 2012).

Remarkably, the B subunit of the cholera toxin (CTB), which is a strong mucosal adjuvant, has been expressed in *C. reinhardtii* as a genetic fusion along with the Pfs25 antigen. It was demonstrated that the algae-made CTB-Pfs25 accumulates as a soluble, properly folded and functional protein. Stability of the antigen at room temperature in the form of freeze-dried algae biomass was also proved. More importantly, oral immunization in mice with freeze-dried algae material resulted in anti-CTB systemic IgG responses as well as intestinal secretory IgA against both CTB and Pfs25 (Gregory et al. 2013).

These studies indicate that algae-based malaria vaccine candidates are a potential source of low cost oral vaccines. Further characterization of the immunogenic and immunoprotective properties of the algae-made antigens will be critical with respect to defining the road ahead.

Staphylococcus aureus

Staphylococcus aureus is the etiologic agent of community-acquired and nosocomial infections (Kluytmans et al. 1997). The D2 fibronectin-binding domain of *Staphylococcus aureus* was produced in the *C. reinhardtii* chloroplast as a fusion protein along with CTB (Dreesen et al. 2010). Immunogenicity evaluation reflected that algae-made CTBD2 is able to elicit specific mucosal and systemic immune responses when orally administered in mice (Fig. 3.3). Of special interest is the result observed in immunoprotection studies where a 80 % protection rate was found in test mice immunized with the algae oral vaccine and challenged with a *S. aureus* lethal dose (Dreesen et al. 2010; Fig. 3.4). This observation augurs promising perspectives for this candidate.

Human Papillomavirus

Human papillomavirus (HPV) constitutes the main cause of cervical cancer. This pathology is the second most common cancer in women worldwide by age-standardized incidence rate. During 2008, a total of 529,000 new cases were estimated with 274,000 deaths due to cervical cancer. At least 85 % of cervical cancer deaths occur in developing countries, where it accounts for 13 % of all female cancers (WHO 2015b). The E7 protein of HPV type 16 is a promising vaccine antigen.

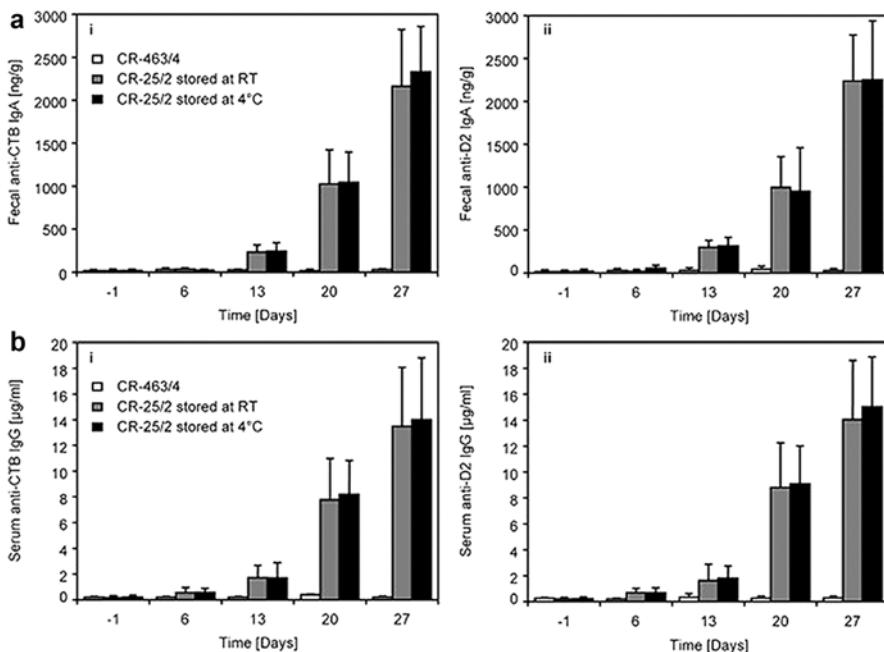


Fig. 3.3 The *C. reinhardtii*–made vaccine against *S. aureus* is orally immunogenic in mice. **(a)** Mucosal humoral responses induced in mice orally immunized with the CTB-D2-expressing algae CR-25/2. The freeze-dried vaccine formulation was stored for 20 months at 4 °C or at room temperature and used to immunize mice by the oral route. **(a)** Anti-CTB (i) or –D2 (ii) antibody levels were determined by ELISA in fluids collected at different days during the immunization scheme. **(a)** IgA levels detected in feces. **(b)** IgG levels detected in serum (Figure taken from Dreesen et al. (2010), permit number 3543841343519)

A therapeutic vaccine targeting E7 has been devised in *Chlamydomonas reinhardtii* by the transplastomic expression of the protein E7GGG, which is an attenuated version of the E7 oncoprotein. Yields were up to 0.12 % TSP. In terms of immunogenicity, the induction of specific anti-E7 IgGs and E7-specific T-cell proliferation was observed in test mice immunized by the subcutaneous route with either alga total extracts or the alga-made purified antigen (Fig. 3.5). Remarkably, high protective effects were observed in a mouse model challenged with a tumor cell line expressing the E7 protein (Demurtas et al. 2013; Fig. 3.6).

Influenza Virus

A remarkable contribution in this field consists of an influenza vaccine prototype produced in *Schizochytrium*. Given the high need of accessible and safe vaccines for massive immunization against high impact diseases, innovating the conventional

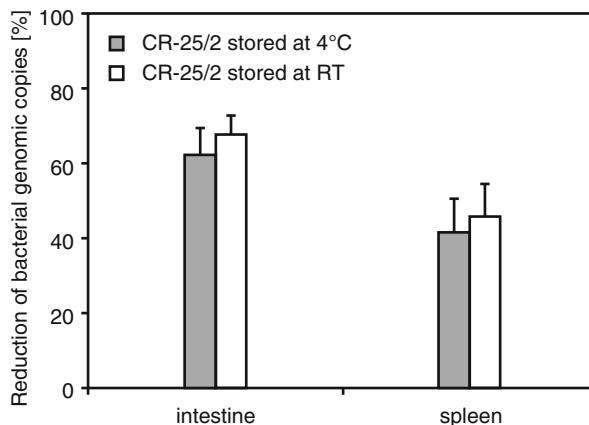


Fig. 3.4 The *C. reinhardtii*-made vaccine against *S. aureus* reduced bacterial load in organs of challenged mice. Mice orally immunized with algae-expressing CTB-D2 (stored for 20 months at 4 °C or room temperature) were challenged with a sub-lethal dose of *S. aureus*. Levels of *S. aureus* genomic DNA were estimated by qPCR in the intestine and spleen (Figure taken from Dreesen et al. (2010), permit number 3543841343519)

production systems based on egg production is needed. Hemagglutinin proteins of influenza virus were produced in *Schizochytrium sp.* taking advantage of the already implemented process for the production of polyunsaturated fatty acids for animal and human health applications. Microalga clones were transformed at the nuclear level with the full-length reading frames of two H1, one H5 and one influenza B hemagglutinins, which were previously codon optimized. Expression occurred at the nuclear level under the control of the *Schizochytrium* EF-1 promoter and PFA3 terminator. The obtained clones were capable of secreting the full-length membrane-bound proteins with hemagglutination activities ranging from 16 to 512 HAU/50 µL of supernatant. The immunogenic properties of the algae-made hemagglutinin from A/Puerto Rico/8/34 (H1N1) Influenza virus were evaluated in mice. Immunoprotection at the rates of 80–100 % from a lethal challenge was achieved following immunization with a single dose of 1.7, 5 or 15 µg rHA with or without adjuvant (Addavax). When a two-dose regimen was followed, full protection (100 %) was achieved (Bayne et al. 2013; Fig. 3.7). Importantly, glycosylation patterns were studied in the algae-made proteins. No fucose or xylose residues were detected among any glycoforms. Sialic acid residues were also not detected. However, high-mannose structures, such as (GlcNAc)₂ (Man)_{5–6}, were observed; opening questions on the biological implications of such glycans since these have been reported as immunogenic in mammals. A promising potential for this platform is identified since the protein was produced at sufficient levels for purification by a simple method and this species is already produced at the industrial level, which will facilitate technology transfer and regulatory approval. An interesting prospect comprises the use of this model for the production of oral vaccine formulations using whole alga biomass.

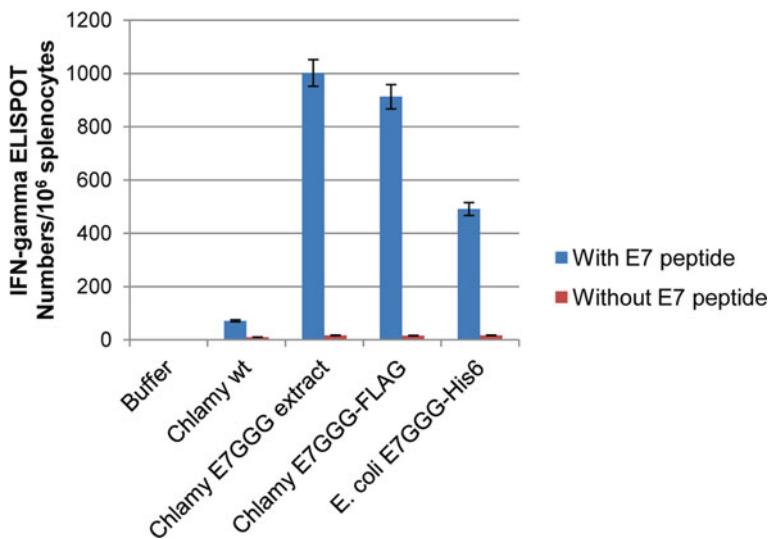


Fig. 3.5 The algae-made vaccine targeting Human papillomavirus induce T cell responses. ELISPOT analysis was performed with splenocytes from mice vaccinated s.c. with the algae-made E7GGG antigen, which were stimulated with a CTL E7 peptide (blue bars) or treated with the vehicle alone (red bars). The number of IFN- γ producing T cells upon E7 stimulus is presented (mean number of spots per 10^6 splenocytes). Error bars represent standard deviation (Figure taken from Demurtas et al. (2013))

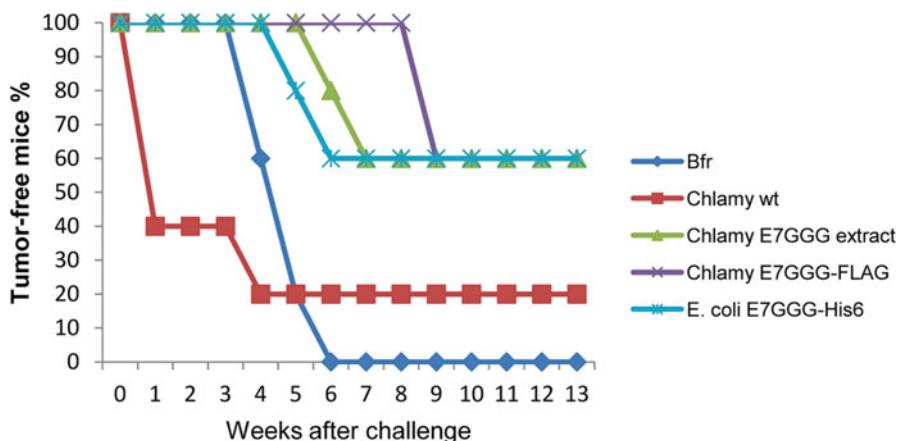


Fig. 3.6 The algae-made vaccine targeting Human papillomavirus protects mice against tumor formation. Mice immunized s.c. with the algae-made E7GGG antigen were challenged by subcutaneous injection of TC-1 cells. Tumor development was monitored by palpation twice a week. Percentage of tumor-free mice is presented (Figure taken from Demurtas et al. (2013))

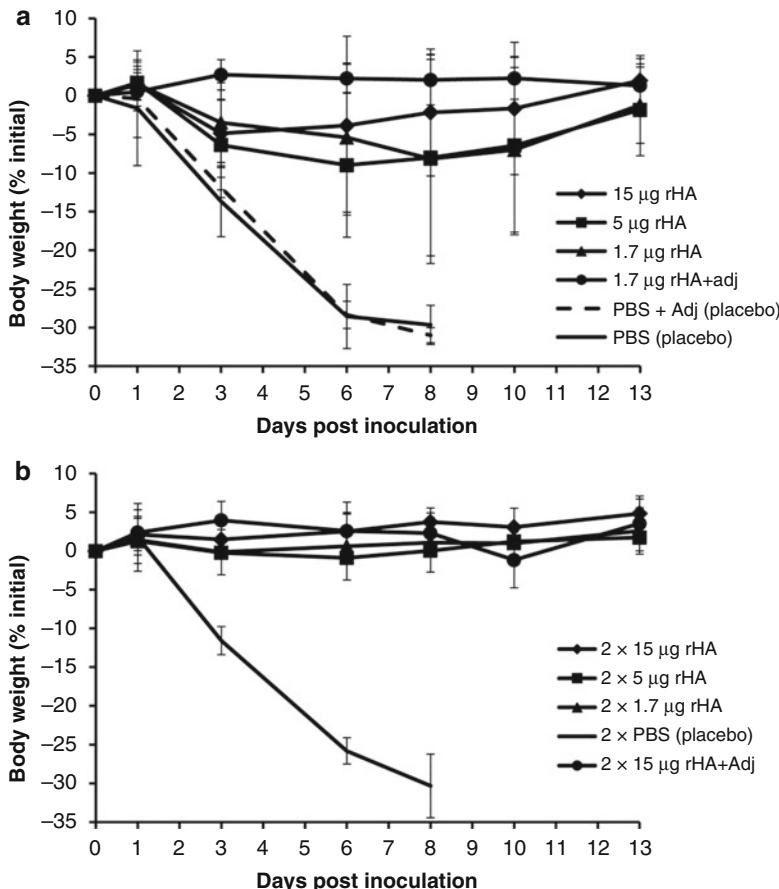


Fig. 3.7 A *Schizochytrium*-made vaccine protects mice against *Influenza virus*. Mice were immunized with the algae-made HA antigen by the i.m. route in schemes consisting of one (a) or two (b) immunizations and a subsequent challenge with a lethal dose of *influenza virus* (Figure taken from Bayne et al. (2013))

Hepatitis B Virus

Hepatitis B causes a potential life-threatening liver infection, whose etiologic agent is the hepatitis B virus (HBV), and constitutes a major global health problem. The chronic liver disease and chronic infection by HBV confers high risk of death by means of liver cirrhosis or cancer. Although a vaccine against hepatitis B has been available since 1982, WHO estimates more than 240 million people with chronic liver infections and more than 780,000 deaths every year due to the

acute or chronic consequences of hepatitis B (WHO 2014). Geng et al. in 2003 reported on the nuclear expression of the surface antigen of the Hepatitis B virus antigen (HBsAg) in *D. salina* as an expression host, under the control of the ubiquitin-Ω promoter. The transgene was introduced into the cells by using electroporation. The best productivity was 3.11 ng HBsAg/mg of soluble protein (Geng et al. 2003). Further studies on the antigenic and immunogenic properties of this alga-made antigen are yet to be conducted to determine its potential as vaccine.

Xue et al. (2003) submitted a patent application claiming the use of *Dunaliella salina* for the expression of a fusion protein comprising the cholera toxin B subunit and the hepatitis B surface antigen comprising 1–226 amino residues of HBsAg and 20–48 amino residues of PreS1Ag. A transformation vector was assembled to mediate a site-directed integration of expression cassettes for the gene of interest and two marker genes for transformants screening (*Bar* gene and nitrate reductase). However no data on the expression efficiency and functionality of the *D. salina*-made antigen were found (Xue et al. 2003).

Human Immunodeficiency Virus

The *Human immunodeficiency virus* (HIV) causes acquired immune deficiency syndrome (AIDS), which has a relevant epidemiological impact worldwide. Although anti-viral drugs provide a means for controlling the disease progression, vaccination is the ideal approach to fight HIV/AIDS. Multicomponent vaccines are proposed as a strategy to induce such broad immune responses capable of preventing viral spread in the host (Nabel et al. 2002). A promising antigen in vaccine development is the p24 antigen, which has been tested in several vaccine candidates in preclinical and clinical levels (Climent et al. 2014; Kulkarni et al. 2013; Omosa-Manyonyi et al. 2015).

Following a nuclear expression approach mediated by an expression cassette derived from the *C. reinhardtii* PSAD locus, Barahimipour et al. (2016) achieved the expression of p24 in *C. reinhardtii*. The innovation in this report was the use of an optimized *nptII* selectable marker gene, which was designed with preferred codons in the nuclear genome of the host having a GC content of 73 %. The *nptII* optimized gene in combination with the expression strain UVM11 (see Chap. 2) allowed for efficient generation of transgenic algal clones on kanamycin concentrations similar to those used in plant transformation (50–75 mg/L). This investigation provides new insights related to the fact that previous studies failed in the efficient use of *nptII* as a selection marker, which is therefore attributed to a poor marker gene expression (Bingham et al. 1989; Hall et al. 1993). Under this optimized approach, expression of the alga-made p24 antigen reached accumulation levels of up to 0.25 % of the total cellular protein. Evaluating the immunogenic properties of the alga-made p24 HIV antigen remains a pending objective.

Vaccines Targeting Non-communicable Diseases

Type I Diabetes

The fight against type 1 diabetes is a priority for health systems at the global level as it is responsible for millions of deaths yearly; the incidence of this pathology is rapidly increasing in children and adolescents, with a reported increase of 3 % annually (Carstensen et al. 2014; Patterson et al. 2009). Since type I diabetes is an autoimmune disorder, immunotherapies targeting endogenous proteins involved in the physiopathology are considered a promising approach (Rigby and Ehlers 2014). Wang et al. reported in 2008 *C. reinhardtii* strains expressing the human glutamic acid decarboxylase as a target antigen in type I diabetes immunotherapy (Bach and Chatenoud 2001). Expression of the full-length *hGAD65* in chloroplasts permitted productivities of up to 0.25–0.3 % TSP. In terms of antigenicity, the algae-made GAD65 was recognized by sera obtained from diabetic subjects and was able to increase the production of spleen cells from non-obese diabetic mice. This evidence suggests proper antigenicity but future evaluations are yet to be conducted to clearly define the safety and potential of this approach to fight autoimmune diabetes.

Atherosclerosis

Atherosclerosis is a key pathology in the development of some cardiovascular diseases and current pharmacologic treatments show significant limitations (Patel and Blazing 2013). Thus, immunotherapies targeting proteins associated with the progression of atherosclerosis have received considerable attention (Fredriksson et al. 2003). Beltrán-López et al. (2016) developed transplastomic *C. reinhardtii* strains for the expression of a chimeric protein comprising CTB (mucosal adjuvant) and the epitope p210 from ApoB100, which is involved in the atherosclerosis progression and has been used for the development of immunotherapies. Clones accumulated the antigen at levels of up to 60 µg per g of fresh biomass weight and also proved to be immunogenic in BALB/c mice when orally administered. Interestingly, the humoral response was followed up for several weeks and it last for at least 80 days after the last boost (Beltrán-López et al. 2016). The atheroprotective effects of this vaccine candidate remains to be tested in rabbits subjected to a high cholesterol diet or transgenic mice models.

Hypertension

Hypertension is a disease affecting around 40 % of adults older than 25 years worldwide, and accounts for 7 % of total disability-adjusted life-years. Immunotherapies against hypertension have been proposed as an approach to induce humoral

responses targeting physiological molecules associated with the pathology (Nakagami et al. 2014). Several vaccine candidates have been developed but the most promising of those directed against Angiotensin II is the candidate vaccine CYT006-AngQb, which is currently in phase II trials (Bairwa et al. 2014; Tissot et al. 2008). This kind of therapies is intended to provide alternatives of lower costs and better compliance to those that are drug-based. A fusion protein based on the nucleocapsid antigen from Hepatitis B virus (HBcAg) as virus-like particles (VLPs)-forming carrier and Angiotensin II was devised and expressed in *C. reinhardtii*. The expected recombinant protein was detected by Western blot and ELISA analysis at accumulation levels of 0.05 % of TSP (Soria-Guerra et al. 2014). VLPs formation and immunogenicity are aspects to be determined for this specific candidate. An algae-made hypertension vaccine would be characterized by low cost and is estimated that the vaccine with even 50 % efficacy could protect about 90 million people from hypertension and its heavy economic burden.

Allergy

Food allergy is mainly caused by milk, egg, wheat, shellfish, tree nut and peanut (Smith et al. 2015). The latter causes allergy cases that affect the population at 1–2 % rate and can lead to fatal anaphylaxis. A therapeutic approach consists of treatment with specific allergens administered by various routes (Liu et al. 2016). In this area, there is the need of robust platforms for producing recombinant food allergic proteins to formulate immunotherapies. Gregory et al. (2016) conducted studies on the expression in *C. reinhardtii* of the proteins Ara h 1 and Ara h 2 as well as a truncated CrAra h 1 consisting of amino acids 171–586 (CrAra h 1-core). Recombinant proteins CrAra h 1-core and CrAra h 2 were produced in algae clones transformed at the chloroplast level under the control of the psbA promoter, while CrAra h 1 expression was not achieved. The authors presented a detailed characterization of the immunotherapeutic effects in a mice model of allergy. Following sensitization with allergens, mice were treated with increasing doses of algae-made Ara h 1 or CrAra h 1-core purified proteins for 4 weeks. CrAra h 1-core-treated mice showed low levels of mouse mast cell protease 7 (mMCP-7), slight drop in temperature, and a reduced basophil activation; indicating a protective effect of the therapy mediated by the algae-made allergen (Fig. 3.8). This pioneering study uncovers interesting perspectives for exploring the production of other allergens in a platform that requires short time for production and no extensive purification since a GRAS host is used for their production.

Prospective View

Given the interesting findings derived from the preclinical evaluations of algae-made vaccines, several interesting perspectives have arisen. Oral immunization with biomass from GRAS organisms is considered a very low cost and convenient

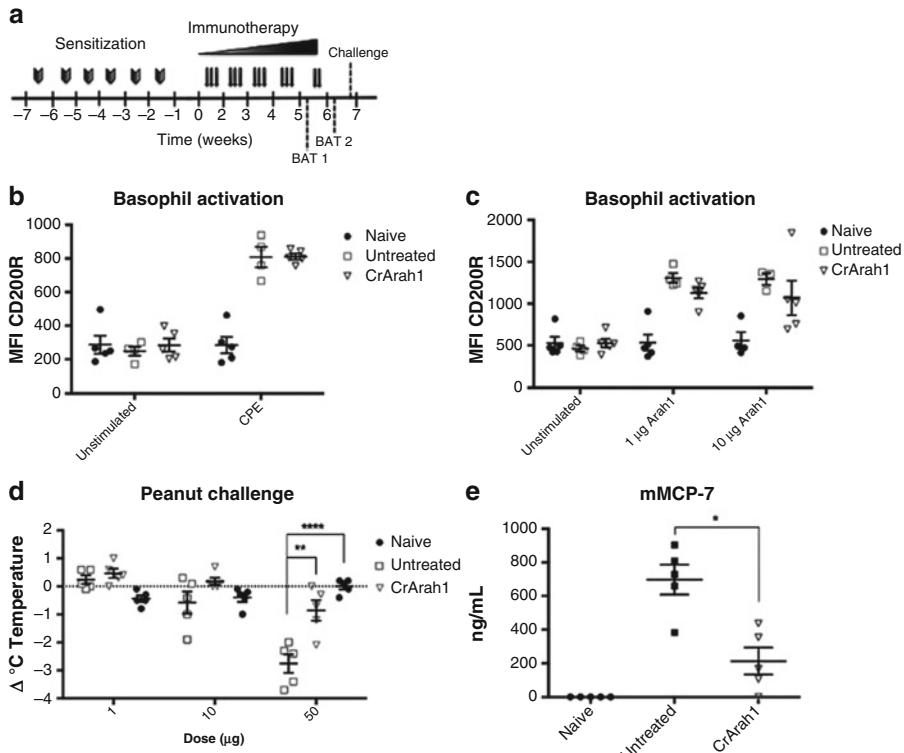


Fig. 3.8 The algae-made allergen can serve as immunotherapy against anaphylaxis. A peanut sensitized mice model was used to evaluate the potential of treatment with algae-made recombinant allergens. (a) Schemes covering sensitization, immunotherapy, basophil activation and challenge in mice. (b–c) Estimation of basophil activation by Ara h 1 (BAT 1) or CPE (BAT 2) in terms of increases in CD200R expression in blood cells using flow cytometry. (d) Challenge with peanut extracts 1 week after completing immunotherapy. Anaphylaxis was measured in terms of droping in body temperature 30 min after each challenge. (e) mMCP-7 levels were measured by ELISA in serum after the last challenge. Statistical significance of challenge data was determined by one-way ANOVA followed by Bonferroni multiple-comparison correction. mMCP-7 levels were compared using a Mann–Whitney test.* $p < .05$, ** $p < .01$, *** $p < .0001$ (Figure taken from Gregory et al. (2016), permit number 3801980880423)

vaccination approach. Algae offer the potential to address this objective. In terms of yields, expression in chloroplasts have proven sufficient yields (average levels of 3–4 % TSP) (Rasala and Mayfield 2011). However, nuclear expression has been characterized by low yields, which is attributed to position effects and transgene silencing. Chapter 2 analyses recently developed approaches that will lead to advances in this regard.

In particular, approaches for multi-gene expression could be applied to achieve formulations for addressing oral co-administration of adjuvants. For example the use of CTB or the B subunit of *E. coli* heat labile enterotoxin (LTB) has been narrowly explored. This is a critical objective given the low immunogenicity often shown

by oral vaccines, requiring accessory adjuvants to reach proper immunogenicity (Holmgren et al. 1993).

Secretion of the recombinant protein also provides interesting perspectives. The use of proper secretion signals (see Chap. 2) could provide an approach for obtaining culture supernatants where the protein would be easily purified for the formulation of parenteral vaccines. This is important for the reason that many efficient immunization schemes comprise a combination of priming and boosting steps though distinct immunization routes. It has been proposed that parenteral priming followed by boostings by mucosal routes (e.g., oral or intranasal) provides convenient immune profiles at both systemic and mucosal compartments (Pniewski et al. 2011).

Considering that poor immunogenicity is frequently observed for antigens administered orally, a fundamental research objective in this field consists of optimizing immunization schemes and vehicles to achieve proper immune responses. Using whole cells that possess cell wall is proposed as a bioencapsulation approach that will protect the antigen from degradation without interfering with bioavailability. These effects have been proposed for the case of plant-based vaccines where some candidates have shown high immunogenic activity even at low doses (e.g. doses of 20 ng p24-HIV or 100 ng of S-HBsAg VLPs given orally). Moreover, some plant-based vaccines have shown higher immunogenic activity than that of pure antigens produced in conventional systems (Lindh et al. 2014; Pniewski et al. 2011). However, systematic studies of the immunogenic properties of plant-based vaccines are still in its infancy (Rosales-Mendoza and Salazar-González 2014).

In addition to bioencapsulation, the adjuvant effects exerted by algae compounds should also be considered since it is well known that several algae compounds act as immunomodulators with anti-tumor activity, wound-healing, among other therapeutic effects. In particular, Guzmán et al. (2003) reported that crude polysaccharide aqueous extracts from the microalgae *Chlorella stigmatophora* and *Phaeodactylum tricornutum* showed immunostimulatory effects. Upon *H. pylori* infection in mice, orally administered *Chlorococcum* sp. extracts reduced bacterial load and gastric inflammation, effects that correlated with changes in the polarization of Th responses (Liu and Lee 2003). Similarly high molecular weight polysaccharide preparations from *Spirulina platensis*, *Aphanizomenon flos-aquae*, and *Chlorella pyrenoidosa* have also been identified as a source of immunomodulatory compounds. In particular, a transcription factor-based bioassay (Nuclear Factor kappa B, NF-kappa B) performed in THP-1 human monocytes/macrophages revealed their immunostimulatory activity in terms of increases in mRNA levels of IL-1beta and TNF-alpha (Pugh et al. 2001). Therefore, algae a potential source of adjuvants that are likely involved in the efficacy of the oral vaccines tested at the preclinical level thus far. In this regard, it should be considered that the singular cell wall composition of alga could exert its adjuvant effects. In fact, algae have distinct cell wall composition than that of plants or yeast. For instance, Chlamydomonas cell wall seems to lack cellulose, being constituted by layers of crystalline Ara-rich, Hyp-rich glycoproteins (Bollig et al. 2007; Miller et al. 1974; Roberts 1974). β -1,3-D-glucan is present in most green algae species but not in Chlamydomonas (Eder et al. 2008). Mannans are also known to occur in green algae, including *Codium fragile* (Eder et al. 2008) and *Acetabularia acetabulum* (Dunn et al. 2007).

Fucoidan is a sulfated polysaccharide from brown algae that exert several immunomodulatory effects such as promotion of antigen uptake and enhancement of both anti-viral and anti-tumor effects. Moreover, fucoidan serves as an adjuvant that induces Th1 immune response and CTL activation, and therefore it has been proposed as a component in cancer vaccines (Jin et al. 2014).

This knowledge will be useful for designing specific evaluations using combinations of algae fractions along with recombinant algae expressing antigens to gain new insights on the potential adjuvant effects and identifying promising formulations to achieve proper immunogenicity, especially for oral vaccines. Therefore, detailed knowledge of the adjuvant effects of algae compounds will provide additional insights on their potential uses in vaccines design and understanding the immunogenic activity of vaccines relying on algae as delivery vehicle. The use a wide range of algae species is very important so as to take advantage of the diverse features of algae species, such as immunostimulatory properties, biosynthetic capacity and growth conditions. The expression of multiepitope vaccines remains a virgin field and offers the potential to achieve effective vaccines against several pathogens, especially those virus that are highly variable (Rubio-Infante et al. 2015).

The data gained in preclinical evaluations augur the implementation of clinical trials in the short term, which is the next step of making the goal of algae-based vaccines a reality. The experience gained in the development and clinical assessment of plant-made vaccines constitutes an important precedent that will facilitate the progress in developing algae-based vaccines (Yusibov et al. 2011). For example the technical strides at the level of expression strategies as well as the experience gained in the regulatory approval of plant-made biopharmaceuticals (BFs) provide a close-related counterpart (Mor 2015, see Chap. 9). Given the potential of algae-based systems for vaccine production, more investment by the industry will be required to accelerate this kind of developments. GRAS category and production under GMPs will greatly facilitate the regulatory frame applicable to BFs. However, the road to algae-made vaccines is still long and several paths should be explored to diversify this field and increase the opportunities of making such kind of vaccines a reality in the clinic.

In conclusion, significant advances have been achieved in the field of developing algae-made vaccines targeting human diseases, generating exciting perspectives on how recombinant algae will result in innovative immunization approaches in the benefit of global health, especially in countries where poverty restricts access to vaccines.

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Chapter 4

Algae-Made Vaccines Targeting Animal Pathogens

Introduction

Livestock, poultry, or fish producers continuously fight against diseases that affect the productivity of these animal species used for food production. Currently infectious diseases are a significant financial risk for the producer and may represent a health concern for the consumer derived from contaminated foods. For instance, around 9.4 million cases of illness derived from contaminated food consumption are reported in the United States per year (Scallan et al. 2011); bovine spongiform encephalopathy (mad cow disease) generated a cost for the Canadian beef and dairy industries surpassing \$5.3 billion in the two subsequent years after the detection of an infected animal (Statistics Canada 2006). In parallel, many tropical diseases are transmitted by animals that are consumed as food or wild animals that infect humans by incidental contact (Mackey et al. 2014). Recent global disease events, such as influenza and Ebola epidemics, highlighted the serious impact of zoonotic diseases (Taylor et al. 2001). In fact, some factors are modifying these events including agricultural intensification, population growth, climate change, and human encroachment into wildlife habitats; which may favor zoonotic disease emergence (Daszak et al. 2013; Woolhouse and Gowtage-Sequeria 2005).

In this context, the One Health Initiative has been generated to emphasize the relatedness of human, animal, and environmental health (Kahn 2011; Rabinowitz et al. 2013); and the importance of transdisciplinary efforts to promote health under an integrative focus (Karesh et al. 2012). This concept has been adopted as a key approach to control and prevent diseases by the Food and Agriculture Organization of the United Nations, the World Organization for Animal Health, and the World Health Organization (www.who.int/influenza/resources/documents/tripartite_concept_note_hanoi/en/index.html); among several other organizations (www.one-healthinitiative.com/supporters.php).

Therefore promoting animal health will favor human public health, animal welfare, and business profitability. One of the key actions to achieve this goal consists in implementing good production practices, which comprise provision of uncontaminated feed and water, adequate ventilation and air quality, biosecurity, robust surveillance of animal health, and the judicious use of antimicrobial agents and parasiticides for disease prevention and treatment, when warranted. However, under many circumstances these actions are not sufficient and newly developed vaccines and immunotherapeutic agents constitute the alternative to those factors that cannot be controlled such as exposure to wild animals, poverty, and antibiotic resistance (Potter et al. 2008).

Vaccination is a major approach in the fight against infectious diseases in livestock, poultry, or fish. The global market for animal vaccines represents \$5.8 billion, approximately (www.researchandmarkets.com). According to a recent report, timely reaction to an outbreak by vector-borne threats is highly dependent on vaccines availability (Dórea et al. 2016). Therefore, the commercialization of new vaccines possessing high efficacy, low cost, and easy administration could increase the benefits of these interventions (MacDonald et al. 2015).

Under this context, the advances in biotechnology have led to new methodologies that are changing the scenario of vaccine production. New platforms for vaccine production and delivery have been generated, offering significant advantages such as decreasing costs, enhancing safety, and making administration easier. The concept of food-grade vaccines has emerged as a convenient approach where organisms that are safe for human or animal consumption are genetically engineered to express and deliver specific antigens (Rosales-Mendoza et al. 2015). For instance, during more than two decades of research, plant-based vaccines have shown to serve as a viable source of convenient vaccines that are near to commercialization (Kolotilin et al. 2014; Phan et al. 2013). On the other hand, given the attractive features of algae species for biopharmaceuticals (BFs) production (see Chap. 1), these organisms have also been adopted as convenient vaccine biofactories and delivery vehicles; especially for oral vaccines. Herein, an outlook on algae-made veterinary vaccines is described and future prospects for this field are identified.

Algae-Based Vaccines

Classical Swine Fever Virus

Classical swine fever virus (CSFV) causes a highly contagious disease responsible for major losses in pig farming and effective live attenuated vaccines against it are currently used in endemic countries (Muñoz-González et al. 2015). However this pathogen has not been eradicated, which demands the development of new vaccines. Since the structural protein E2 carries critical epitopes, it is a relevant target in vaccine development. Researchers in China have developed *Chlamydomonas reinhardtii* transplastomic strains expressing the E2 gene, obtained by a particle

bombardment method. The transformed clones successfully produced the E2 protein, yielding up to 1.5–2 % of the total soluble protein (TSP). The immunogenicity of the algae-made E2 protein was assessed in test mice immunized three times by either the oral or s.c. routes. Oral immunized animals received by intragastric intubation 1 mL of extracts containing approximately 10 µg of algae-made E2 without accessory adjuvants while subcutaneous administration consisted of extracts containing approximately 20 µg of algae-made E2 emulsified in complete Freund's adjuvant (for priming) or incomplete Freund's adjuvant (for boosting). The authors stated that humoral responses were elicited in the s.c. immunized group but the data was not presented (He et al. 2007). A systematic evaluation on the immunogenic properties of this candidate vaccine is required to better determine its potential for vaccination.

White Spot Syndrome Virus

White spot syndrome virus (WSSV) causes significant losses of shrimp yields (Lightner and Redman 1998). It is well established that the VP28 protein from WSSV possesses immunostimulatory activity that provides protection against this pathogen. Interestingly, *Dunaliella salina* was engineered to achieve the nuclear-mediated expression of VP28. Using the glass bead transformation method, transformed clones were generated and ELISA assays confirmed the expression of the recombinant protein at levels of up to 3.04 ng/mg of total protein; which was equivalent to 780 µg of recombinant VP28 protein per liter of algal culture. The immunostimulatory activity of the algae-made VP28 was investigated by feeding shrimp with lysates from transgenic clones, observing that transgenic algae-treated shrimp showed a reduction of 40 % in mortality in comparison to the control group (Feng et al. 2014; Fig. 4.1). This study indicates the potential of algae to contribute in the fight of diseases that cause significant problems in aquaculture.

Another research group studied the expression of VP28 in *C. reinhardtii* (Surzycki et al. 2009). The authors determined the influence of codon optimization, proteases activity, and protein toxicity on the productivity. Outstandingly, yields of VP28 protein reached levels >20 % of TSP; which is the highest level of recombinant protein for algae (previous studies with high expression reported 5 % of TSP; Manuell et al. 2007). The authors confirmed that codon optimization is an important step to maximize protein production. The control of the activity of proteases, in particular ATP-dependent proteases, was identified as an important factor. The toxicity of heterologous proteins was proven and the use of inducible gene expression is proposed as an approach to produce BFs that are toxic for algal hosts.

Interestingly, in this report the concept of the transformosome was established to refer the generation of transformed lines that possess unique characteristics, or a transformation-associated genotypic modification. This concept could explain the existence of recalcitrant or low yielding transgenic lines with different mechanisms for specific incompatibility between genes, genetic elements, and insertion

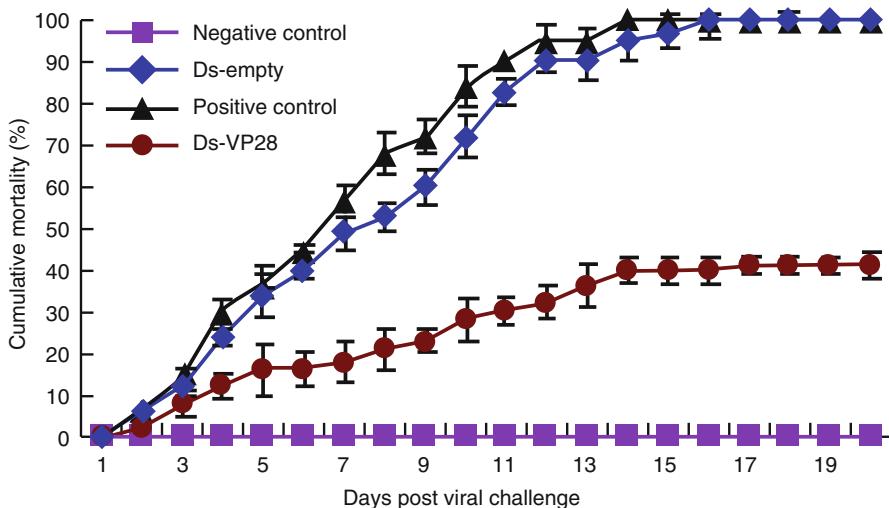


Fig. 4.1 Protective effect in crayfish of an algae-made vaccine against *White spot syndrome virus*. Cumulative mortality rates of crayfish challenged with WSSV are presented. Challenged experimental groups were previously vaccinated during 10 days by feeding crayfish with algal extracts from a strain expressing VP28 (Ds-VP28), or a wild type strain (Ds-empty). Negative (unchallenged) and positive (challenged) control groups were fed with the vehicle alone (Figure taken from (Feng et al. 2014), permit number 3795551234349)

site; which could comprise the occurrence of some other genetic events in the transgenic strain. For instance, the authors postulated that “it is possible that in addition to inserting a transgenic gene into the chloroplast genome target site, additional insertions of the vector or DNA fragments also occur within the nuclear genome; this could result in a change in the function of nuclear gene (s) that regulate protein yield (such as proteases) or gene (s) that increase recombinant protein expression”. Therefore, the analysis of a very large group of candidate clones is recommended to increase the probability to rescue higher expressing clones. Chapter 2 discussed in detail the factors involved in protein productivity. Overall, these outcomes indicate a potential for applying this technology in the development of vaccines against WSSV.

Taenia Solium

Cysticercosis, caused by accidental ingestion of eggs of *Taenia solium*, is spreading all over the world due to globalization and it is one of the most neglected tropical diseases. In addition, in the US it is recognized as a relevant disease (Ito et al. 2015; O’Keefe et al. 2015). Vaccination of pigs, which are the intermediary host, may provide a convenient approach in the fight against this zoonosis. Among the vaccines under development, the S3Pvac vaccine has shown protective efficacy. S3Pvac

is composed of three peptides: KETc12 (8 aa), KETc1 (12 aa), and GK1 (18 aa). The S3Pvac protective capacity was initially described against *T. crassiceps* mice infection (Toledo et al. 1999, 2001). Since these antigens are also present in several structures of *T. solium*, it was proposed as vaccine against this species; providing significant protection in pigs under experimental and field conditions (Sciutto et al. 2007). In particular, the peptide GK1 has been also characterized as an adjuvant to stimulate immune responses to unrelated parenterally administered vaccines (Segura-Velázquez et al. 2006). Interestingly GK1 is also orally immunogenic (Betancourt et al. 2012).

Our group is working in the development of *C. reinhardtii* clones expressing a chimeric protein, called polyGK1, consisting of tandem repeats of GK1 along with enterokinase recognition sites as spacers. Transplastomic approaches have allowed obtaining clones that are resistant to the selection process following particle bombardment. This approach is intended to provide a low cost source of GK1 since it is typically obtained by synthesis resulting in a very expensive approach for veterinary vaccine production. Once orally delivered, the polyGK1 protein is expected to be digested by the host's enterokinase; releasing the functional GK1. This model will be useful in the development of oral cysticercosis vaccine prototypes as well as a new adjuvant co-administered along with unrelated antigens (unpublished).

Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus (FMDV) infects a wide variety of cloven-hoofed domesticated and wild animal species, causing an acute disease that implies vesicular lesions of the tongue, snout, buccal cavity, feet, and teats (Grubman and Baxt 2004). Even though this infection usually resolves without the need for treatment (Arzt et al. 2011), its highly contagious nature, wide dissemination, and significant economic impact makes this pathology one of the most feared livestock diseases. Progress on vaccine development is still required, especially considering that low cost and facilitated logistics for administration are critical factors to exploit the benefits of vaccines. A research group from China aimed at expressing the VP1 protein in *C. reinhardtii* as a fusion with the cholera toxin B subunit (CTB) (Sun et al. 2003). This CTB-VP1 design is intended to lead to a highly effective mucosal vaccine since CTB serves as a transmucosal carrier allowing an efficient antigen transport to the submucosa thanks to its binding to the GM1 ganglioside at the epithelium surface. Once at the submucosa, the antigen can be efficiently processed by antigen presenting cells (dendritic cells) with the subsequent induction of adaptive immune responses; comprising mucosal IgA and systemic IgG responses (Langridge et al. 2010). *C. reinhardtii* transplastomic clones were obtained by particle bombardment-mediated transformation. The CTB-VP1 fusion protein was produced at levels of up to 3% TSP. One important aspect for CTB-based chimeric immunogens is the retention of the oligomeric structure (pentamers) since this is the

form that binds the GM1 and thus biologically active. Interestingly an ELISA based on GM1-ganglioside binding affinity confirmed the presence of the pentameric form, suggesting that CTB-VP1 is functional. Antigenic determinants of both CTB and VP1 were also detected in these assays. However, the immunogenic activity of this candidate was not assessed and thus constitutes the next critical step for its preclinical evaluation.

A patent application has also been submitted claiming the expression of codon-optimized *P1* and *3C* genes from the FMVD in *Schizochytrium sp.* using a promoter from a *Schizochytrium sp.* native gene. Transformants were obtained by the electroporation method. Both soluble and insoluble protein fractions were fraction were used for conducting expression analysis and an animal challenge study, nevertheless no detailed data was provided on the immunogenic potential of the algae-made antigen (Guo et al. 2011).

Porcine Circovirus

Porcine circovirus type 2 is the causative agent associated with several diseases collectively called porcine circovirus-associated disease (PCVAD), comprising post-weaning multisystemic wasting disease (Chae 2005). Vaccination is a major tool to control *Porcine circovirus* type 2 infections and prevent losses in pig farms. Interestingly, Yang et al. (2013) were granted a patent on the production of *Porcine circovirus* vaccine by the expression of the *Porcine circovirus* type 2 antigen gene in *C. reinhardtii*. The use of a chloroplast transformation vector transferred by particle bombardment allowed for the rescue of transformed clones expressing the corresponding viral antigen.

Prospective View

The field of veterinary vaccines is deriving benefits from the biotechnology developments. For instance a veterinary vaccine produced in a next generation platform was approved for commercialization, consisting of a plant-made vaccine against Newcastle disease in poultry. The vaccine was approved for commercialization by the U.S. Department of Agriculture-Center for Veterinary Biologics in 2006 (Mihaliak et al. 2005) and produced in a suspension-cultured tobacco cell line expressing hemagglutinin/neuraminidase (HN) of *Newcastle disease virus*. This case illustrates the potential of photosynthetic organisms to be used as a novel source of convenient recombinant vaccines (Kolotilin et al. 2014). Sadly, the adoption of the technology is always subjected to several factors and the company decided not to commercialize the vaccine. No specific technical reasons for this decision have been communicated.

The current developments of algae-made vaccines targeting veterinary diseases indicate that these organisms have an attractive potential for producing functional antigens from distinct pathogens. However, this group of vaccine prototypes is reduced in number when compared to the magnitude of health problems caused by infectious diseases in animals of economic interest and in humans affected by zoonosis. Thus, interesting perspectives arise in this field.

Key zoonotic diseases that should be considered as a priority in the development of veterinary algae-made vaccines include Brucellosis, chlamydophilosis, Q fever, Orf, *Escherichia coli* O157 infection, Rift valley fever, and Bovine Spongiform Encephalopathy (Ganter 2015). Remarkably, the sustained ongoing research on the development of vaccines against *Brucella abortus* (Avila-Calderón et al. 2013), *Chlamydia* sp (Manam et al. 2013), *Coxiella burnetii* (Schulze et al. 2016), *Orf virus* (ovine ectyma; Onyango et al. 2014), *Escherichia coli* O157 (Cai et al. 2015), *Rift Valley fever Virus* (Laughlin et al. 2016), and prions (Mabbott 2015); will generate valuable knowledge that will facilitate the development of algae-based vaccines as a promise to improve, at low costs, the health of animals of economic interest and, more importantly, the human health by preventing zoonosis.

Targeting tropical neglected diseases (TNDs) through algae-made vaccines is another priority goal. Since this group of diseases affects human beings in developing countries where health resources are limited, low cost vaccination strategies are needed (Hotez and Ferris 2006). Most of the diseases included in this category are zoonosis, therefore the production of low cost algae-vaccines could enable a broad vaccination coverage in host animals in which vaccination is feasible; thus blocking the transmission of TNDs. Interestingly photosynthetic organisms, such as superior plants, have been exploited for that purpose targeting echinococcosis, cysticercosis, rabies, dengue, and helminthiases (Rosales-Mendoza et al. 2012). However, the development of recombinant vaccines against some TNDs diseases in innovative hosts is limited to the case of cysticercosis and thus algae-based vaccines could be widely applied as an attractive source of low cost vaccines targeting these diseases. Among TNDs, the following are considered preventable through animal vaccination: Human hidatidosis (transmitted by dogs), cisticercosis (transmited by pigs), and Rabies (mainly transmitted by dogs). Therefore, the immunization of the intermediary host may provide a viable intervention to block disease transmission. In the case of Chagas disease, leishmaniasis, filariasis, onchocerciasis, African tripanosomiasis, fascioliasis, and dengue; which are vector-borne diseases, human vaccines targeting proteins from the infectious agents or vectors could be contemplated (see Chap. 3).

Remarkably, the industry is adopting the technology of algae-made vaccines to fight veterinary vaccines. For instance DSM Nutritional Product, a subsidiary of Dutch Royal DSM N.V., established a Development and Option to License Agreement with the animal health specialist Merial Limited; which is part of the US-subsidiary of French Sanofi S.A with the aim to produce animal health vaccines in a DSM's proprietary algal expression system (<http://www.european-biotechnology-news.com/news/news/2012-04/dsm-wants-algae-made-vaccines.html>; www.merial.com).

Another prominent example of companies with the interest to use algae as biofactories of proteins used to promote animal health is Triton Algae Innovations, Ltd. Although vaccines are listed in the interests of the company, the initial developments are rather an approach to prevent diarrheal diseases; which generate losses of more than \$5 billion per year globally. The production of the protein bovine mammary-associated serum amyloid (M-SAA) is proposed as an alternative to antibiotic treatments since M-SAA is normally delivered to newborn animals through the mother's colostrum. As a gut-active protein, M-SAA stimulates mucin production providing physical blocking of bacterial and viral infections. Therefore, oral supplementation with M-SAA leads to beneficial effects of great economic impact for the agricultural industry and could also be used in humans for similar diseases. Similarly, the companion animal market is always searching for new products to improve pet health without compromising safety (<http://www.tritonhn.com/>). Chap. 8 contains a detailed analysis on the algae-made nutraceuticals.

In terms of the expression approaches in algae species, optimization of recombinant antigen yields is still a pending objective. Interestingly, an aspect that remains unexplored is the implementation of viral vector-based expression systems in algae; which in theory may allow for extremely high productivity and the expression of chimeric viruses having high immunogenicity. This methodology has shown a remarkable success in the case of plant-made BFs. For example many vaccines expressed in *Nicotiana* plants using a deconstructed viral vector (MagnIcon technology) has allowed for the purification of virus-like particles (VLPs), which are currently under clinical trials as vaccine candidates against influenza (Salazar-González et al. 2015). Therefore, adopting this concept with viruses that naturally infect algae species is a relevant idea that in the future might allow expanding the use of these organisms in the BFs production field. Interestingly, it is known that specific viruses infect algae species. For instance, the virus family Phycodnaviridae comprises a large number of species able to infect algae species; which typically possess up to 600 protein-encoding genes. Therefore viruses from Phycodnaviridae constitute an exceptional source of elements to implement innovative expression approaches (Van Etten et al. 1991; Van Etten and Dunigan 2012). In fact, some biotechnology procedures for biofuel production take advantage of viral infection of an alga species (*Chlorella*) to facilitate extraction of the compounds of interest (Cheng et al. 2013). It is expected that the implementation of viral-mediated expression approaches for BFs production could lead to improvements in yields and new antigenic configurations (for a wide context on expression approaches see Chap. 2).

In conclusion, although few reports exist in the literature on algae-made vaccines targeting animal diseases; this topic will have with no doubt a tremendous impact in the animal health industry as well as in public health agencies since the prevention of zoonosis can be achieved by intermediary host immunization. The fact that some companies are adopting this technology generates promising prospects. The establishment of product development public-private partnerships with innovative developing countries where the target diseases are prevalent will be critical to exploit the

maximum benefits of algae-made vaccines. Considering that regulatory approvals are less strict for veterinary use compared to vaccines for human use, it is expected that animal health will be the first field deriving benefits from algae-made vaccines approved for commercialization.

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Chapter 5

Algae-Made Antibodies and Immunotoxins

Introduction

Antibodies are complex proteins secreted by B cells and constitute the secreted form of the B-cell receptor, formed by paired heavy (H) and light (L) polypeptide chains. The Y-shaped antibody complex has three equal-sized portions connected by a flexible tether. The Y ends has regions that vary between different antibody molecules called variable regions (V regions) whereas the stem of the Y is far less variable and thus called the constant region (C region). Antibody molecules achieve dual tasks: they bind to a highly diverse group of antigens and also specifically interact with effector molecules and cells. These functions are mediated by distinct parts of the molecule: the two arms of the Y end correspond to V regions that bind to antigens (Fab region), whereas the stem of the Y is the C region that interacts with effector cells and molecules (Fc region). The generic term immunoglobulin is used to refer antibody molecules, however five different classes of immunoglobulins exist and are identified by their C regions (IgM, IgD, IgG, IgA, and IgE). In addition, highly refined differences located at the V region confer the specificity of antigen binding (Janeway et al. 2001).

Mabs are outstanding tools in biotechnology and conventional methods for production are mainly based on mammalian cell lines obtained through the hybridoma technology established in 1975 (Köhler and Milstein 1975). Since then, modern biotechnology have led to innovative methods to manipulate and produce antibodies as well as new types of molecules derived from them; which facilitates production and specific applications of the recombinant molecules.

Large single-chain (lsc) antibodies consist of a genetic fusion of the entire heavy chain to the variable region of the L chain with a flexible linker between them. The main advantage of this approach is that only a single gene is engineered to achieve the production of functional recombinant antibodies, greatly simplifying the genetic engineering approach.

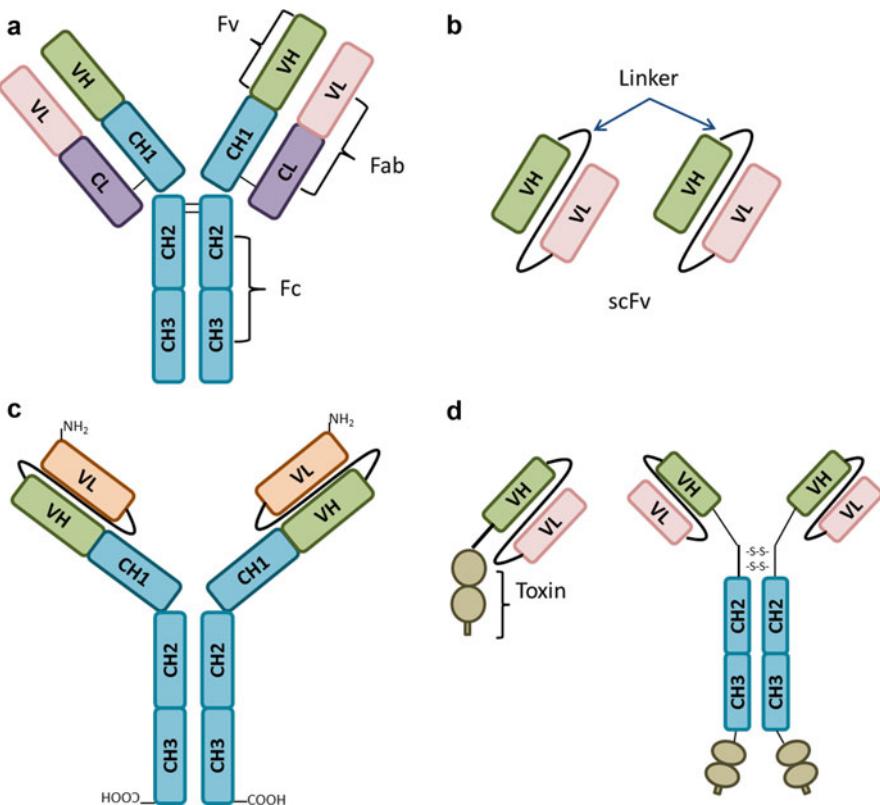


Fig. 5.1 Schematic description of a full-length antibody (a), scFvs (b), large single (lsc) antibody (c), and immunotoxin (d)

Single chain antibodies consist of a fusion protein comprising antibody variable light (VL) and heavy (VH) fragments (Raag and Whitlow 1995), commonly known as single chain variable fragments (scFvs). A convenient feature of scFvs is that they constitute small antibody fragments encoded by a single gene carrying the entire antigen-binding region but lacking the functions of the Fc region, which is desirable in some cases to avoid undesired reactions (Bird et al. 1988). Through genetic engineering the region coding for the carboxyl terminus of one variable fragment is linked to the amino group of the other fragment via a polypeptide linker (Fig. 5.1). An important aspect in the design of scFvs is the linker selected to join the two variable chains since major steric obstructions can be avoided thanks to the length and conformation of the linker (Takkinen et al. 1991).

Immunotoxins are antibody-toxin bifunctional complexes designed to kill specific cells by the action of an intracellular toxin. Their specificity is given by the binding properties of the selected antibody. Immunotoxins have been mainly used in the development of cancer therapies; however, they have had also implications in immune regulation and treatments against viral or parasitic diseases (Antignani and Fitzgerald 2013).

Mammalian systems are currently used for the production for 60–70 % of the recombinant protein pharmaceuticals; however cultivation is very expensive and the risk of contamination with human pathogens exists (Farid 2006). Considering these limitations, alternative expression systems have been developed to account with better attributes during production; which include bacterial systems for the expression of antibody fragments as well as yeast, insect cells, transgenic plants, and moss for antibody production (Chadd and Chamow 2001; De Muynck et al. 2010; Reski et al. 2015; Sack et al. 2015; Verma et al. 1998). The challenges in this topic include using more attractive expression hosts, achieving improved yields, as well as proper glycosylation patterns that are related to safety and efficacy of the biopharmaceutical (BF).

Among the next generation expression hosts, plants offer a very convenient set of advantages for antibody production (Streatfield et al. 2015); however, the development of transgenic plants is a laborious process and plant biomass also takes longer timer for production than microalgae. A second limitation found in the plant systems is related to the potential for gene flow (via pollen) to surrounding crops (Gressel 2010), which will have implications in the regulatory approval. Since algal systems possess a great potential for large-scale production of biopharmaceuticals (BFs) (see Chap. 1), the present chapter summarizes the effort made thus far to implement platforms for the production of antibodies and immunotoxins in alga species (Table 5.1).

Current Developments on Algae-Made Antibodies and Immunotoxins

Large Single-Chain (lsc) Antibody Against Herpes Simplex Virus (HSV) Glycoprotein D

Mayfield and coworkers reported in 2003 a seminal study on the expression of antibodies in algae. A lsc antibody was successfully assembled in the *C. reinhardtii* chloroplast. A gene coding for an lsc targeting the HSV glycoprotein D was used, comprising the entire IgA heavy chain fused to the variable region of the light chain with a flexible linker between them. *C. reinhardtii* was transformed at the chloroplast level with the expression driven by the chloroplast *atpA* or *rbcL* promoters and 5' untranslated regions. Remarkably, the lsc antibody accumulated as a soluble protein in the transplastomic strains and showed a positive reactivity against Herpes virus proteins in ELISA assays. Interestingly, the algaе-made lsc antibody assembled into dimeric structures *in vivo* with no posttranslational modifications, aside from the disulfide bonds associated with dimerization, detected in the recombinant protein. Therefore, the lsc antibody will avoid concerns related to the differential glycosylation that occur in other expression hosts associated to undesired immunogenic activity.

Table 5.1 Compilation of the antibodies and immunotoxins produced in algal systems

| Molecule of interest | Species used as host | Transformation/expression approach | Findings | Yields | Reference |
|--|-----------------------|-------------------------------------|---|-----------------------------|-------------------------|
| Large single-chain antibody against Herpes simplex virus glycoprotein D | <i>C. reinhardtii</i> | Particle bombardment-Transplastomic | Human antibody was successfully expressed as soluble protein able to bind the specific target | ≈1 % TSP | Mayfield et al. (2003) |
| Human IgG1 monoclonal antibody 83K7C targeting the PA83 anthrax antigen | <i>C. reinhardtii</i> | Particle bombardment-Transplastomic | Human antibody was successfully expressed and the affinity is similar to the same antibody expressed in mammalian cells | Not reported | Tran et al. (2009) |
| Camelid VH _H domains targeting the botulinum neurotoxin | <i>C. reinhardtii</i> | Particle bombardment-Transplastomic | Algae-made nanobodies were able to specifically bind the target toxin and prevented death of neurons in vitro, the dimeric nanobodies showed enhanced activity and were successfully delivered at the gastrointestinal tract of mouse using whole algae cells administered orally. This is a promising approach for intoxication therapies and oral delivery of intact antibodies using whole cells | 4.6 % TSP | Barrera et al. (2015) |
| Human monoclonal IgG antibody CL4mAb targeting Hepatitis B virus surface protein | <i>P. tricornutum</i> | Particle bombardment-nuclear | Functional IgG antibody was expressed and secreted into the culture medium | 2.5 µg/mL in culture medium | Hempel and Maier (2012) |

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|---|-----------------------|-------------------------------------|---|--|----------------------|
| Human monoclonal IgG1 antibody CL4mAb against the Hepatitis B Virus surface antigen | <i>P. tricornutum</i> | Particle bombardment-nuclear | Functional antibody was expressed and assembled in the endoplasmatic reticulum of <i>P. tricornutum</i> using the endoplasmic reticulum retention signal (DDEL) at the C-terminus of both antibody chains. The use of a nitrate-inducible promoter allowed to produce HBsAg in <i>P. tricornutum</i> more efficiently than in other plant systems such as <i>N. tabacum</i> | 8.7% TSP | Hempel et al. (2011) |
| Immunotoxin (αCD22PE40) comprising the domains II and III of Exotoxin A (PE40) from <i>Pseudomonas aeruginosa</i> and a scFv targeting CD22 | <i>C. reinhardtii</i> | Particle bombardment-Transplastomic | Algae-made immunotoxin was able to specifically bind the target cells in vitro and induced apoptosis, inhibiting tumor growth in a tumor mice model The dimeric form (αCD22HCH23PE40) showed enhanced activity This is a promising approach for therapies in B-cell chronic lymphocytic leukemia | αCD22PE40: 0.3–0.4 % TSP αCD22HCH23PE40 (dimeric form): 0.2–0.3 % TSP | Tran et al. (2013a) |

Full-Length Antibodies Against the Anthrax Protective Antigen 83

The production of a full-length human monoclonal antibody in algae was reported for the first time by Tran et al. (2009). The variable region from the human monoclonal antibody 83K7C targeting the anthrax protective antigen 83 (PA83) was fused to the constant domain of the human IgG1. This therapeutic antibody is of relevance due to its blocking activity against the anthrax toxin. The corresponding H and L chain proteins were successfully expressed in the *C. reinhardtii* chloroplast under the control of the psbA promoter. The rescued transplastomic clones accumulated these proteins, which were successfully purified (Fig. 5.2). Proper antibody assembly was proven in PA83 binding assays (ELISA) with positive findings. Interestingly, affinity of the algae-made antibody was similar to that of the mammalian-made 83K7C antibody (Fig. 5.3). Following the same methodology, the authors reported the expression of two additional antibodies against unspecified targets (a human IgG1 and a mouse IgG1 antibodies), thus proving the flexibility and capability of algal cells for the expression of a wide range of antibodies in the chloroplast.

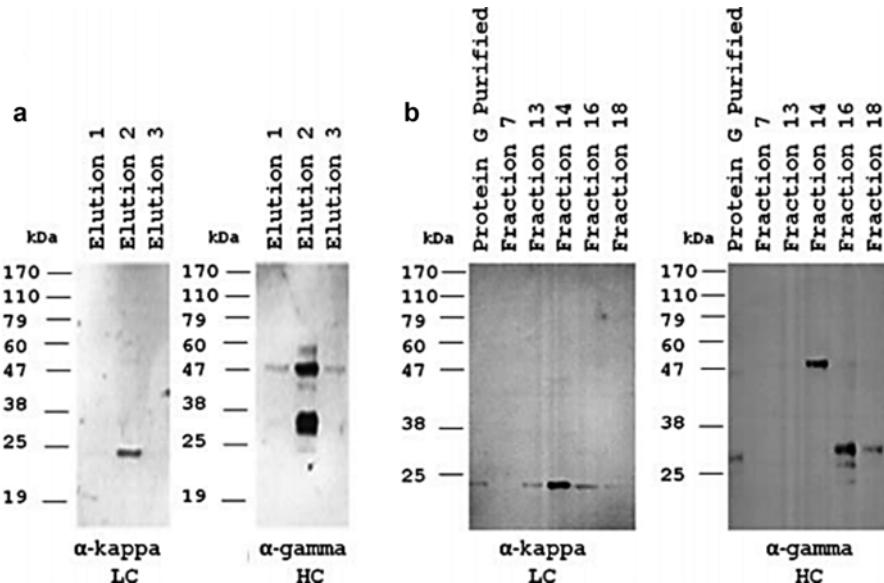


Fig. 5.2 The alga-made antibody targeting the anthrax antigen was successfully produced and purified from *C. reinhardtii* extracts. A Protein G-based purification methodology allowed for the capture of the antibodies via Fc region binding (a) Immunoblots showing the stoichiometric amounts of LC protein co-purified along with the HC protein when using protein G. (b) Immunoblots showing that the purification process allowed for the separation of the antibody partial degradation products using size exclusion chromatography (Figure taken from Tran et al. (2009), permit number 3795580253155)

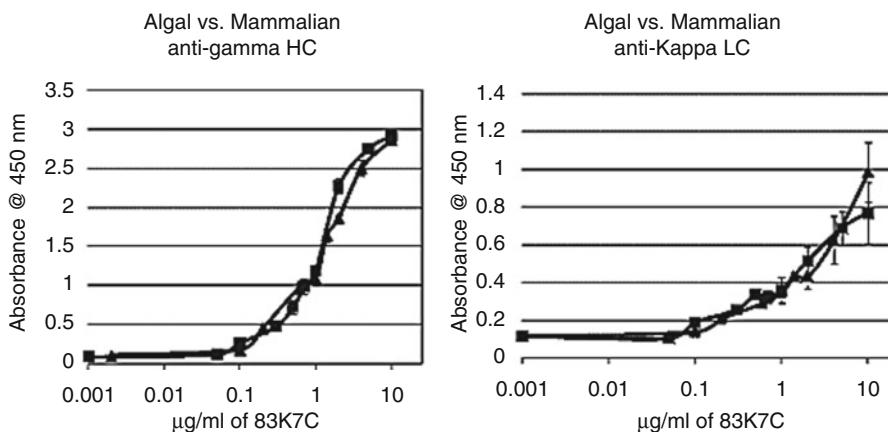


Fig. 5.3 Evidence on specific binding of the algae-made antibody to the target antigen PA83. ELISA analysis targeting PA83 performed with either algae- or CHO cell-made antibodies. All measurements were done in triplicate from a range of 0.001 to 10.0 mg/mL for both the algal (&) and mammalian (~) expressed antibody. Binding was determined using an anti-kappa LC and an anti-gamma HC secondary antibody. Algal expressed 83K7C was shown to have similar binding characteristics as the CHO expressed antibody (Figure taken from Tran et al. (2009), permit number 3795580253155)

An Anti-hepatitis B Surface Protein Antibody Produced in *Phaeodactylum tricornutum*

Hempel and coworkers in 2011 reported the expression in *Phaeodactylum tricornutum* of a monoclonal human IgG antibody against the Hepatitis B surface protein. Expression of the recombinant antibody was driven by the nitrate reductase promoter, which is induced by the presence of nitrate and down regulated by ammonia. The recombinant antibody was accumulated into the endoplasmic reticulum since a retention signal was fused to it, thus complex glycosylation that occurs in the Golgi apparatus is avoided under this approach.

According to ELISA assays the algae-made antibody positively reacted with the specific target and the yields attained were of up to 8.7 % of the total soluble protein (TSP) (ca. 1.6 mg per liter of culture). Hempel and Maier (2012) subsequently explored the production of the same antibody but in absence of the reticulum endoplasmic retention signal to secrete the recombinant protein into the culture medium. Such approach would allow for an easy purification of the recombinant antibody since *P. tricornutum* essentially does not secrete endogenous proteins and thus antibody separation is easier than purifying it from total cellular extracts (Hempel and Maier 2012). The overall yield for the production of this antibody was higher (2250 ng per mL) than those attained with the endoplasmic reticulum (ER) retention approach. Interestingly, the *P. tricornutum*-made antibody remained stable in the culture medium for at least 2 days and supplying fresh media allowed to restore antibody production and thus continuous cultivation seems viable (Fig. 5.4).

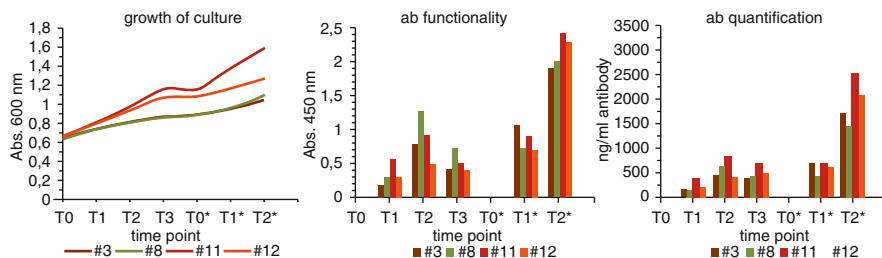


Fig. 5.4 Yield and functionality analysis of the alga-made secreted antibodies targeting Hepatitis B virus antigen. Inducible expression was performed in cell lines #3, #8, #11, and #12. Samples were taken over 3 days (T1, T2, T3) with a subsequent medium exchange (T0*) and two additional samples were taken (T1*, T2*). ELISA assay targeting the Hepatitis B virus antigen allowed to detect antibody binding. Antibody accumulates within the first 2 days of induction (450–850 ng/mL) with a subsequent decrease that correlates with the observed binding efficiency. After medium exchange the levels of the recombinant antibody are restored (productivity of 1550–2550 ng/mL at day T2*) (Figure taken from Hempel and Maier (2012))

A recent study aimed at characterizing, at the biochemical level, the *P. tricornutum*-made antibody (Vanier et al. 2015). The sequencing analysis revealed that the DDEL retention signal on both light and heavy chains is preserved in the recombinant molecule. Moreover, the analysis confirmed the presence of the C-terminal lysine; demonstrating that recombinant antibodies preserving the C-terminal end can be produced in this host. This is in contrast with mAbs secreted from Chinese hamster ovary (CHO) cells that show high heterogeneity in this respect. In addition, the removal of the signal peptide was also proved. The authors concluded that the microalga is able to cleave the signal peptide present in the heavy and light chains by a signal peptide peptidase mechanism similar to that occurring in mammalian cells. However, in the case of the light chain a second N-terminal peptide resulting from a cleavage after the amino acid 31, instead of the expected processing at the amino acid 22, was found in about 90 % of the molecules. Furthermore high mannose-type *N*-glycans were identified in the alga-made antibody, which could lead to a low half-life modifications of the effector functions mediated by the Fc region (Loos and Steinkellner 2012). Therefore, the authors suggested implementing glycoengineering approaches in this species to avoid this concern.

Camelid Antibodies Against Botulinum Neurotoxin Serotype A (BoNT/A)

Camelid V_HH domains are attractive antibody-like molecules for developing anti-toxin therapies due to their simple structure comprised of a single chain of small size (12–15 kDa) (De Genst et al. 2006), high water-solubility, heat-stability, pepsin-resistance, and high binding capacity. Camelid V_HH domains were used to target

botulinum neurotoxin (BoNT), which is a potent toxin with a LD₅₀ of 1.3–2.1 ng/kg in primates (Arnon et al. 2001) that play a key pathogenic role in *Clostridium botulinum*-derived intoxication. BoNT impairs neurotransmission capabilities in the intoxicated nerve cells leading to a potentially lethal flaccid paralysis that demands artificial ventilation and feeding. A therapeutic approach for this condition consists in the neutralization of the toxin by antibodies present in antisera.

Barrera et al. (2015) have reported the expression in the *C. reinhardtii* chloroplast of three V_HH domains derived from alpacas immunized with BoNT/A (two monomer V_HH domains, called C2 and H7; and a heterodimer comprised of two V_HH domains separated by a flexible spacer, called H7-fs-B5). The psbA promoter drove the expression of the transgenes. The corresponding transplastomic *C. reinhardtii* clones were able to accumulate soluble and functional V_HH domains, which were subsequently purified by affinity chromatography at a similar yield than that observed in *E. coli*. The expression levels, in general, were of up to 4.6 % of TSP. In ELISA assays, the algae-made V_HH domains were able to bind the BoNT/A in the subnanomolar range; which is similar to the behavior of the *E. coli*-made domains (Mukherjee et al. 2012). In vitro testing using rat primary cerebellar neurons revealed that the algae-made VHH domains are capable of protecting the cells from BoNT/A inactivation at similar potency when compared to *E.coli*-made molecules.

Interestingly, the multivalent nanobody H7-fs-B5 containing two binding domains showed a superior binding avidity and efficacy in the cell protection assay than V_HH monomers. Moreover, the authors explored the ability of whole algae cells to serve as an oral delivery vehicle by administering algae biomass by the oral route. Algae cells released intact neutralizing antibodies at the stomach and small intestine following oral administration of a transplastomic clone expressing H7-fs-B5 (Fig. 5.5). This approach will obviously greatly simplify the production of antibodies with implications in treatments at the intestinal level since algae would serve as antigen biofactory and delivery vehicle, avoiding the purification process needed for conventional products. Moreover considering the successful approaches using plant cells for the delivery of BFs to the systemic compartment following oral administration, algae could be used in a similar way to treat diseases whose targets are in the systemic level (Kohli et al. 2014).

Immunotoxins Targeting CD22+ Cells

Microalgae have been proposed as attractive hosts for the production of immunotoxins. A seminal work in this topic was reported by Tran et al. (2013a), in which algae were assessed to produce fully functional immunotoxins. The authors designed a gene encoding the immunotoxin called α CD22PE40 consisting of a single chain antibody (scFv) targeting CD22, a B-cell surface molecule, fused to domains II and III of Exotoxin A (PE40) from *Pseudomonas aeruginosa*. PE40 induces apoptosis via inhibition of translation in mammalian cells by ribosylating eukaryotic elongation factor 2 (eEF2) (Kondo et al. 1988). This modified toxin is unable to bind cells,

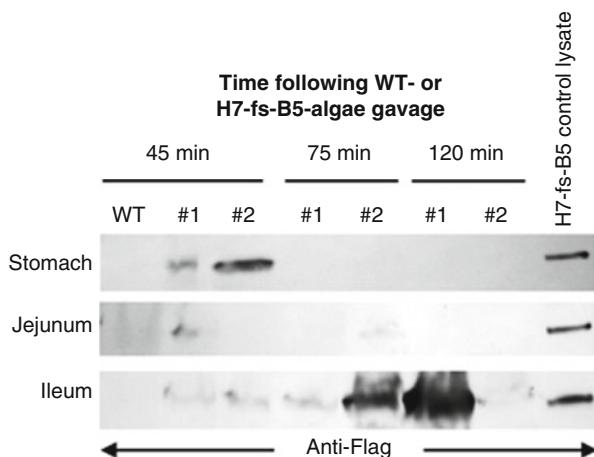


Fig. 5.5 *C. reinhardtii* cells serve as the biofactory and oral delivery vehicle of nanobodies. Whole *C. reinhardtii* biomass expressing the camelid was orally administered and camelid antibodies detected by immunoblot analysis of samples from mouse stomach and small intestine at distinct time points (45, 75, or 120 min post-administration). #1 and #2: two test mice. WT: mouse given wild type algae. The control lysate was obtained from the biomass administered to test mice. Antitoxin detection is positive in the stomach 45 min post-administration. The signal is subsequently detected in the jejunum and ileum at 75 min and accumulated at a major degree in the ileum 120 min post-administration (Figure taken from Barrera et al. (2015), permit number 3795570920978)

resulting in a 100-fold less toxic molecule than the native exotoxin A (Frankel 1992). Once the immunotoxin is internalized in the target cell, the domain II is cleaved in acidified endosomes by a furin protease; liberating the cytotoxic domain III from the antibody, which is targeted for degradation (Gordon et al. 1995).

In addition considering that a limitation known for immunotoxins, similar to α CD22PE40, is the short serum half-life which is consequence of their small size (Kreitman et al. 1999); a more complex chimeric immunotoxin was designed (“ α CD22CH23PE40”) to improve this aspect. The design of “ α CD22CH23PE40” relies in the inclusion of the hinge and CH2 and CH3 domains of a human IgG1, which were located between the α CD22 scFv antibody and PE40. This configuration is intended to produce a complex having higher molecular weight since dimerization of the complex is induced by two disulfide bonds formed in the hinge region.

The authors placed the chimeric genes under the control of the promoter and 3' UTR from the psbA gene and transferred this heterologous DNA into the *C. reinhardtii* chloroplast genome. Transplastomic clones were able to produce both monomeric and dimeric immunotoxins in an active form since they possessed enzymatic activity, bind specifically to cells displaying the CD22 molecule, and induce apoptosis. The dimeric form showed higher activity as expected. Remarkably, *in vivo* evaluations of the algae-made toxins revealed that these significantly inhibit tumor growth in a subcutaneous tumor cell xenograft mice model (Tran et al. 2013a; Fig. 5.6).

Similarly, the same research group headed by Mayfield (Tran et al. 2013b) produced another immunotoxin in *C. reinhardtii* called α CD22Gel, which comprises a scFv targeting the B-cell surface antigen CD22 and gelonin; a eukaryotic ribosome

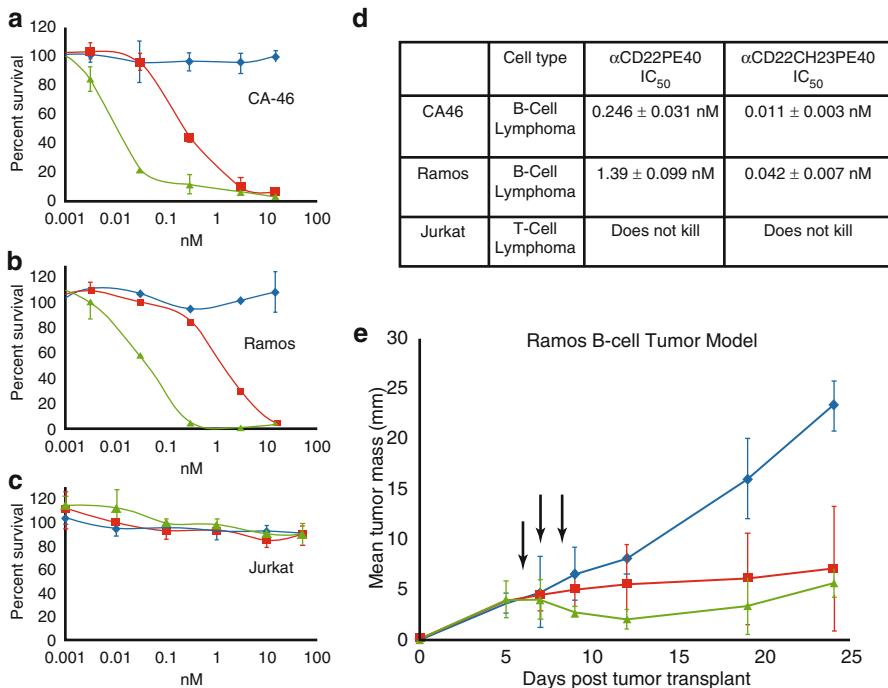


Fig. 5.6 Evidence on the in vitro and in vivo activity of the algae-made immunotoxins against cancer cells. α CD22 (blue traces), α CD22PE40 (red traces), and α CD22CH23PE40 (green traces) were incubated with CA-46 B cells, Ramos B cells, and Jurkat T cells to determine their cytotoxic activity. (a) α CD22PE40 and α CD22CH23PE40 were effective at killing CA-46 B cells, but α CD22 alone was incapable of killing CA-46 cells. (b) Additionally, α CD22PE40 and α CD22CH23PE40 were able to kill Ramos cells, but α CD22 was unable to inhibit Ramos cell proliferation. (c) α CD22, α CD22PE40, and α CD22CH23PE40 were unable to kill Jurkat T cells. (d) Immunotoxin IC₅₀ values determined in vitro. Both immunotoxins killed B cells, observing a higher efficacy for the dimeric α CD22CH23PE40 in comparison to that of the α CD22PE40 immunotoxin. (e) In vivo activity was assessed by transplanting Ramos cells into Rag^{-/-} × gc^{-/-}. After the development of tumors of approximately 4 mm in diameter mice were treated each day for 3 day with 240 µg/kg of α CD22, α CD22PE40, or α CD22CH23PE40. Both α CD22PE40 and α CD22CH23PE40 were capable to inhibit tumor proliferation when compared to α CD22 alone (Figure taken from Tran et al. (2013a))

inactivating protein from *Gelonium multiflorum*. Following the previous work, a dimeric form of the immunotoxin (α CD22CH23Gel) was successfully produced by the inclusion of the constant domains 2 and 3 of a human IgG1 (CH23) between the α CD22 and gelonin genes. Transplastomic clones were successfully generated accumulating the expected recombinant proteins. According to ELISA assays, CD22 accumulated at approximately 0.6–0.7% of TSP, α CD22Gel at 0.2–0.3% of TSP, and α CD22CH23Gel at 0.1–0.2% of TSP.

In terms of functionality, the algae-made immunotoxin bound cells expressing CD22. Interestingly, the divalent α CD22CH23Gel showed higher cytotoxic activity against CA-46 and Ramos B cells than that showed by the monovalent α CD22Gel (Tran et al. 2013b). This is relevant since the dose to attain therapeutic effects could

be reduced and thus a reduction of side effects of these types of molecules (e.g. vascular leak syndrome) may be achieved (Liu et al. 2012).

This system based in algae-made immunotoxins offers significant advantages over conventional methods. Antibody drug conjugates (ADCs) are promising therapies able to inhibit cancer-cell proliferation (Polakis 2016), however their production requires the purification of an antibody using a mammalian expression platform with a subsequent chemical coupling of a toxin molecule (Shen et al. 1988). The only marketed ADCs namely brentuximab vedotin and trastuzumab emtansine (T-DM1), have proven efficacy against haematological and solid malignancies; respectively. Most ADCs are designed with special linkers that are cleaved upon acidification in the endosome, thus the toxin is released after uptake by the target cells (Bradley et al. 2013). ADCs typically are highly heterogeneous in terms of drug-to-antibody ratios and drug load distributions, which hamper their stability and efficacy (Sochaj et al. 2015). Another limitation consists in the undesired immunogenic activity of nonhuman or even humanized antibodies, as well as the limited access to tumors due to the large size of the antibodies (Peters and Brown 2015). Therefore, fully human, small, and effective immunoagents are needed. In parallel, having low cost methods for the production of these complexes are necessary to meet the demand of such therapeutics.

The use of genetic engineering approaches enables the production of immunotoxins in a straightforward manner at lower costs. Immunotoxins can be produced through chimeric genes comprising scFvs genetically fused to a protein with toxic activity. However, the expression of functional immunotoxins is not possible in the eukaryotic cells typically used for antibody expression (e.g. CHO cells) because of their toxicity in that host (FitzGerald and Pastan 1991; Pastan and FitzGerald 1991). For this reason immunotoxins have been produced in *E. coli*, which allowed for efficient production of a homogeneous product. However, this production system involves the rescue of insoluble aggregates that should be subjected to refolding to obtain the functional immunotoxin (Mansfield et al. 1997a, b) and possesses a reduced ability to fold proteins with multiple domains efficiently and form disulfide bonds within proteins (Yin et al. 2007).

In contrast, algae-made immunotoxins avoid the use of specific linkers and chemical coupling since they rely on the recognition of a cleavage site included in the chimeric protein. Additionally, the ability to produce genetically coupled immunotoxins eliminates the possibility of unstable linkers (Xie et al. 2004) and reduces off-target toxicity. Moreover unlike bacteria, algae chloroplasts efficiently assembled functional, soluble immunotoxins.

Prospective View

MAbs and immunotoxins are of great relevance in medicine and thus new attractive sources for these molecules are needed to expand their applications and increase coverage in the benefit of human health at a global level. Some alternative platforms

for the production of antibodies have been proposed during the last decades. Among them, superior plants and moss are promising approaches (Reski et al. 2015). However, some challenges in the field exist. In the case of stable-transformed plants, protein expression levels and growth rates are still a limiting aspect. Furthermore, plant cultivation in open fields implies the use of agricultural areas and the spreading of transgenic plants represents a biosafety concern. Transient expression systems in plant tissues are considered more promising and these systems are under industrial adoption for the production of vaccines (Gleba et al. 2014; Salazar-González et al. 2015). A drawback of this system is the lack of seed stocks. In contrast, algae systems offer preservation of recombinant clones and can be grown in a short period of time.

The reports analyzed in the present chapter indicate that algae-based systems possess particular attributes with interesting perspectives to the scenario of platforms for antibody production. An attractive biosynthetic capacity has been found in algae for antibody production, especially in the chloroplast where disulfide bounds can be formed and thus full-length antibodies can be successfully assembled. This approach offers convenient yields and avoids the differential glycosylation that would occur when nuclear expression is followed. In the case of nuclear expression, assessing the expression of antibodies in *C. reinhardtii* remains a pending objective. Recent advances in the development of more efficient nuclear expression approaches in *C. reinhardtii* will allow exploring this aim (Rasala et al. 2014; see Chap. 2). Also glycoengineering approaches in algae are of relevance to confer specific glycosylation patterns with implications in safety and stability. The implementation of such approaches will add important prospects for the field and it should be considered that such kind of approaches already showed to be feasible in plant systems (Bakker et al. 2006; Cox et al. 2006; Decker and Reski 2012; Schahs et al. 2007; See Chap. 9) broadening the possibilities for using algae-based systems. This aspect acquires special importance in the case of antibodies for cancer therapy since recent studies have proven a clear influence of glycans on the antibody ability to access tumors (Venetz et al. 2015).

In terms of yields, algal system cannot currently compete with mammalian systems that have been engineered to produce high amounts of antibodies with transient expression levels for recombinant antibodies of 100–1000 mg/L (Zhang and Shen 2012). However, an overall analysis of the current advances highlights the substantial potential of microalgal expression systems; thus improvements in expression strategies are considered viable.

In terms of specific targets a number of interesting perspectives are identified. MAbs are of special importance in immunotherapy against cancer due to their specificity and an overall lack of multidrug resistant effects. The efforts to initiate clinical trials with algae-made immunotoxins will be critical in this field.

Since the toxicity and immunogenicity of the bacterial or plant toxins have caused some problems during the development of immunotoxins (Schindler et al. 2001; Weiner et al. 1989), the use of more compatible immunoagents is desirable. ImmunoRNases are complexes comprising a ribonuclease as the toxic component having the advantage of not being immunogenic and not systemically toxic since

they are pro-toxins that become toxic after antibody-mediated internalization into the target cells (Rybak and Newton 1999). Therefore, the production in algae of ImmunoRNases is an interesting path to be explored looking to improve immunotherapies produced in low cost systems. As an example, compact human antibodies (scFv-Fc) targeting ErbB2, which is overexpressed in cancer cells, have been fused to human RNase as the effector moiety. The scFv-Fc consisted of anti-tumor ErbB2-directed scFv fused to a human IgG1 Fc domain and it was proposed as a poorly or not immunogenic molecule in humans that mediate both antibody-dependent and complement-dependent cytotoxicity. In addition, the molecule could be of efficient tissue penetration due to its compact molecular size (De Lorenzo et al. 2004). ImmunoRNases showed to be functional in terms of enzymatic activity, and positive effects were observed using *in vitro* and *in vivo* assays with ErbB2-positive tumor cells (Borriello et al. 2011; D'Avino et al. 2014).

Another important perspective for this field contemplates the use of whole algae biomass for the delivery of functional antibodies at either the intestinal or the systemic levels as required for specific pathologies. This will dramatically simplify the production process decreasing the cost of BFs. The production and delivery of antibodies targeting proinflammatory factors that mediate intestinal pathogens is a potential application for the technology of algae-made antibodies. In plant systems, a product called PRX-106 (Protalix 2015) consists of a recombinant anti-TNF protein comprising the binding domain of the human TNF receptor fused to the Fc component of a human antibody. After efficacy was proven in test animals a phase I clinical trial of the plant-made PRX-106 has been conducted in which safety and accepted tolerability were proven since biological activity in the gut and induction of regulatory T cells were found. The development of such therapies in algae systems will lead to an expansion of the opportunities for achieving efficacious and low cost therapies against several diseases, being the primary targets inflammatory intestinal conditions, e.g. inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (Berns and Hommes 2015).

Another key perspective emerges in the topic for the case of anti-venoms. Current anti-venom therapies consist of serum-derived products containing antibody fragments that inactivate and accelerate the clearance of relevant venom components. Although efficient, the production of these products is complex and their safety is considered improvable (Alvarenga et al. 2014). Alternative developments consisting of recombinant antibodies and smaller functional units are emerging approaches in the development of anti-venom therapies (Roncolato et al. 2015). It is interesting to note that the production of venom neutralizing antibodies in algae systems has been neglected and thus constitutes a relevant perspective for this field.

In conclusion, the current evidence accumulated in preclinical trials indicates that the use of microalgae as attractive biofactories of antibodies and antibody-like molecules is feasible and clinical trials for some candidates are expected in the near future. Therefore, there are interesting perspectives for advancing in the development of biosimilars, biobetters, or next generation molecules based in algae-made antibodies or immunotoxins.

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Chapter 6

Algae-Made Cytokines and Growth Factors

Introduction

Cytokines are a diverse group of signaling molecules that act as regulators of cellular function during normal, developmental, and pathological conditions (Dinarello et al. 1990; Meager 1998). Cytokines exert their actions locally as autocrine, juxtacrine, or paracrine response modifiers; which are triggered by binding specific receptors on the cell membrane in the target cells (Miyajima et al. 1992). The etymology of cytokine is from the Greek *kytos* meaning ‘hollow’ or ‘vessel’ and *kinein* meaning ‘to move’ and was adopted for a group of immunoregulatory proteins, such as interleukins, to be distinguished from other molecules known as growth factors that modulated the proliferation and bioactivation of nonimmune cells. However with the increase in knowledge on these proteins, it is clear that the distinction of these two terms is not accurate since many of the classical immunomodulatory cytokines modulate the proliferation and differentiation of non-immune cells. In addition to this, many typical growth factors regulate immune competent cells.

With some exceptions, such as the platelet-derived growth factor (PDGF) and the transforming growth factor- β (TGF- β), cytokine expression is highly regulated and inducible; resulting in cellular activation after sensing specific signals. For instance, the epidermal growth factor (EGF) is constitutively expressed in some cell types (Wong and Wright 1999). Cytokines typically exert several biological activities in multiple cell types and the overlapping activity from different cytokines is a common phenomenon called cross-talk (Paris et al. 1988; Sun et al. 1999).

Most cytokines show stimulatory or inhibitory activities and may synergize or antagonize the actions of other cytokines and hormones (Matsumoto and Kanmatsuse 2000). An important feature of their action is that a single cytokine may induce a specific reaction under a particular circumstance and elicit an entirely opposite reaction under a different set of circumstances (Sun et al. 2000). This has particular relevance in their therapeutic use. The growth state of the recipient cells, the environment in terms of neighboring cells, the cytokine concentration, and the presence

of other cytokines can influence the type, duration, and extent of the elicited response (Hasbold et al. 1999).

The current platforms for the production of recombinant cytokines including *Escherichia coli*, insect cells, and mammalian cell lines face some limitations such as high production cost, risk of contamination with pathogens or endotoxins, and non-optimal post-translational processing. For instance, the human recombinant IFN α 2b currently used in the clinic is produced using recombinant *E. coli* cultures. Although recombinant proteins are conveniently obtained (in the range of 3–5 g per liter of culture), they are unfolded and thus refolding steps are required to produce the functional molecule (Beldarrain et al. 2001). In addition to the cost of this complex processing, the recovery rates are below 20 % and the specific protein activity is diminished. Another limitation is given by the absence of the *O*-glycosylation existing in the native molecule leading to a shorter serum half-life (Youngster et al. 2002). The costs for these products derived from processes that require fermenters are high as well as for the downstream processing.

Therefore new production platforms with attractive features overriding the above-mentioned limitations are required. Cells having the ability to produce soluble, properly folded glycosylated cytokines and growth factors at low costs would be the ideal case. Herein, the scenario on the use of algae species as convenient biofactories of cytokines and growth factors is presented (Table 6.1), and the potential of these platforms is discussed.

Section I

This section describes the efforts conducted by the research group headed by Mayfield on the expression of human cytokines and a growth factor in *Chlamydomonas reinhardtii* chloroplasts. The recombinant algae strains were generated by particle bombardment with expression vectors containing codon-optimized genes under the control of the *psbA* promoter (Rasala et al. 2010).

Human Interferon β 1

Interferon β (IFN- β) is used as treatment for multiple sclerosis (Freedman et al. 2014) and as antiviral therapy since it has comparable antiviral activity to IFN- α (Lin and Young 2014). For instance IFN- β is an alternative therapeutic option for *Hepatitis C virus* infection (Ahn et al. 2009; Inoue et al. 2009; Ishikawa et al. 2012). However, the IFN- β treatment is rather unwieldy since only a recombinant form of IFN- β is available for treatment; which has a shorter half-life imposing a frequent administration scheme. Under the approach implemented by Rasala et al. (2010), IFN- β was not expressed at significant levels; probably due to stability problems, therefore further strategies to achieve efficient expression of this protein need to be elucidated.

Table 6.1 Compilation of cytokines and growth factors produced in algal systems

| Molecule of interest | Species used as host | Transformation/ expression approach | Findings | Yields | Reference |
|---|--------------------------|--------------------------------------|---|---|----------------------|
| Tumor necrosis factor α (TNF- α) | <i>Dunaliella salina</i> | Particle bombardment- Transplastomic | Not reported | Not reported | Xue et al. (2003) |
| Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) | <i>C. reinhardtii</i> | Particle bombardment- Transplastomic | Proteins detected in Western blot | 0.43–0.67 % TSP | Yang et al. (2006) |
| Human interferon $\beta 1$ (IFN- $\beta 1$) | <i>C. reinhardtii</i> | Particle bombardment- Transplastomic | Protein was not expressed at relevant levels | Not observed | Rasala et al. (2010) |
| Human vascular endothelial growth factor (VEGF) | <i>C. reinhardtii</i> | Particle bombardment- transplastomic | The protein was expressed in a soluble form and purified by affinity chromatography Algae-made VEGF showed similar results to the <i>E. coli</i> -made VEGF in yields following purification and competitive binding assay | 2.5 % TSP | Rasala et al. (2010) |
| High mobility group protein B1 (HMGB1) | <i>C. reinhardtii</i> | Particle bombardment- transplastomic | The protein was expressed in a soluble form and purified by affinity chromatography Algae-made HMGB1 is functional in a fibroblast chemotaxis assay with similar potency to the <i>E. coli</i> -made HMGB1 | 2.5 % TSP | Rasala et al. (2010) |
| VEGF secreted in a scaffold engrafted in mice | <i>C. reinhardtii</i> | Glass beads method/nuclear | Scaffolds containing whole algae cells secreting VEGF were safe (non-immunogenic) and algae secreted functional VEGF in physiologically relevant amounts | Secreted into the biomaterial at levels of $\approx 0.6 \text{ pg}/\mu\text{g}$ protein | Chávez et al. (2016) |

Human Vascular Endothelial Growth Factor

The human vascular endothelial growth factor (VEGF) is one of the most important proangiogenic factors that potentiates microvascular hyperpermeability, which can precede or accompany angiogenesis. VEGF is a 34- to 42-kDa, dimeric, disulfide-bound glycoprotein that is implicated in the treatment of erectile dysfunction (Condorelli et al. 2013), depression (Udo et al. 2014), and emphysema (Tuder and Yun 2008). VEGF can also be used to formulate vaccines against pathologies in which the effect of this pro-angiogenic factor is involved, such as cancer (Mamdani et al. 2015; Pérez Sánchez et al. 2015; Semerano et al. 2015).

VEGF was successfully expressed by Rasala et al. (2010) as a soluble molecule and purified by affinity chromatography targeting the C-terminal FLAG epitope (Fig. 6.1). The algae-made VEGF accumulated at levels of up to 2 % of total soluble protein (TSP) and retained its antigenic properties as revealed by an

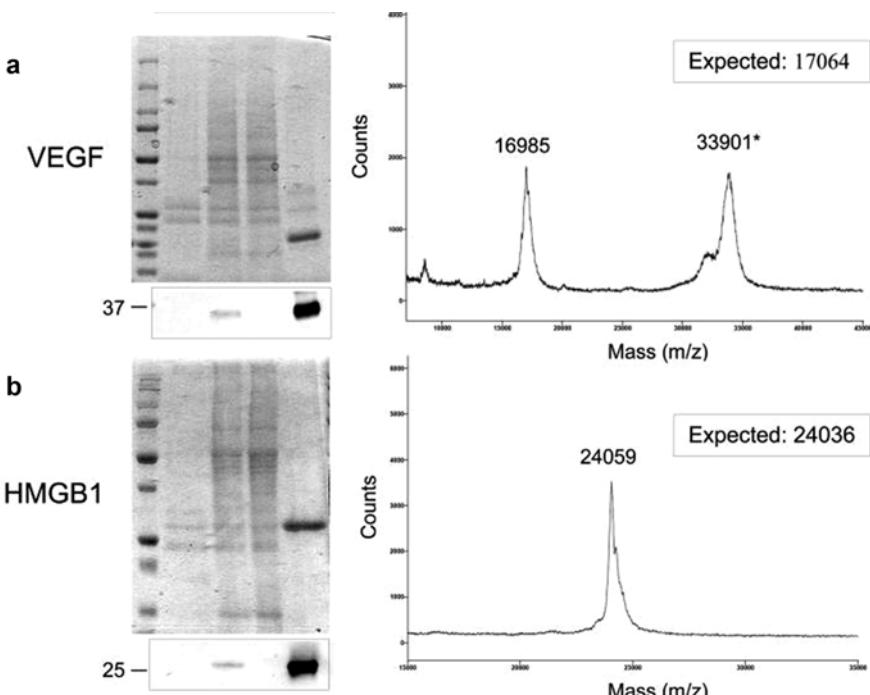


Fig. 6.1 Purification of algae-made VEGF (a) and HMGB1 (b) by affinity purification. Coomassie staining is derived from SDS-PAGE (*top left panel*) and immunoblot (*boxed bottom panel*) analyses. Lanes, receiving samples in equal volumes, from left to right correspond to the following fractions: insoluble fraction (Ins), total soluble protein (TSP), column flow through (Flow), and the eluate (Elu). 3 µg of purified protein were loaded in coomassie gels, while 500 ng of Elu were used for immunodetection. The *right panels* contain the MADLI-TOF MS results for the purified proteins. Asterisk (*) in (a) indicates the predicted dimer form of VEGF (Figure taken from Rasala et al. (2010), permit number 3795581298668)

ELISA assay, suggesting a proper folding. After determining the concentration of VEGF using *E. coli*-derived VEGF as a standard, the receptor binding assay indicated that the algae-made VEGF has a dose dependent binding to the VEGF receptor with a slightly lower affinity in comparison to the bacterial-made protein. The authors hypothesized that this phenomenon might be due to fractions of misfolded or truncated VEGF present in the samples. After performing a VEGF-receptor binding competition assay, it was observed that *E. coli*-derived VEGF competed with algae-made VEGF for receptor binding. In accordance with the previous results from affinity assays, the *E. coli*-derived VEGF displaced the algae-made VEGF (200 ng/mL) from VEGFR with an IC₅₀ of approximately 40 ng/mL (Fig. 6.2).

High Mobility Group Protein B1

The high-mobility group box 1 protein (HMGB1), which previously was thought to function only as a nuclear factor that enhances transcription, is currently recognized as a crucial cytokine that mediates the response to infection, injury, and inflammation (Gardella et al. 2002; Lotze and Tracey 2005). These observations have led to the emergence of a new field in immunology focusing on understanding the mechanisms of HMGB1 release, biological activities, and pathological effects in sepsis, arthritis, cancer, and other diseases (Lee et al. 2014). Therefore

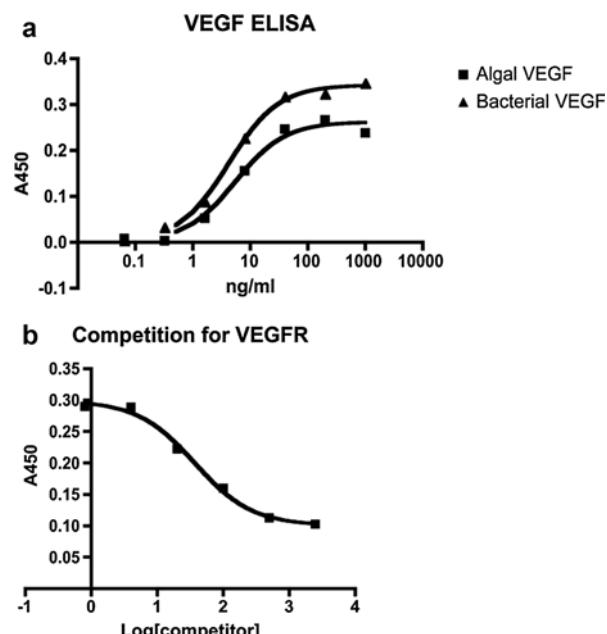


Fig. 6.2 Bioactivity of the alga-made VEGF. (a) Comparison of the VEGF levels with bacteria-derived VEGF using ELISA. (b) VEGF competitive binding assay. A fixed concentration of binding alga-made VEGF was applied to VEGFR-coated plates in the presence of distinct levels of *E. coli*-made VEGF (Figure taken from Rasala et al. (2010), permit number 3795581298668)

HMGB1 has a potential therapeutic use in several conditions. HMGB1 accumulated in soluble form in the *C. reinhardtii* chloroplast at levels of up to 2.5 % of TSP and its purification by affinity chromatography through the C-terminal FLAG epitope was successful (Fig. 6.1). Interestingly, the functionality of the algal-derived HMGB1 was demonstrated using a fibroblast chemotaxis assay, observing that it was comparable with the *E. coli*-made HMGB1 (Rasala et al. 2010; Fig. 6.3).

Section II

Herein other key developments reached by other groups are presented, which comprise the production of recombinant proteins intended for purification and parenteral administration; as well as systems that rely in whole algae cells included in scaffolds for regenerative medicine applications.

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the superfamily of the tumor necrosis factor (TNF). The human TRAIL protein contains 281 amino acid residues and belongs to the type II membrane protein, which includes an N-terminal transmembrane domain (15–40 amino acid residues) and a C-terminal extracellular domain (114–281 amino acid residues).

The biologic actions of TRAIL involve the induction of apoptosis of cancer cells following binding with specific receptors (Allen and El-Deiry 2012). TRAIL-mediated protection against diabetes has also been reported (Bossi et al. 2015). Due to the specific killing capacity against tumor or virus infected cells; TRAIL is of great interest in the development of new therapies. Interestingly, the extracellular domain of TRAIL comprising amino acids 114–281 can be cleaved by metalloproteinases resulting in a homotrimeric and soluble complex (sTRAIL) that preserve the biological activity of the full-length TRAIL (Gura 1997).

Yang et al. (2006) expressed the human sTRAIL in the *C. reinhardtii* chloroplast under the control of the *atpA* promoter. The transplastomic clones were successfully rescued and had accumulated the expected recombinant sTRAIL as revealed by Western blot analysis. The accumulation levels of the algaе-made TRAIL reached up to 0.67 % of TSP. Characterizing the functionality of the sTRAIL produced in *C. reinhardtii* remains a pending objective that will be critical to advance the development of novel therapies. The authors propose the optimization of the sTRAIL coding region according to codon usage in *C. reinhardtii* chloroplast to increase its expression and studying the accumulation mechanism of foreign proteins in this organelle (Yang et al. 2006).

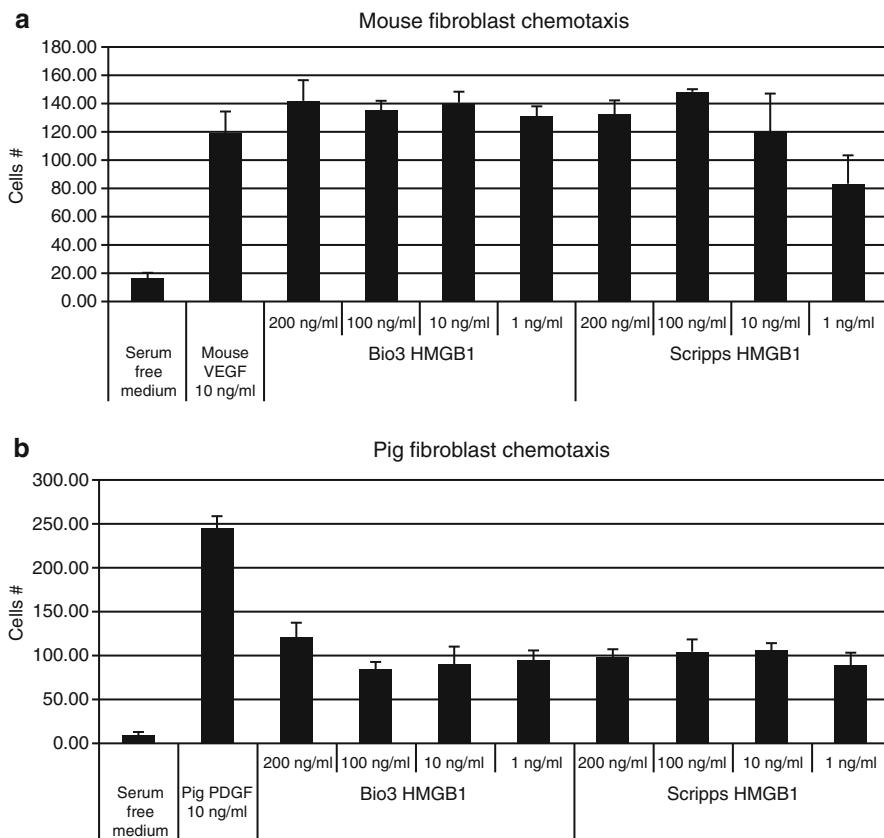


Fig. 6.3 Activity of the algae-made HMGB1. A fibroblast chemotaxis assay was performed to measure the number of mouse (a) or pig (b) fibroblasts migrating towards chemokines. In this assay the activity of algae-made HMGB1 (Scripps) is compared to that of commercial HMGB1 (Bio3), and to the mouse control VEGF (a) or pig PDGF (b) (Figure taken from Rasala et al. (2010), permit number 3795581298668)

Tumor Necrosis Factor Alpha Produced in *Dunaliella salina*

Xue et al. (2003) submitted a patent application claiming the use of *Dunaliella salina* for the expression of tumor necrosis factor alpha (TNF- α). The region clpP-trnl-petB on the chloroplast genome was chosen for the insertion of an expression cassette containing the two promoters (*chlL* and *atpA*) and the 3' terminator sequences of the *chlL* gene. *D. salina* was transformed following three different methodologies and the selection of transformed clones was based on spectinomycin resistance that is conferred by the *aadA* gene included in the vector. However, no data on the expression efficiency and functionality of the algae-made TNF- α was found.

C. reinhardtii Secreting VEGF for the Development of Photosynthetic Biomaterials in Tissue Engineering

Chávez et al. (2016) developed a breakthrough application of a recombinant alga expressing VEGF: *C. reinhardtii* cells that were seeded in a dermal scaffold and evaluated in a full-skin defect mouse model becoming a prototype of new biomaterials for regenerative medicine. The rationale of this approach implies that *C. reinhardtii* can serve as a source of oxygen derived from photosynthesis, thus solving hypoxia at the engrafted scaffolds; and at the same time providing a growth factor that promotes angiogenesis. The initial studies performed by the same research group proved the capability of *C. reinhardtii* to serve as oxygen provider in mammalian tissues maintained *in vitro* (Hopfner et al. 2014). Therefore, the use of collagen scaffolds *in vivo* in a model of full-skin defect mouse model in the presence of *C. reinhardtii* has ensured constant oxygen supply without inducing native immune response in the host. Interestingly, algae survived for at least 5 days *in vivo* and the chimeric tissues which comprise algae and murine cells were successfully generated. This study has opened the path for the development of autotrophic engineered tissues (Schenck et al. 2015). The most recent study on this topic (Chávez et al. 2016) was based on the use of a genetically engineered *C. reinhardtii* strain designed to secrete VEGF, which has a role in the re-establishment of functional vascular network during wound repair. The strain was engineered to express the VEGF gene fused with the leader peptide of the *C. reinhardtii* extracellular enzyme arylsulfatase (ARS2) for protein secretion. Gene expression was driven by the HSP70/RBCS2 promoter regions and the first intron of the RBCS2 gene. The safety of the treatment was confirmed since the material was engrafted in the back of fully immunocompetent mice, with no immune responses in the host organism observed. The authors presented evidence on the secretion of functional VEGF in physiologically relevant amounts (Fig. 6.4). Although no significant effect on vascularization due to VEGF secreted by *C. reinhardtii* was observed, this study has opened the path for the development of new biomaterials. The authors propose assessing the expression of other therapeutic proteins such as antibiotics, enzymes, or immune-modulatory molecules to optimize the induction of a pro-regenerative microenvironment in the wound area. In addition, the use of inducible promoters could allow the system to couple the secretion of necessary therapeutic molecules at specific stages of wound healing.

Prospective View

Cytokines and growth factors play significant roles in the biomedical field since they are part of the treatment of a myriad of pathologies. The proved concept of producing functional cytokines in algal species has opened the path for implementing these systems in a larger scale and evaluating them at the clinical level. Recently,

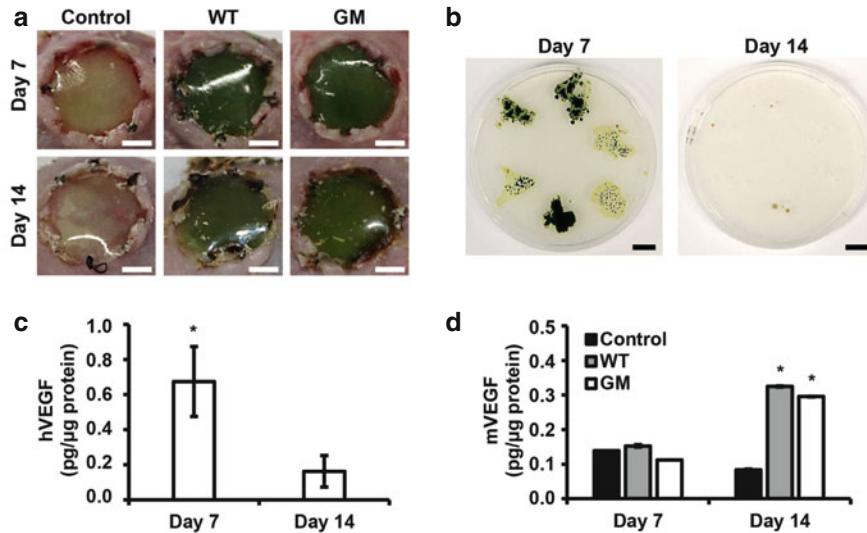


Fig. 6.4 Use of transgenic algae cells in regenerative medicine. Algae cells secreting VEGF are used to provide both oxygen and induction of angiogenesis in engrafted scaffolds in the test mice. Control scaffolds and scaffolds seeded with wild-type (WT) or VEGF-secreting (GM) *C. reinhardtii* strains were engrafted in the back of Skh1-mice (a). Viable algae were rescued from biopsies of the scaffolds at 7 days post-implantation but not at 14 days post-implantation (b). ELISA analysis revealed the presence of VEGF in extracts from explanted scaffolds (c). Levels of VEGF increased in mice engrafted with photosynthetic scaffolds (d). * $p < 0.05$. Scale bar = 2.5 mm in (a), = 1 cm in (b) (Figure taken from Chávez et al. (2016), permit number 3780491266407)

the production of biopharmaceuticals (BFs) at the pilot scale has been implemented (see Chap. 1) and will be useful to advance the industrial adoption of algae-based platforms. From the regulatory point of view the adoption of algae-based BFs is still in its infancy and progress is expected as the production platforms matured.

Although chloroplast has proven to be an attractive expression host with a more complex machinery than bacteria to accomplish disulfide bonds, the nuclear expression offers the possibility of protein secretion; which greatly facilitates purification and complex post-translational modifications (e.g. glycosylation). The use of recent nuclear expression approaches is a crucial objective to improve yields especially when the nuclear-based expression is implemented (See Chap. 2). Since the species *Phaeodactylum tricornutum* has been successfully applied for the production of secretory complex BFs (e.g. full-length antibodies; Hempel and Maier 2012). Thus the use of this platform to produce cytokines and growth factors is a key perspective.

The implementation of glycoengineering approaches is a crucial pending objective. Glycosylation is associated with the advantages of both stability and biological activity (Chamorey et al. 2002). Therefore, although the lack of glycosylation has not hampered the use of cytokines in the clinic, having human glycosylation patterns constitute the ideal situation. However, glycosylation in algae species differs

from that of humans and alterations in stability and biological activity may occur. Moreover, differential glycosylation could confer immunogenic activity on the molecule, leading to the generation of blocking antibodies and side effects (hypersensitivity). Recent advances in describing the glycosylation machinery in algae have been achieved (See Chap. 9), which constitute a relevant knowledge for exploring the viability of generating algae strains with glycoengineered patterns. The experience on implementing glycoengineering in yeast (Piirainen et al. 2014) and superior plants (Loos and Steinkellner 2014) will be useful. The case of moss is also interesting since it has an efficient homologous recombination in the nucleus and has become a model of glycoengineering in a photosynthetic organism by specific gene targeting (Decker et al. 2015). It should be considered that other post-translational modifications can be of relevance in functional interleukins. For instance, phosphorylation is critical for the anti-cancer effect of IL-24 (Panneer selvam et al. 2015).

A list of important therapeutic cytokines and growth factors is identified and constitutes a field of opportunity to generate new algae-made BFs. This includes the following cases: IL-35 is an interleukin associated with benefits in atherosclerosis (Bobryshev et al. 2015; Lin et al. 2015); IL-21, which mediates anti-viral and anti-inflammatory responses is of relevance in HIV and cancer therapies (Croce et al. 2015; Micci et al. 2015); IFN- α 2, IL-2, IL-15, IL-21, IL-12, IL-27, and IL-24, which are relevant in cancer treatment (Floros and Tarhini 2015; Panneer selvam et al. 2015; Yoshimoto et al. 2015). In addition, the production of immunocytokines in algal systems is a relevant aim in this field. Immunocytokines consists of fusion proteins comprising a cytokine with therapeutic activity and an antibody that directs the therapeutic action to specific target tissues. Immunocytokines have been evaluated at both preclinical and clinical levels for the treatment of cancer and inflammatory diseases (Pasche and Neri 2012; Schrama et al. 2006). The development of new immunocytokines, which are produced in conventional hosts, is a growing field. Recently the production of immunocytokines based on murine IL-1 β , IL-13, and IL-6 were described (Hess and Neri 2014, 2015).

On the other hand, the use of algae in generating photosynthetic biomaterial opens exciting perspectives in the field of regenerative medicine since this approach represents attractive possibilities in the fight of degenerative diseases. Stem/progenitor cell transplantation has acquired a remarkable relevance in this field; however the success is hampered by a suboptimal host environment. Oxygen diffusion and the presence of appropriate growth factors are critical during tissue regeneration (Bakshi et al. 2006; Stiers et al. 2016; Wang et al. 2016). Therefore, the design of composite scaffolds including whole algae as the source of both oxygen and recombinant molecules has the potential to significantly improve the current approaches.

Another important aspect to explore is oral delivery using whole algae cells. The research group headed by Henry Daniell (Xiao et al. 2015) and the research team from Protalix (Shaaltiel et al. 2015) have provided extensive evidence on the delivery of functional BFs using whole plant cells. The implementation of such strategy using algae species will expand the possibilities and be especially important considering the fact that algae can be rapidly transformed and grown, and may possess differential properties as delivery vehicles due to the peculiar com-

position of the cell wall (Rosales-Mendoza 2013). Of particular interest is the expression of anti-inflammatory cytokines and their evaluation in intestinal inflammatory disease models. For instance, the production and oral delivery of alpha-melanocyte-stimulating hormone (α -MSH) mediated by algae could lead to novel and effective therapies against colitis (Wei et al. 2015). Another interesting case could be the expression and oral delivery of IL-10 in a model of bowel disease (Asadullah et al. 2003).

In conclusion algae have proven capabilities for the synthesis of functional cytokines and growth factors, and may serve as a new host in the biopharmaceutical industry. Advances in developing new recombinant molecules as well as entering to clinical trials are envisioned in this field.

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Chapter 7

Other Biopharmaceuticals Produced in Algae

Introduction

The use of conventional hosts for the production of recombinant biopharmaceuticals (BFs) including *Escherichia coli*, insect cells, and mammalian cell lines; faces the limitations of high production cost, risk of contamination with pathogens, prions, or endotoxins; and non-optimal post-translational processing (Celik and Calik 2012; Fischer et al. 2015; Terpe 2006). As shown in the previous chapters of this book, microalgae species have emerged as new production platforms with attractive features overriding the above-mentioned limitations since they are safe and possess the ability to produce, at low costs, soluble and properly folded BFs. The group of BFs expressed is diverse and there is a realistic potential to use these products in the benefit of the human and animal health. The group of BFs produced in algae is expanding and therefore myriad of applications for algae-made BFs are expected (Rosales-Mendoza et al. 2012). Herein the efforts conducted by several research groups around the globe to explore the production of a diverse group of BFs in algae species are summarized and discussed.

Antimicrobial Peptides

Rabbit Neutrophil Peptide-1

The increase of resistance to antibiotics by pathogenic bacterial species demands alternative approaches to fight them. An alternative consists in the use of antimicrobial peptides, which can show a broad-spectrum of antimicrobial activities. Interestingly, the discovery and evaluation of such peptides is a growing field (Brogden 2005; Hancock 1997). Defensins are cysteine-rich, 3- to 4-kDa cationic antimicrobial peptides that induce membrane permeabilization in pathogenic

organisms (Agerberth and Gudmundsson 2006). In particular, the rabbit neutrophil peptide-1 (NP1) is capable of inhibiting the growth of a diverse group of pathogens (Miyakawa et al. 1996; Sinha et al. 2003). However, exploiting this peptide at the clinic requires cost effective platforms for its production at a large scale. Recombinant bacterial systems have been explored but fusion to other proteins is required because of the toxicity of NP1 in the bacterial expression host, imposing complex processing to remove the partner protein and isolate the target peptide (Sun et al. 2010).

In algal systems, NP1 was expressed in a nitrate reductase (NR)-deficient *Chlorella ellipsoidea* (*nrm-4*) strain; which was previously generated using an X-ray mutagenesis method (Wang et al. 2005). This strain is able to grow in medium containing nitrite, while dies in nitrate containing medium; thus it can be used for transformation experiments where the NR gene is used as the selective marker in combination with the use of nitrate as the selection agent. The employed expression vector comprised a mutated *NP-1* gene (a mature α -defensin *NP-1* gene from rabbit with an additional start codon) under the control of the Ubiquitin (*Ubi1*) gene promoter from maize as well as the *nptII* and *NR* genes as the selection markers. Following an electroporation procedure, transgenic lines were rescued under selection with G-418 and NaNO₃. Remarkably, the algae-made mNP-1 was isolated from transgenic clones and confirmed by N-terminal sequencing. Moreover, mNP-1 was produced at high levels (up to 11.42 mg/L). In terms of functionality, the algae-made mNP-1 showed strong activity against *Escherichia coli* at 5 μ g/mL (Fig. 7.1). Therefore, the authors postulated this platform as a promising approach to substitute the use of antibiotics in aquaculture (Chen et al. 2001).

Lactoferricin

The use of antibiotics in the clinic faces important challenges such as the cost and the generation of resistant strains. An alternative is the use of antimicrobial peptides. Lactoferricin (LfB) is a 25-residue peptide with antimicrobial activity, which is released from bovine lactoferrin following pepsin digestion. However, the production of this peptide under conventional methodologies represents a high-cost product whose use is unfeasible. For instance in aquaculture, anti-microbials against bacterial pathogens are needed but the cost determines the feasibility of their use. The microalga *Nannochloropsis oculata* was used as an attractive alternative host to produce LfB. This is a promising approach since *N. oculata* is part of the phytoplankton used to feed fish larvae and thus can be used as a convenient antimicrobial agent in the form of food.

An algae-codon-optimized gene coding for LfB was obtained in a fusion form with the red fluorescent protein (DsRed). The gene was expressed under the control of the heat-inducible promoter from the heat shock protein 70A along with the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2' promoter from *C. reinhardtii*. Electroporation was performed to develop transformed clones and after extensive analyses of candidates two stable transformed lines were selected for

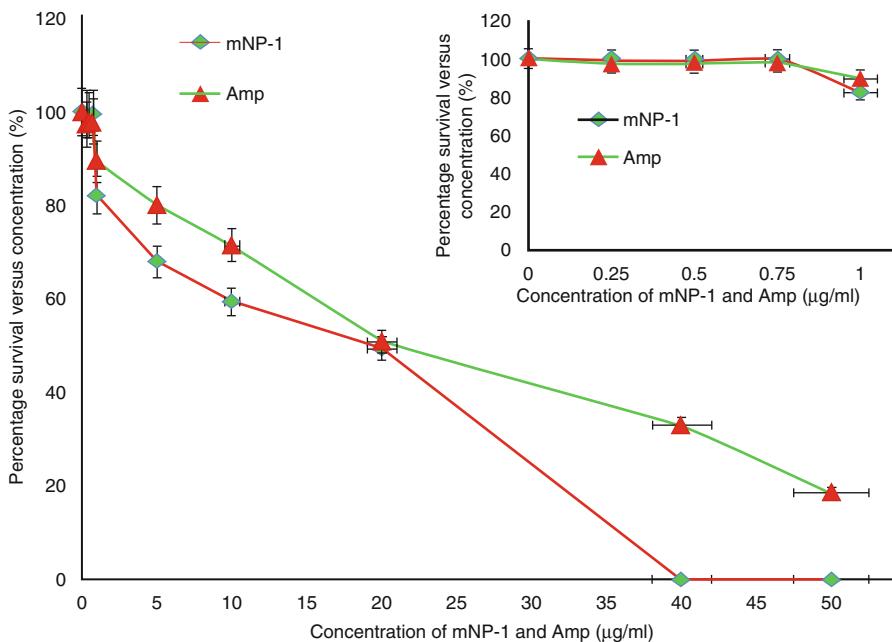


Fig. 7.1 Comparative analysis of mNP-1 and Amp against *E. coli* ATCC 25922 (Figure taken from Bai et al. (2013))

characterization. The detection of both the transcript by RT-PCR and the LfB-DsRed protein by fluorescence confirmed the expression of the product of interest upon induction by setting the cultivation temperature at 42 °C (Fig. 7.2). The activity of the LfB-DsRed protein was evaluated in medaka fish (*Oryzias latipes*), which were fed with the transgenic algae by the oral-in-tube delivery method. An infectious challenge with *Vibrio parahaemolyticus* was subsequently applied, observing a higher survival rate for the group treated with the transgenic algae when compared to that of the wild type algae-treated group. Therefore these algal strains are proposed as convenient biofactories and oral delivery vehicles of LfB, constituting an attractive approach to fight infectious diseases in aquaculture (Li and Tsai 2009).

Fibronectin Domains

Fibronectin (FN) is an adhesion protein that specifically interacts with several growth factors including the transforming growth factor beta 1 (TGF- β 1), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF)-A (Hoshijima et al. 2006; Mooradian et al. 1989; Rahman et al. 2005; Smith et al. 2009; Xu et al. 2004). In particular, it has been

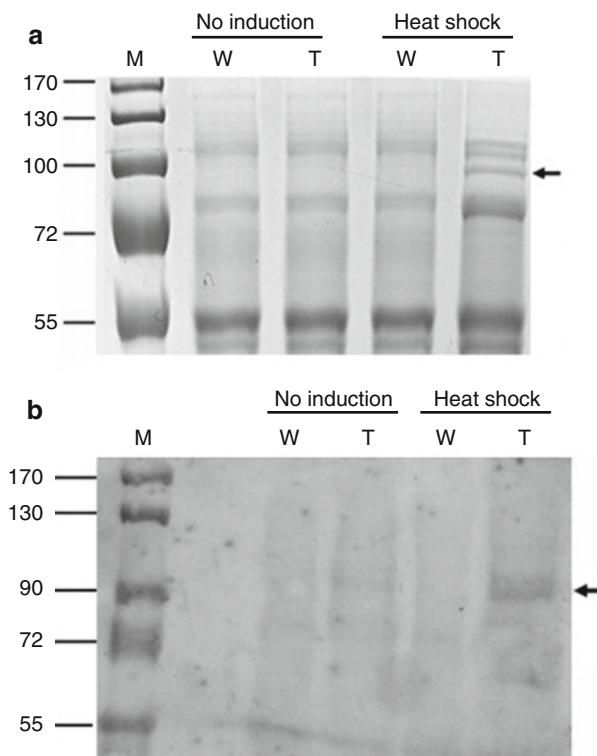


Fig. 7.2 Protein analysis of transgenic *C. reinhardtii* strain expressing lactoferricin (LfB). Cultures of wild type (*W*) and transgenic (*T*) strains were treated at 42 °C for 16 h to induce LfB expression and proteins were subsequently extracted and SDS-PAGE (a) or western blot labelling with anti-DsRed antibodies (b) were performed. The arrow indicates the tetramer of recombinant fusion protein LfB-DsRed (Figure taken from Li and Tsai (2009), permit number 3810490111826)

demonstrated that fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor-A165 (VEGF-A165) bind with high affinity to a domain consisting of the 12th to 14th type III repeats of FN (FN III12–14), also known as the FN heparin-binding domain II (Bossard et al. 2004; Wijelath et al. 2006).

The fibronectin-type III domain (FNfn10) of human fibronectin is a small β -sandwich protein domain with a similar structure to that of immunoglobulins (Koide et al. 1998; Koide and Koide 2007) and thus can be used as antibody mimic in several applications. Interestingly, FNfn10 has the following attractive features: possesses a high physical stability and has the ability to select ligand-binding proteins with nanomolar affinity, contains three structural loops (BC, DE, FG) that can be diversified either alone or in combination and thus both linear and discontinuous structures can be generated on the scaffold surface, and serves as a monobody of smaller size and more efficient expression in contrast to antibodies (Lipovsek 2010; Richards et al. 2003). Therefore FNfn10 has many potential biomedical applications, e.g. in the development of a class of target antigen-binding

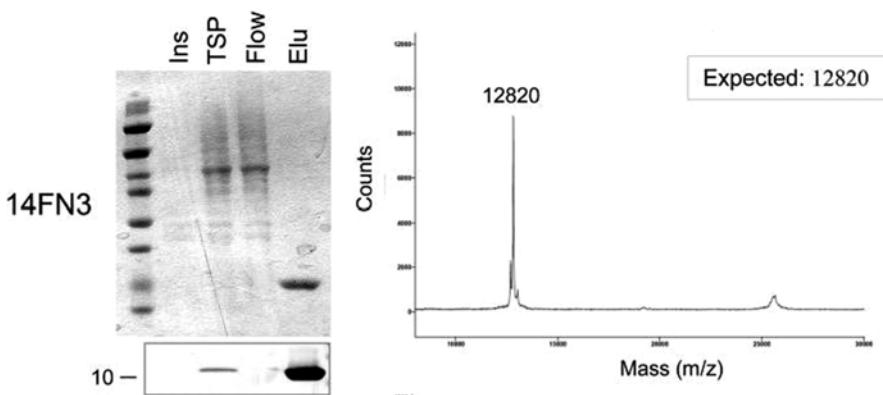


Fig. 7.3 Findings from the expression and purification of 14FN3 (fibronectin domain) in *C. reinhardtii*. Transplastomic clones expressing 14FN3 were used to isolate the recombinant protein by affinity chromatography. Pure protein (3 µg) was analyzed in coomassie-stained gels after SDS-PAGE (top left panel) or western blot (500 ng, boxed bottom panel). Lanes, from left to right, correspond to: insoluble fraction (Ins), total soluble protein (TSP), column flow through (Flow), and the eluate (Elu). The right panel contains the MADLI-TOF MS results for the purified protein (Figure taken from Rasala et al. (2010), permit number 3795581298668)

BFs that could complement or be superior to recombinant antibodies (Heinzelman et al. 2015).

By using a codon-optimized gene for expression in *C. reinhardtii*, Rasala et al. (2010) assessed the expression of 14FN3 and 10FN3. The corresponding coding region was fused with the FLAG-tag at the C-terminus and assembled in an expression vector under the control of the *psbA* promoter. The algae-made 14FN3 accumulated in transplastomic clones as soluble protein to approximately 3 % of the total soluble protein (TSP) and these yields allowed to successfully purify it by affinity chromatography mediated by the C-terminal FLAG epitope (Fig. 7.3). In contrast 10FN3 was not expressed at significant levels, probably due to stability problems and therefore further strategies to achieve the efficient expression of these proteins remain to be elucidated. It is then expected that the production in algae of the 14FN3 will lead to low cost approaches for developing therapies aiming at specific targets.

Soybean Kunitz Trypsin Inhibitor

Soybean Kunitz trypsin inhibitor (SKTI) is a serine proteinase inhibitor against the activities of both trypsin and chymotrypsin, leading to anti-inflammatory and anti-carcinogenic activities (Kobayashi 2013). The conventional sources of trypsin inhibitors comprise soybean and pumpkin. In the interest of developing a low cost and fast source of SKTI, the inhibitor was expressed in *Dunaliella salina* by Chai

et al. (2013). The binary vector pCAM2201, in which the transgene *skti* was cloned under the control of the 35S promoter, was used to transform *D. salina* following the acetate/polyethylene glycol method. The yields of SKTI in the transformed clones reached up to 0.68 % of the TSP in transformation events that were stable since the heterologous DNA was present after 35 subcultures. The functional evaluation of this biopharmaceutical (BF) remains to be assessed.

Hormones

Erythropoietin

The viability, proliferation, and differentiation of hematopoietic stem and progenitor cells are controlled by several factors (pleiotropic and blood cell lineage). Erythropoietin (Epo) is a hormone that regulates the production of red blood cells (RBCs), thus it contributes to maintain the blood hemoglobin in normal levels whereas its deficiency causes anemia (Jelkmann 2013). Human Epo is an acidic glycoprotein of 30.4 kDa constituted by a 165 amino acid chain that forms four antiparallel α -helices, two β -sheets, and two intra-chain disulfide bridges (Cys⁷–Cys¹⁶¹, Cys²⁹–Cys³³). Epo is a highly glycosylated protein (the carbohydrate fraction is 40 % of the molecule) comprising three N-glycans (at Asn²⁴, Asn³⁸, and Asn⁸³) and one O-glycan (at Ser¹²⁶). The N-glycans are involved in the protection of Epo from proteases and the affinity of the receptor binding (Elliott et al. 2003). Since Epo deficiency causes anemia in chronic kidney disease (CKD), the treatment with recombinant human Epo (rhEpo, epoetin) is required in this and other indications, e.g. primarily anemia in patients under chemotherapy against cancer.

Transfected Chinese hamster ovary (CHO) cells are currently used for the large-scale production of recombinant human Epo (rhEpo). Two brands of innovator CHO cell-derived rhEpo, namely epoetin alfa and epoetin beta, were launched as anti-anemic agents about 25 years ago. After patents expiration, ‘biosimilars’ have been developed (Jelkmann 2010) using cost-saving processes. Since Epo is a BF of complex structure, it cannot be exactly copied; and thus the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) has elaborated guidelines for the approval of biosimilars and product-specific requirements for epoetins (www.ema.europa.eu/ema/). Similar regulatory pathways exist in many other regions of the world (USA, Canada, Australia, Japan, etc.) (reviewed in Jelkmann 2010).

In an effort to establish the production of Epo in algal systems, the expression of Epo in *C. reinhardtii* chloroplasts was explored by Rasala et al. (2010). Transformed clones were generated by particle bombardment with expression vectors containing codon-optimized genes under the control of the *psbA* promoter. However Epo was not expressed at significant levels, probably due to stability problems; therefore further strategies to achieve the efficient expression of this hormone in the chloroplast remain to be elucidated.

Interestingly, some improvements were achieved for the production of Epo in *C. reinhardtii* following nuclear expression. Eichler-Stahlberg et al. (2009) used a codon-optimized *Epo* gene fused to the leader peptide of Chlamydomonas arylsulfatase gene *ARS2* to mediate the secretion of Epo, as well as a C-terminal hexahistidine-tag for isolation. The gene was expressed under the regulatory sequence of the *RBCS2* gene (promoter, three introns and 3'UTR) using the strain cw15arg⁻, which possesses only a rudimentary cell wall (de Hostos et al. 1988) and thus an efficient secretion of Epo is expected. The endogenous intronic sequences into the expression cassette aimed at enhancing the expression of transgenes in the nucleus. Interestingly, this approach allowed for the detection of recombinant Epo protein in the culture medium. However, the yields were very low (100 µg/L of recombinant erythropoietin in the supernatant or 0.03 % of the dry weight). The protein was partially isolated from the culture medium by means of affinity chromatography (Fig. 7.4). While the expected molecular weight for an unmodified Epo was 19 kDa, the recovered protein was 33 kDa; which is interesting since the size of the native Epo is 34 kDa. Previous studies reported the production of Epo in tobacco cells with a 30 kDa molecular weight (Matsumoto et al. 1995). Although the authors did not provide an analysis of the glycans profile in the algae-made Epo, it seems that it is produced in a glycosylated form but further analyses are required to determine the functionality and the convenience of the glycosylation patterns conferred by the algal host (see Chap. 9).

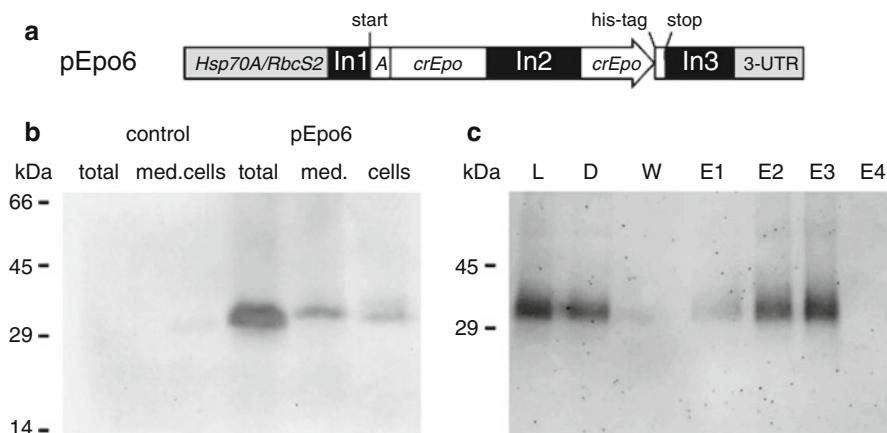


Fig. 7.4 Analysis of *C. reinhardtii* strains expressing erythropoietin. (a) Description of the Epo expression cassette. A Epo gene artificially divided into two exons and optimized for codon usage was used. Introns 1–3 of the RBCS2 gene were included in the construct. The ARS2 signal peptide (A) and a C-terminal hexa-histidine-tag were included. (b) Immunodetection by western blot of the algae-made Epo in medium and algal cells. (c) Results from affinity chromatography to isolate the algae-made Epo from the culture medium. The eluted fractions were subjected to western blot analyses using an anti-erythropoietin antibody. *L* resuspended lyophilisate, *D* flow through, *W* washing fraction, *E1–E4* elution fraction 1–4, respectively (Figure taken from Eichler-Stahlberg et al. (2009), permit number 3810490785062)

Human Growth Hormone

The Human growth hormone (hGH) is a therapeutic agent for hGH deficiency in children and adults that leads to improvements in height, body composition, bone density, cardiovascular risk factors, physical fitness, and quality of life. Effective schemes comprise daily subcutaneous injections that represent a costly therapy (Høybye et al. 2015). Chlorella has been engineered to produce hGH (Hawkins and Nakamura 1999) as an attractive platform looking to reduce the production cost. Transformed strains were generated by treating protoplasts with PEG/DMSO and the expression vector, which was designed to code the hGH fused to the signal sequence from plant α -amylase allowing for an efficient secretion to the culture medium. Two strains of Chlorella with high growth rate were used (*C. vulgaris* strain C-27 and *C. sorokiniana* ATCC-22521). Transformed clones were rescued in G-418 containing medium showing similar hGH yields when CaMV35S, a synthetic Chlorella Virus promoter, or rbcS2 promoters driven the expression (Jones and Robinson 1989). The average yields of hGH in the culture supernatants were 200–600 ng/mL, which are comparable to those obtained in *E. coli* or *Bacillus brevis* platforms (0.5–2.4 μ g/ml, Goeddel et al. 1979; Kajino et al. 1997). However, the functional evaluation of the algae-made hGH was not reported.

Flounder Growth Hormone

An attractive application on aquaculture is the administration of growth hormones to promote a faster growth of fish increasing productivity. Interestingly, the growth hormone genes of many fish species have been described and are able to exert growth-enhancing effects upon oral administration (Moryyama et al. 1993). Nonetheless to achieve this application in a feasible manner, low cost sources of growth hormones and efficient delivery vehicles should be developed. Kim et al. (2002) explored the expression of the flounder growth hormone gene in Chlorella as a convenient host and delivery vehicle since it is widely used in the aquaculture and food industries. Protoplasts from a *C. ellipsoidea* strain were transformed with a vector carrying the flounder growth hormone gene (*fGH*) under the control of the CaMV 35S promoter as well as a selection cassette based in the *Sh-ble* gene under the control of the Chlamydomonas RBCS2 gene promoter, which confers phleomycin resistance. Evidence on the expression of *fGH* was generated using Western blot analysis. According to ELISA analyses, the observed yields were up to 400 μ g *fGH* per L of culture. Remarkably, evidence of the activity and delivery of the algae-made hormone was generated in an experiment where flounder fry were fed with the transformed *C. ellipsoidea* strain; leading to a 25 % increase in the growth rate after a 30 day treatment (Kim et al. 2002; Fig. 7.5).

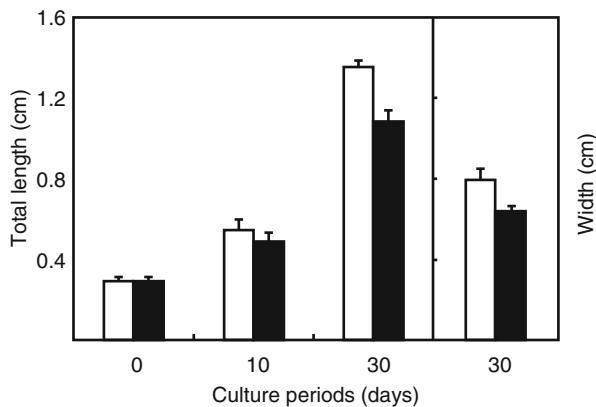


Fig. 7.5 Effect of flounder growth hormone orally delivered by transgenic Chlorella. Four-day-old flounder fry were subjected to feeding with rotifer and brine shrimp enriched with transformed (open bars) and wild type Chlorella (filled bars). Length (left panel) and width (right panel) were determined in 150 randomly selected fish (Figure taken from Kim et al. (2002), permit number 3810491098601)

Prospective View

Algae species possess a wide biosynthetic capacity since several recombinant BFs, varying in complexity, have been produced in transgenic algae thus far. However, some challenges are pending objectives in this field. For instance, the production of Epo is an interesting case in which success is still pending due to the complexity of the molecule. Although chloroplast-based expression offers in general acceptable yields, complex proteins such as antibodies and Epo should be approached by nuclear expression to access the complex post-translational processing that mainly occurs in the endomembrane systems (ER and Golgi apparatus). In this way glycosylation can be accomplished in molecules that require it for proper function.

Interestingly, the production of Epo has also been explored in other photosynthetic hosts. For instance the expression in tobacco cells resulted in a yield of 0.0026 % of the total extractable protein (Matsumoto et al. 1995), while expression in leaves of whole tobacco and Arabidopsis plants reached higher levels but led to phenotypic alterations (retarded vegetative growth and male sterility, Cheon et al. 2004). The most successful case for this topic is the moss platform that allowed for the production of Epo with the flexibility to implement glycoengineering approaches (Weise et al. 2007). However, the protein was decorated with the so-called Lewis A (Le^a) structures, which was in fact observed in the *Nicotiana benthamiana*-made Epo (Castilho et al. 2013). Since Le^a epitopes are biomarkers for certain types of cancer (Rho et al. 2014), subsequent glycoengineering approaches were implemented to avoid them in the recombinant Epo (Parsons et al. 2012). Moreover, the

safety and efficiency of moss-made asialo-EPO was improved by eliminating the gene responsible for undesired non-human prolyl-hydroxylation (Parsons et al. 2013). This evidence indicates the relevance of implementing glycoengineering approaches in algal species to improve the quality of BFs in which glycosylation may seriously affect their safety and efficacy. Applying the recently developed algal genetic engineering tools (see Chap. 2) will greatly facilitate this goal. In Chap. 9 of the present book relevant perspectives for the field of producing BFs in algal systems are presented. Given the promising findings reported thus far for the production of a diverse group of BFs, an expansion on the targeted molecules is expected in the following years; which will accelerate the developments that could result in a market reality in the midterm.

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Chapter 8

Algae-Made Nutraceuticals Produced Using Genetic Engineering Approaches

Introduction

According to the World Health Organization (WHO), 38 million deaths occurred in 2012 from non-communicable diseases (NCDs) including cardiovascular diseases, diabetes, cancer, and chronic respiratory diseases. From these deaths more than 40 % were premature, affecting people under 70 years old and there is encouraging evidence that premature NCD deaths can be significantly reduced. Since an unhealthy diet is one of the four main behavioral risk factors for NCDs, functional foods and nutraceuticals are agents that may be included in actions to prevent and control them (WHO 2014).

Although there is certain controversy about definitions, nutraceuticals can be defined as “a food or a part of a food for oral administration with demonstrated safety and health benefits beyond the basic nutritional functions to supplement the diet, which are administered in a nonfood matrix or nonconventional food formats and contain specific compounds in a quantity that exceeds those that could be obtained from normal foods” (Prasad et al. 2010). Under this definition, nutraceuticals are of great chemical diversity comprising polysaccharides, biopeptides/proteins, secondary metabolites, and lipids; among others.

Genetic engineering techniques, which are currently available for many food-grade organisms, have opened the path for the generation of genetically modified organisms producing enhanced amounts of endogenous bioactive compounds or synthetizing compounds that do not naturally occur in these organisms. A classic example of a genetic engineering approach with implications on the production of functional foods and nutraceuticals is Golden Rice, a genetically engineered variety that is used as a source of carotenoids to fight Vitamin A deficiency in poor countries (Beyer 2010; Sommer 1997). The Golden Rice technology relies on the expression of two provitamin A pathway genes in rice endosperm to achieve the production of lycopene, which is the provitamin A precursor (Al-Babili and Beyer 2005). One of the genes codes for the enzyme phytoene synthase, which is the major rate-limiting

step in carotenoid production in most plants. The second gene, *CrtI* from the bacterium *Erwinia uredovora*, codes for a carotene desaturase that obviates the need for engineering the four genes that plants normally use to achieve the synthesis of lycopene (two carotene desaturases and two carotene *cis-trans* isomerase). Since the rice endosperm possesses the rest of the enzymes required in the carotenoid pathway, this approach results in rice seeds that serve as a source of provitamin A (Schaub et al. 2005). A further improvement of this technology consisted in using the phytoene synthase from maize instead of the one from daffodil, which resulted in increased provitamin A formation (Paine et al. 2005). Interestingly, the trait was transferred into selected locally adapted rice varieties showing acceptable provitamin A bioavailability (Tang et al. 2009).

In this context the attention is turn to algae, which can be engineered to enhance the accumulation of bioactive compounds in these attractive organisms characterized by a fast grow rate, short time for generating transformed strains, low cost, and feasibility for growth in contained systems avoiding the concerns related to the cultivation of genetically engineered crops in open fields (Lucht 2015). The present chapter contains a description of the current approaches under development to achieve the production of nutraceuticals in algae species using genetic engineering (Table 8.1).

Proteins

Bovine Milk Amyloid A Produced in C. reinhardtii

Childhood mortality due to diarrhoea remains high, thus renewed actions are required to diminish its impact in global health. Therefore, new affordable diarrhoeal treatments are required to be applied in the fight against this group of diseases. A key challenge consists in achieving successful treatment scale-up (Unger et al. 2014). Moreover, traveller's diarrhoea has also a great impact in persons visiting developing countries, affecting 20–50 % of the estimated 35 million travelers each year (Lima 2001). Among infectious diseases, diarrhoeal diseases are conditions of significant epidemiologic impact in which antibiotic treatments are frequently administered; nonetheless most patients do not required these treatments that are associated to side effects and antibiotic resistance in bacterial species (Zollner-Schwetz and Krause 2015).

Bovine Milk Amyloid A (MAA) is a protein from the milk colostrum and possesses interesting activities (Wheeler et al. 2012) including the prevention of enteropathogenic bacteria adherence to intestinal cells (Larson et al. 2003), anti-microbial activity (Molenaar et al. 2009), and modulation of macrophages activity (Domenech et al. 2012). Thus, the diet supplementation with MAA has been proposed as an approach to prevent infectious diarrhoea in both animals and humans. Although MAA is a potentially viable solution for reducing the risks of infectious diarrhoea (Walker et al. 2012), a low cost source of this protein is required to make viable its

Table 8.1 Compilation of the most advanced cases of bioactive compounds produced in algae species by genetic engineering approaches

| Compound of interest and algae species | Transformation and expression approach | Main findings | Reference |
|--|--|---|---|
| Bovine Milk Amyloid A (MAA), <i>C. reinhardtii</i> | Particle bombardment mediated transformation to achieve chloroplast expression of the MAA gene under the <i>psbA</i> promoter | Functional MAA is expressed in the chloroplast. Photosynthetic strains cultivated in 100 L plastic bags in greenhouse condition produced up to 2.27 mg MAA per L of culture | Gimpel et al. (2015a, b) Manuell et al. (2007) |
| Carotenoids/ <i>Chlorella zofingiensis</i> | Nuclear expression of phytoene desaturase gene with a single amino acid substitution (L516F) under the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RBCS) promoter | Increases of up to 32.1 % in the accumulation of total carotenoids and 54.1 % in the production of astaxanthin were achieved in the engineered strain | Liu et al. (2014) |
| Carotenoids/ <i>Chlamydomonas reinhardtii</i> | Glass bead-mediated transformation to achieve nuclear expression of a phytoene desaturase gene with a single amino acid substitution (L505F) | Increases in lutein, β -carotene, and violaxanthin were found in the engineered strains | Liu et al. (2013) |
| Eicosapentaenoic acid (EPA)/ <i>P. tricornutum</i> | Electroporation-mediated transformation to achieve nuclear expression of the $\Delta 5$ desaturase (<i>PtD5b</i>) driven by the <i>fopC</i> promoter | Endogenous $\Delta 5$ desaturase (<i>PtD5b</i>) achieving a 58 % increase in EPA levels. The strains also showed enhanced levels of poly and mono unsaturated FA | Peng et al. (2014) |

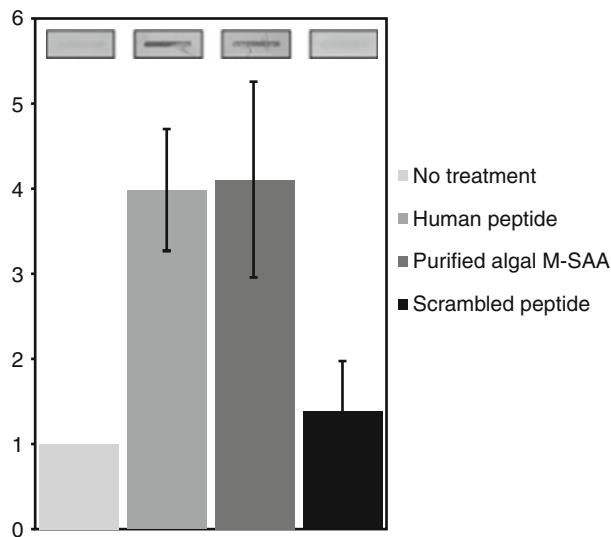


Fig. 8.1 In vitro functional evaluation of the algae-made mammary-associated serum amyloid (M-SAA). HT29 cells were incubated every hour in a 4 h period with one of the following: a human peptide corresponding to the amine terminus of M-SAA (human peptide), pure algae-made purified M-SAA digested with protease to release an 18-amino-acid peptide from the amine terminus of M-SAA, a non-related peptide (same amino acid content as the human peptide but having a random sequence; scrambled peptide), or the vehicle alone. Mucin (*muc3*) accumulation was estimated by slot blot analysis. Both the human peptide and purified algal M-SAA induce *muc3* at significant levels ($P < 0.05$) (Figure taken from Manuell et al. (2007))

large scale use. Studies to generate a platform based in *C. reinhardtii* for the production of MAA have been reported by the group headed by Mayfield.

Strains transformed at the chloroplast genome level were generated to produce MAA. Initial studies revealed that the best yields are attained when the *psbA* promoter and the 5' untranslated region (UTR) drive MAA gene expression. Up to 10% of total soluble protein (TSP) yields were achieved (Manuell et al. 2007; Fig. 8.1). In fact, since MAA showed notable accumulation levels, the fusion of MAA to other biopharmaceuticals of interest has been explored as an approach to enhance their accumulation. In the study reported by Rasala et al. (2010) the MAA protein was fused to 10FN3, 14FN3, VEGF, and HMGB1. In the case of HMGB1, the expression decreased when fused to MAA. However in the case of the fibronectin 10FN3, which did not accumulate when expressed alone, the fusion with MAA allowed its accumulation in the chloroplast; suggesting that MAA fusion is a feasible approach to facilitate the expression of some proteins whose expression in the chloroplast of *C. reinhardtii* is difficult in its native form (Rasala et al. 2010).

Furthermore, the production of MAA mediated by the *psbA* regulatory sequences is an approach that renders *psbA*-deficient strains that are non-photosynthetic. This approach makes the production unviable under greenhouse conditions, in which the maximal benefit of the photosynthetic host can be exploited since natural sunlight

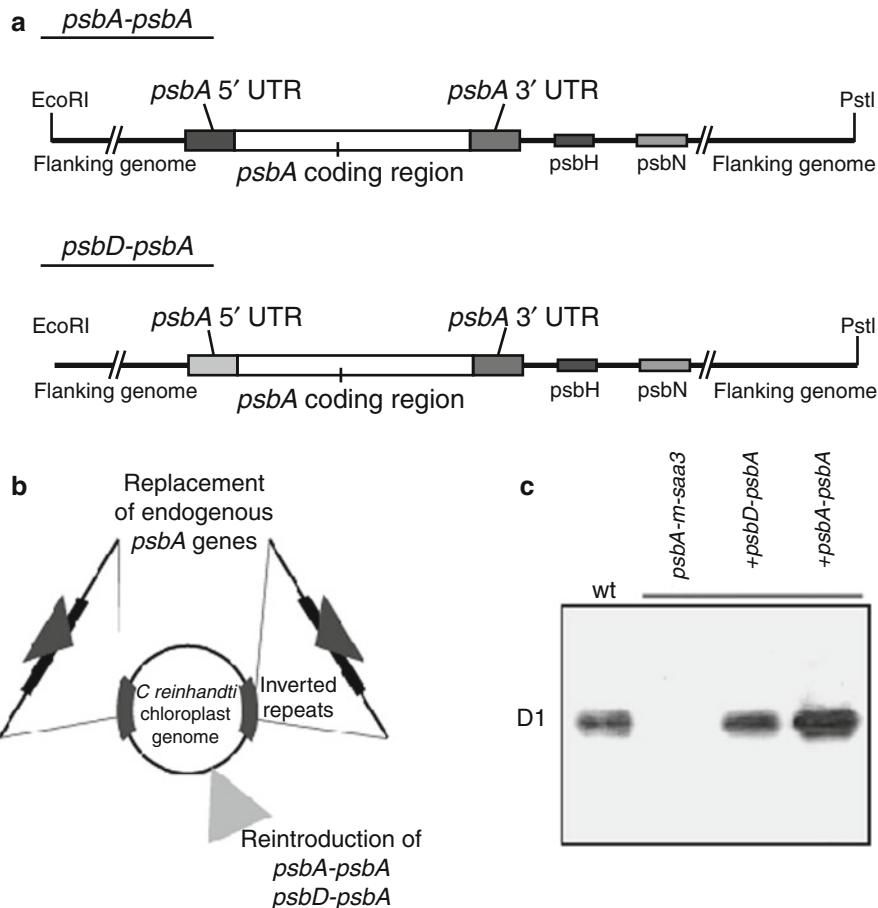


Fig. 8.2 Study conducted to re-establish photosynthesis in the algae strains producing MAA under the *psbA* promoter. The *psbA* was introduced at a distal site from the MAA gene on the chloroplast genome. (a) Two strategies to restore photosynthesis were explored: reintroduction of the *psbA* gene (top) under its endogenous regulatory sequences or the *psbD* 5'UTR promoter (bottom). (b) Approach to reintroduce the *psbA* gene in the modified plastome to produce MAA by the presence of the *psbA-m-saa* cassette. (c) Evidence on the production of the D1 protein encoded by the *psbA* gene (wt wild-type) (Figure taken from Manuell et al. (2007))

drives the biomass generation employing low cost minimal media. To override the limitations of non-photosynthetic strains, an initial approach consisted in placing the endogenous *psbA* coding sequence under the *psbD* promoter and 5'UTR; observing photosynthetic activity and at the same time maintaining the MAA production. However, since MAA production levels decreased in these strains when compared to the photosynthesis deficient strains and a dependency on dark to light shifts to favor expression was observed; thus the process still requires further optimization (Fig. 8.2). The authors hypothesized that the rapid increase in light intensity induces

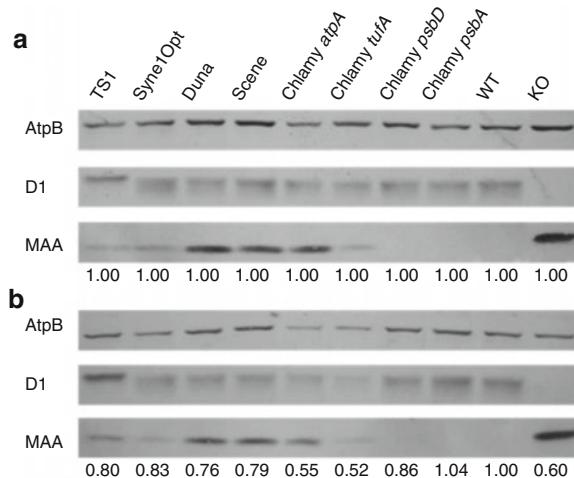


Fig. 8.3 MAA levels expressed in strains in which photosynthesis was reestablished. In panel (a), equal amounts of total protein were loaded (10 mg per well); while in panel (b) equal amounts of culture volume were loaded (biomass from 75 mL of algae culture were processed and loaded per well). Blots were labeled with anti-MAA, -D1, or -AtpB antibodies; the latter as loading control. Underneath values correspond to the amount of total protein loaded relative to the WT (Figure taken from Gimpel et al. (2015b), permit number 3795590420298)

the expression of MAA (under the control of the *psbA* promoter) to a higher rate than the D1 protein (under the control of the *psbD* promoter), avoiding the D1-induced auto-attenuation of MAA (Manuell et al. 2007). In an effort to implement an optimized system in which D1-induced auto-attenuation of MAA is avoided, Gimpel and Mayfield (2013) tested distinct combinations of endogenous and heterologous promoters and 5'UTRs driving different *psbA* coding sequences (Gimpel and Mayfield 2013). The authors observed that the heterologous coding sequence was expressed in *C. reinhardtii*, but full genes were not. The authors subsequently examined the activity of six selected heterologous *psbA* promoters, observing that all of them drove the transcription of the *C. reinhardtii* *psbA* gene and accumulated the D1 protein; leading to reestablished photosynthesis. The authors suggested that “promoter recognition might be facilitated in this case by the existence of an ancient *psbA* core promoter that is apparently conserved from Glaucoophyta to Streptophyta”.

In a further report, the expression of MAA was assessed in the complemented-photosynthetic strains under greenhouse conditions (Gimpel et al. 2015b). Six of the strains reported by Gimpel and Mayfield (2013) expressed MAA at detectable levels (TS1, Syne1Opt, Duna, Scene, Chlamy *atpA*, and Chlamy *tufA*), from which the strains Duna, Scene, and Chlamy *atpA* resulted in the highest MAA accumulation in terms of protein amount per biomass unit. However when the strains were evaluated in terms of productivity, by detecting MAA in the same equivalents of culture medium; the Duna and Scene strains showed the highest productivity, which correlated with their higher photosynthetic capability (Fig. 8.3). The Scene MAA-

Fig. 8.4 Aspect of the photosynthetic *C. reinhardtii* cultures producing the MAA protein cultivated under greenhouse conditions. These strains showed an attractive productivity and additionally possess photosynthetic activity, thus sunlight drives the generation of biomass in a low cost culture medium (Figure taken from Gimpel et al. (2015b), permit number 3795590420298)



Table 8.2 Growth parameters and MAA expression data collected from the cultures grown in 100 L bags under greenhouse conditions

| Bag | Dry weight ^a (g/L) | Max. productivity ^b (g/L/day) | T°C ^c | pH ^c | MAA ^d (mg/L) | MAA ^d % expression |
|----------------------|----------------------------------|--|------------------|-----------------|----------------------------|----------------------------------|
| D7 | 0.265 | 0.047 (11) | 23.0±4.6 | 6.6±0.2 | 1.86±0.07 | 1.21±0.05% |
| D9 | 0.256 | 0.037 (11) | 23.1±4.6 | 6.5±0.2 | 1.66±0.06 | 1.12±0.04% |
| D11 | 0.278 | 0.069 (9) | 23.4±4.9 | 6.6±0.2 | 3.28±0.21 | 1.86±0.12% |
| Average ^e | 0.267±0.011 | 0.051±0.016 | 23.2±0.2 | 6.6±0.1 | 2.27±0.88 | 1.40±0.40 |

^aAchieved on day 12 of the culture

^bParentheses show the day in which the maximum productivity was attained

^cThe uncertainty (except for the “Average” row) corresponds to the standard deviation from all daily measurements

^dThe uncertainty (except for the “Average” row) corresponds to the standard deviation from the triplicate wells in the ELISA plate

^eThe uncertainty represents the standard deviation between the three bags without error propagation

producing strain was selected by the authors to further perform the MAA production in a larger scale under greenhouse conditions. The average MAA yield achieved in 100 L-plastic bags was 2.27 mg/L of culture (Fig. 8.4), corresponding to an accumulation level of 1.39 % of TSP. Considerable differences in growth and MAA production between the bags were observed and are attributed to variations in light irradiation and to the position of the bags in the greenhouse (Gimpel et al. 2015b; Table 8.2). In this regard, in Chap. 9 of the present book perspectives on bioreactor design and operation are presented.

Therefore, the use of the *psbA* promoter-mediated expression in strains with re-established photosynthesis provides a model for the robust expression of recombinant proteins in the chloroplast of *C. reinhardtii*. Under this approach, the transgene is constitutively expressed but not dependent upon induction derived from shifting the light conditions as previously approached (Manuell et al. 2007).

Moreover, under this strategy translation of the gene of interest is avoided by placing the *psbA* gene under the heterologous 5'promoter/UTR to restore the *psbA* function.

Interestingly, this case of the algae-made MAA protein is under industrial adoption. Triton Algae Innovations, Ltd. is a company currently investing in this MAA produced in algae as a low cost biofactory and oral delivery vehicle (<http://www.tritonhn.com/>). Thus, this platform is promising and has the potential to reach wide applications in the benefit of human and animal health.

A Chimeric Protein Carrying Bioactive Peptides Produced in *C. reinhardtii*

Bioactive peptides and protein hydrolysates have the potential to serve as important agents in the fight against NCDs. However, achieving a wider exploitation of bioactive peptides requires to decrease the cost and simplify the production process. An alternative in this field consists on the design of new proteins that release ‘cryptides’ with target bioactivity (Li-Chan 2015). One alternative consists in the *in silico* design of chimeric proteins that can be produced in convenient recombinant hosts and serve as precursors of bioactive peptides following digestion *in vivo*. Therefore, the released bioactive peptides become bioavailable and exert a biologic activity in the target organism after ingestion. Campos-Quevedo et al. (2013) reported the design of a chimeric protein called NCQ carrying a combination of peptide sequences with antihypertensive, antimicrobial, opioid, and hypocholesterolemic activities. Linkers were used to join each of the peptides and served as cleavage sites for gastrointestinal proteases (pepsin, chymotrypsin, elastase, and trypsin). The expression of the codon-optimized gene was driven by the *rbcL* or the *atpA* promoters. Transplastomic clones were generated following particle bombardment. The clones carrying the gene under the control of the *rbcL* promoter accumulated the NCQ protein in a range of 1.8–2.4 % of TSP, while clones carrying the *atpA* promoter accumulated between 0.16 and 1.5 % of TSP. The functional evaluation of the algae strains expressing the NCQ protein is a pending objective. The NCQ design is intended to serve as a multifunctional approach to fight chronic diseases. For instance the metabolic syndrome comprises atherogenic dyslipidemia, elevated blood pressure, insulin resistance and elevated glucose, a pro-thrombotic state, and a pro-inflammatory state. This condition is mainly derived from an excess in energy intake as well as concomitant obesity, and is an important risk factor for atherosclerosis disease and type 2 diabetes (Grundy 2015). Therefore the approaches to produce nutraceuticals in feasible systems with multiple therapeutic effects, such as anti-hypertensive and anti-cholesterolemic activities, are of particular relevance.

In plants, several developments for producing heterologous proteins or peptides with antihypertensive properties have been performed (for a review see Rosales-Mendoza et al. 2013). For instance tomato plants were engineered to produce the

acidic-subunit of amaranthin, which is the main seed storage protein of *Amaranthus hypochondriacus* and carries four antihypertensive biopeptides Val-Tyr. Stably transformed plants accumulated the recombinant protein at levels up to 12.71 % of TSP, with a remarkable increase in total protein content (5–22 % increase) and in content of essential amino acids with respect to the wild type (WT) line. The functional activity of the tomato-made acidic-subunit of amaranthin was positive in assays where protein hydrolysates were tested in their ability to inhibit the angiotensin-converting enzyme in vitro (IC_{50} values ranged from 0.376 to 3.241 $\mu\text{g/mL}$) (Germán-Báez et al. 2014). Following similar approaches, algae could serve as a feasible and safe source of proteins related to nutraceuticals production.

Lipids

Some of the ω -3 and ω -6 fatty acids, which are polyunsaturated fatty acids (PUFA), are required in the human body for important functions; however they cannot be synthesized in the organism and thus are called essential fatty acids (Lauritzen 1994). In the case of the arachidonic acid (all-cis-5,8,11,14-eicosatetraenoic acid, or ARA), it can be obtained from linoleic acid; however it can be considered an essential fatty acid because of the dependence on the ability for the linoleic acid conversion or because linoleic acid availability. Essential fatty acids contribute to the integrity of several tissues. It is known that linoleic and linolenic acids are involved in the synthesis of the cell membrane prostaglandins, via cyclo- and lipoxygenase, and also participate in processes implicated in the immune system function and tissue regeneration (Gabbs et al. 2015). Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) are associated to a lower risk of developing cardiovascular strokes and arthritis, they also have anti-hypertensive activity (Bonafini et al. 2015; Lorente-Cebrián et al. 2013; Ruggiero et al. 2009). These compounds also improve lipid serum profiles in terms of decreasing triglyceride levels and increasing HDL, and exert anti-inflammatory activity. In addition, DHA plays an important role in the development and function of the nervous system and normal growth (La Rovere and Christensen 2015).

Seafood, especially oily fish, currently serves as the main source of PUFAs. However the intake of these compounds is suboptimal due to cultural or individual preferences, convenience, geographic location, or awareness of risks associated to fatty fish consumption; thus alternative sources are needed (Martins et al. 2013). Several approaches have been implemented to engineer the production of PUFAs in algae species with the goals to exploit these organisms as a convenient biofactory. ω -3 PUFAs in fact are of much higher value than biodiesel. Algae as platforms for the production of PUFA would offer a much shorter time for production when compared to plants. To engineer the production of PUFAs, overexpression of specific fatty acid desaturases and elongases as well as transesterases has been implemented.

Studies performed with *C. reinhardtii*, based in the overexpression of the endogenous $\Delta 4$ desaturase ($Cr\Delta 4FAD$), have revealed that it is possible to increase the

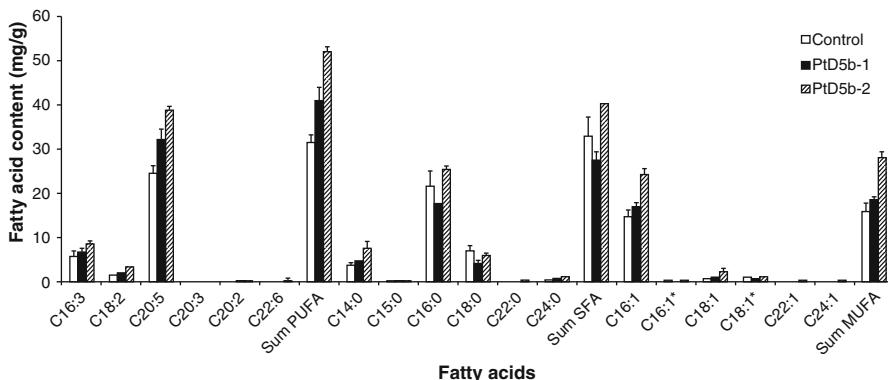


Fig. 8.5 Fatty acid composition in genetically engineered *Phaeodactylum tricornutum* strains overexpressing a delta 5 desaturase, which plays a key role in EPA biosynthetic pathway. A 58 % increase in EPA levels was achieved. The strains also showed enhanced levels of poly and mono unsaturated FA (Figure taken from Peng et al. (2014))

production of hexadeca-4,7,10,13-tetraenoic acid (16:4). The opposite effect was observed when the gene was silenced through iRNA (Zäuner et al. 2012). *P. tricornutum* was also engineered to overexpress an endogenous $\Delta 5$ desaturase (PtD5b) under the control of the *fcpC* promoter from the fucoxanthin chlorophyll a/c binding protein gene of *P. tricornutum*. Remarkably a 58 % increase in EPA levels was achieved. The strains also showed enhanced levels of poly and mono unsaturated FA (Peng et al. 2014; Fig. 8.5). Niu et al. (2013) developed *P. tricornutum* strains overexpressing an isoform of diacylglycerol acyltransferase (DGAT), which is a key enzyme that catalyzes the last step of triacylglyceride biosynthesis. An increase in the polyunsaturated fatty acids levels was achieved (e.g. EPA levels increased by 76.2 %). Hamilton et al. (2014) have also explored approaches to increase lipid production in algae. Improved levels, an eight-fold increase, of DHA were achieved in *P. tricornutum* strains by the expression of two recombinant fatty-acid modifying enzymes: a D5-elongase and an acyl-CoA-dependent D6-desaturase, both from the green alga *Ostreococcus tauri* using the promoter of the *FcpA* gene. Another study reported by Ren et al. (2015) consisted in the expression of the ω -3 desaturase gene from *Saprolegnia diclina* driven by the ubiquitin promoter. The transgene was transferred by the 18S homologous sequence to *Schizochytrium* sp. following an electroporation method. When compared to the WT strain, the transformed clones improved the ω -3/ ω -6 ratio from 2.1 to 2.58 and converted 3 % of the docosapentaenoic acid (DPA) to DHA. On the other hand, a reduction in squalene and sterol contents of 37.19 and 22.31 %, respectively, was observed in the transformed strains.

This topic is in its infancy and the main bottleneck is given by the complexity of the lipid biosynthesis. The generation of fundamental knowledge on the regulation of biosynthesis pathways will allow the implementation of optimized metabolic engineering approaches redirecting lipid biosynthesis in a more integral manner. Considering that in green algae the initial pathways for FA synthesis occur in the chloroplast, engineering the expression of lipogenic enzymes into the chloroplast

genome is a relevant approach to be explored. For instance, a recent analysis performed by Mühlroth et al. (2013) generated a model pathway for the n-3 LC-PUFA synthesis in *P. tricornutum*, which was derived from transcriptome data comprising the co-expression network of 106 genes involved in the lipid metabolism; and supported by molecular biological and metabolic studies. The limiting steps of the n-3 LC-PUFA synthesis by enzymes such as thioesterases, elongases, acyl-CoA synthetases, and acyltransferases have been discussed and metabolic bottlenecks hypothesized. From this analysis it has been hypothesized that the supply of the acetyl-CoA and NADPH will be critical factors to consider in metabolic engineering approaches.

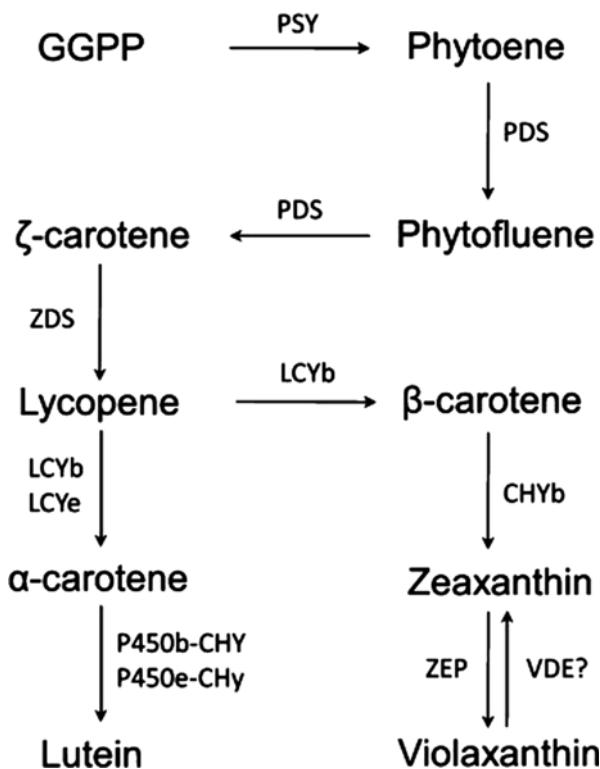
Carotenoids

Carotenoids are pigments responsible for the red, yellow, and orange colorations in a myriad of organisms. Carotenoids are synthesized by bacteria, fungi, yeast, higher plants, and algae; and are valuable compounds due to their attractive colors and, most importantly, potential health benefits. Due to these attractive properties carotenoids are frequently used in the production of food products and the formulation of cosmetics and pharmaceuticals (Sandmann 2015). In terms of health benefits, carotenoids have strong antioxidant activity and are associated to cancer prevention (Gammone et al. 2015; Soares Nda et al. 2015). It is recognized that lutein and zeaxanthin carotenoids are bioavailable compounds that localize in the eye as protective macular pigments; in fact the frequent dietary intake of carotenoids is associated to a lower risk of age-related macular degeneration (Wu et al. 2015).

The orange carotenoid, β-carotene, has a particular relevance as the precursor of vitamin A. Since Vitamin A is involved in critical physiological processes related to immunity, tissue development, as well as iron metabolism; its deficiency causes blindness and make infants more susceptible to several diseases such as measles and chronic diarrhea, thus leading to high mortality in poor countries (Mason et al. 2016). Phytoene is a colorless carotenoid of nutritional value since its intake decreases the inflammatory response to UV light and provides protection against oxidative stress (Fuller et al. 2006). β-cryptoxanthin, which also has provitamin A activity, has anti-inflammatory properties (Liu et al. 2015) as well as an anabolic effect on bone that may be associated to bone loss reduction (Uchiyama et al. 2004).

Excepting the aphids, animals are unable to synthesize carotenoids and thus they must be obtained from the diet (Moran and Jarvik 2010). Because of the cost associated with the chemical synthesis, the keto-carotenoids produced by this approach are of unviable use, thus alternative platforms for their production are needed (Serra 2015). Some studies on the production of carotenoids have been conducted based in astaxanthin-producing algae, such as *Haematococcus pluvialis* (Li et al. 2011). Genetically engineered microorganisms producing carotenoids have also been generated, including *Escherichia coli* (Scaife et al. 2009) and yeast species (Ukibe et al. 2009; Zhu and Jackson 2015). In the case of plants, soybeans producing canthaxanthin have been generated (Pierce et al. 2015).

Fig. 8.6 Description of the carotenoid biosynthesis pathway in Chlamydomonas. GGPP geranylgeranyl pyrophosphate, PSY phytoene synthase, PDS phytoene desaturase, ZDS ζ -carotene desaturase, LCYb lycopene β -cyclase, LCYe lycopene ϵ -cyclase, P450b-CHY cytochrome P450 β -hydroxylase, P450e-CHY cytochrome P450 ϵ -hydroxylase, CHYb β -carotene hydroxylase, ZEP zeaxanthin epoxidase, VDE violaxanthin de-epoxidase, not found in Chlamydomonas (Figure taken from Liu et al. (2013), permit number 3787841415702)



Algae can be genetically engineered to establish or enhance carotenoid production at high levels in a type of biomass that is safe for oral consumption by human and animals, thus constituting a low and fast source of these valuable compounds. For instance, several efforts on this direction have been conducted in the model micro-alga *C. reinhardtii*. Strains expressing in the chloroplast a heat-stable geranylgeranyl-pyrophosphate synthase (involved in the early steps of carotenoid biosynthesis) were generated by Fukusaki et al. (2003), nevertheless this approach did not lead to significant changes in the isoprenoid profiles. In another approach, the production of keto-carotenoids (e.g., astaxanthin) was investigated by developing strains that expressed at the nuclear level the beta-carotene ketolase genes from *H. pluvialis* (*bkt3*) and *C. reinhardtii* (*CRBKT*). However, this approach also failed to achieve keto-carotenoids biosynthesis (Wong 2006). In contrast León et al. (2007) explored the expression of the *bkt1* gene from *H. pluvialis*, observing a discrete accumulation of 4-keto-lutein with no astaxanthin production. The phytoene desaturase gene (*pds*, coding for the second step of carotenoid biosynthesis) has been silenced through RNAi, nonetheless no significant changes in carotenoid content were observed; thus it was hypothesized that additional rate-limiting processes exist (Vila et al. 2008). Remarkably, an approach that led to an increase in carotenoid accumulation is the expression at the nuclear level of the phytoene synthase gene (*psy*); which is required for carotenoid synthesis (Cordero et al. 2011; Couso et al. 2011; Fig. 8.6). Another

approach has consisted in the nuclear expression of a point mutant version of the *pds* gene, which codes for an enzyme that has an increased activity by 27%. This approach led to higher accumulation of lutein, beta-carotene, zeaxanthin, and violaxanthin (Liu et al. 2013; Fig. 8.7).

The genes from *H. pluvialis* coding for the β -carotene ketolase and β -carotene hydroxylase were expressed in a cell wall deficient *C. reinhardtii* strain (CC-849)

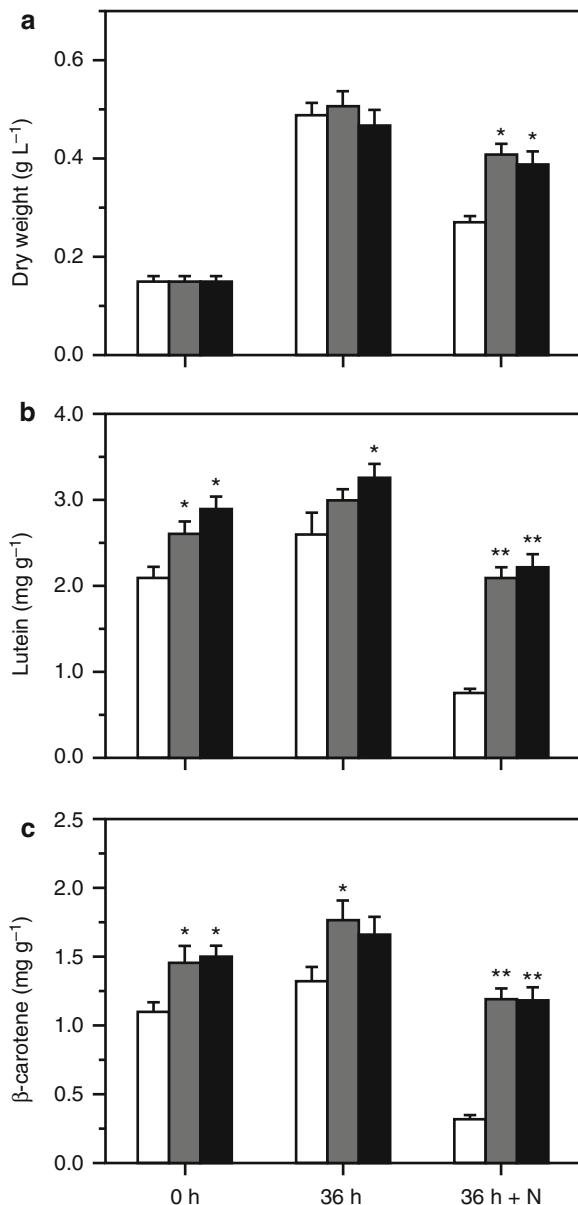
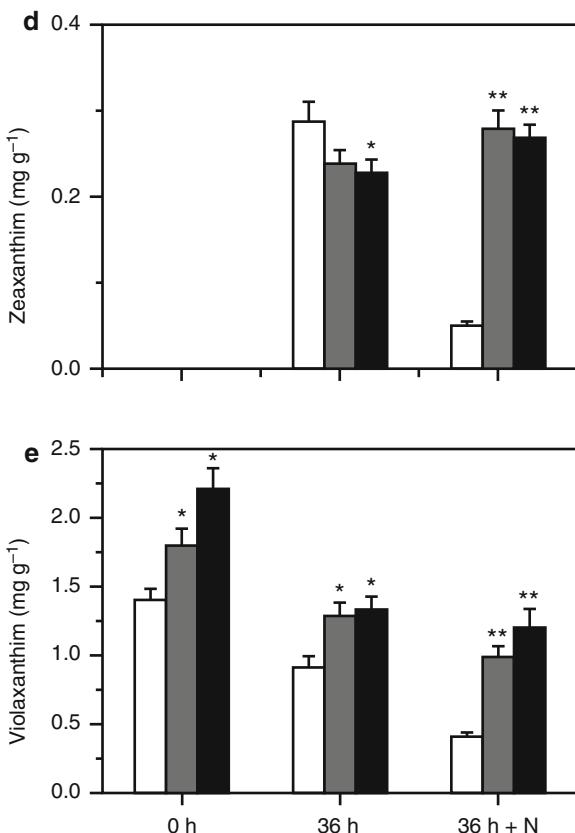


Fig. 8.7 Growth patterns and carotenoids profiles of *C. reinhardtii* strains engineered to express a phytoene desaturase (PDS) with a single amino acid substitution (L505F). Biomass (a), lutein (b), beta-carotene (c), zeaxanthin (d), and Violaxanthin (e) levels are presented. White box WT, gray box T1, black box T8. Cells were harvested at different time points after exposure to light during 36 h. +N, addition of 0.5 μM norflurazon. * and ** indicate the statistical difference versus the WT at the levels of 0.05 and 0.01, respectively (Figure taken from Liu et al. (2013), permit number 3787841415702)

Fig. 8.7 (continued)

following a nuclear expression approach mediated by the hsp70A-RBCS2 promoter. When compared to the WT strain, the transformed strains obtained by a bead-based transformation method exhibited a 29.04 and 30.27 % increase in carotenoids and xanthophylls; respectively (Zheng et al. 2014).

Studies performed in other algae species are of outstanding interest. For instance *H. pluvialis* was engineered at the nuclear level with the gene coding for a mutant *pds* gene, leading to an increase in astaxanthin accumulation of up to 26 % (Steinbrenner and Sandmann 2006). In a silencing approach, the targeting of the *D. salina pds* gene was achieved but no data on carotenoid content are presented (Sun et al. 2008). *C. zofingiensis* was transformed at the nuclear level with a mutant version of the endogenous *pds* gene, which codes for an enzyme with increased (33 % higher) activity. The genetically engineered strains showed an increase of up to 32.1 % in the accumulation of total carotenoids and 54.1 % in the production of astaxanthin (Liu et al. 2014; Figs. 8.8 and 8.9).

Since carotenoid metabolic engineering is still in its infancy, Gimpel et al. (2015a) suggested that the targeted enzymes do not constitute the bottleneck steps; or there are several rate-limiting reactions in the pathway, thus the simultaneous

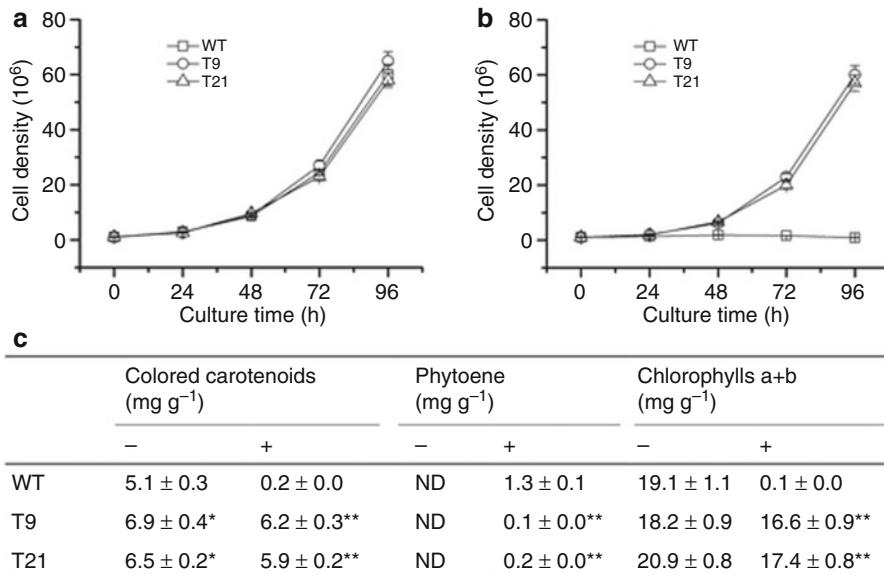


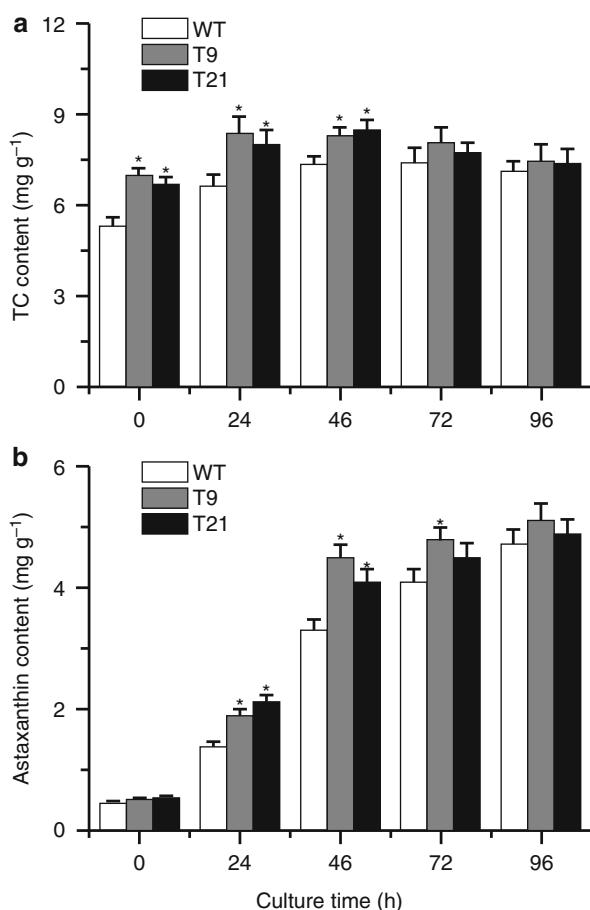
Fig. 8.8 Characterization of the *Chlorella zofingiensis* strains genetically engineered with the phytoene desaturase (PDS) gene with a single amino acid substitution (L516F) under the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RBCS) promoter. (a, b) cell growth under normal growth conditions in absence or presence (0.5 μ M) of norflurazon. (c) Accumulation levels of coloured carotenoids (phytoene and chlorophylls) in strains grown in absence (-) or presence (+) of 0.5 μ M norflurazon. Single and double asterisks denote statistically different values versus the WT strain at the levels of 0.05 and 0.01, respectively. ND not detected (Figure taken from Liu et al. (2014), permit number 3795590768811)

transformation of three or more enzymes to strengthen the desired metabolic flow is proposed. A promising prospect consists in the discovery and engineering of microalgae transcription factors that regulate terpenoid synthesis. Additional enzymes to consider in this area comprise the following from the terpenoid biosynthetic pathway: deoxyxylulose 5-phosphate synthase, deoxyxylulose 5-phosphate reductoisomerase, phytoene synthase, ζ -carotene desaturase, and lycopene β -cyclase (Ferhatoglu and Barrett 2006; Sandmann 2002).

Prospective View

A myriad of bioactive compounds have been characterized thus far offering an opportunity to generate new nutraceuticals of relevance in the fight against NCDs. Algae species constitute convenient biofactories for such compounds. An overall successful modification of the metabolism in algae species has been achieved by genetic engineering strategies at both nuclear and chloroplast levels. The production of bioactive proteins and peptides has also proven to be a feasible approach,

Fig. 8.9 Levels of total carotenoids (**a**) and astaxanthin (**b**) in *Chlorella zofingiensis* strains genetically engineered with the phytoene desaturase (PDS) gene with a single amino acid substitution (L516F) under the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RBCS) promoter. A single asterisk denotes significant statistical difference versus the wild type strain ($p < 0.05$) (Figure taken from Liu et al. (2014), permit number 3795590768811)



especially following chloroplast expression. However although a substantial progress in this field was achieved, considering the diversity of compounds that may serve as nutraceuticals, several avenues are yet to be explored to achieve a wide exploitation of the potential of algae organisms.

Among the groups of compounds that could be explored in this field are alkaloids, terpenoids, vitamins, and amino acids. Alkaloids are nitrogen-containing compounds of low molecular weight, most of them derived through decarboxylation of amino acids (Minami et al. 2008). Alkaloids possess several attributes such as antimicrobial activity (e.g. berberine) (Kong et al. 2004) and therapeutic effects in cancer (e.g. sanguinarine) (Kemeny-Beke et al. 2006), autoimmune disorders, hypertension (bisbenzyliso-quinoline alkaloid tetrandrine) (Kwan and Achike 2002; Lai 2002), diabetic retinopathy, and Parkinson's disease (e.g. indolocarbazole alkaloids) (Butler 2005). Since the high structural diversity and molecular complexity makes the chemical synthesis of alkaloids difficult, metabolic engineering strategies have been implemented in plants to increase alkaloid production (Allen et al. 2004; Fujii et al. 2007; Sato et al. 2007); however the outcomes are limited by the complexity of

alkaloid biosynthetic pathways and their regulation (Zulak et al. 2007) and the dilution of the synthetic intermediates in the distinct organelles (Leonard et al. 2009). The reconstitution of alkaloid biosynthesis in algae would have several advantages including: rapid growth and biomass accumulation, abundant availability of genetic tools for pathway expression and optimization, and easier characterization and isolation of final products and key intermediates due to a relative simpler background with respect to other hosts (Hawkins and Smolke 2008).

Terpenoids (also called isoprenoids) constitute perhaps the largest group of compounds with medicinal and industrial applications. These compounds derived from five-carbon isoprene units are formed from two basic isoprene building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Chang and Keasling 2006; Gershenson and Dudareva 2007). Among the approaches to produce such type of compounds by genetic engineering, *S. cerevisiae* has been engineered to produce artemisinic acid, a key precursor for the anti-malarial drug artemisinin (Ro et al. 2006); and taxadiene, which is the precursor of the anticancer compound taxol (Engels et al. 2008). As described above, some research has been conducted to engineered the production of carotenoids in algae; however given the diversity of terpenoids, several compounds of relevance are still pending to be targeted by genetic engineering.

Vitamins and amino acids also play an important role in the nutraceuticals field. In microorganisms, genetic engineering approaches have allowed the production of such compounds (Burgess et al. 2009; Dong et al. 2011). Some of the tools applied in this field comprise engineering the carbon storage regulator (Csr), a global regulatory system of *E. coli* which was engineered to improve phenylalanine biosynthesis (Tatarko and Romeo 2001); mutations in csrA and csrD as well as csrB overexpression, and overexpression of *tktA* gene (Yakandawala et al. 2008).

Therefore the molecular approaches previously implemented in other microorganisms, such as *E. coli* and *S. cerevisiae*, constitute a key reference to assess whether algae species can serve as efficient biofactories (Minami et al. 2008). The availability of recently developed tools for gene expression represents an important field of opportunity to expand the developments in this arena (see Chap. 2). In addition the generation of new knowledge in the biosynthetic pathways of carotenoids and lipids, especially PUFAs, will allow for a more precise targeting of metabolic engineering approaches. Future research in the upcoming years will be critical to get the inflection point in terms of industrial application and impact the scenario in human and animal health. On the other hand, robust clinical trials to evaluate efficacy and metabolic fate of the developed products will be of critical importance to justify their impact on human and animal health.

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Chapter 9

Perspectives for the Algae-Made Biopharmaceuticals Field

Introduction

Microalgae possess a high degree of application in biotechnology (Gimpel et al. 2015a, b). The current book has provided the recent achievements on the field of algae-made biopharmaceuticals (BFs). Microalgae are promising expression hosts due to unique advantages such as low production cost, high growth rates, high yields, better safety, and the ability to accomplish post-translational modifications. The adoption of these approaches by some companies highlights the potential of algae-based platforms (Scranton et al. 2015). The production of antibodies, immuno-toxins, cytokines, and hormones in algal systems, as well as vaccines for human and animal health has been explored by several research groups. Figure 9.1a contains the cumulative scientific articles related to this topic since 1990, whereas Fig. 9.1b presents a classification of the BFs produced in algae systems thus far. Under this sustained research context, most of these candidates showed attractive *in vitro* and *in vivo* bioactivities with the perspective to be evaluated at the clinical level (Rosales-Mendoza 2013). Although a decade of research have led to important advances, the exploitation of algae for generating new candidates should be expanded and industrial scale production and regulatory approvals should be accomplished such that BFs become a reality in the market. Therefore, despite the proof of concepts available for the production of BFs in algae species, extensive research to expand the applications and the potential for achieving the commercialization of the first algae-made BFs is still required. Herein key perspectives for this field are identified and discussed (Fig. 9.2). Plant-based production of BFs, which was started a decade before, have reached a notable acceptance by the industry and a product is currently commercialized (for a review see Rosales-Mendoza et al. 2016; Streatfield et al. 2015). Due to the similarities between these organisms in terms of sharing some biological aspects and advantages as hosts, this chapter presents perspectives for algae-made BFs under a comparative focus with those produced in plant systems. The experience gained in the use of plants will be without a

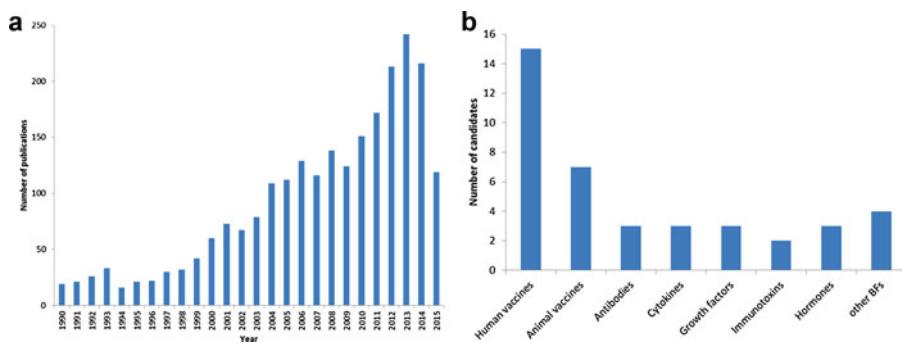


Fig. 9.1 (a) Pubmed search result for citations from 1990 to 2015 containing the words ‘algae’ and ‘biopharmaceutical’. **(b)** Classification of the BFs produced in algae-based platforms thus far

doubt a valuable resource to optimize and expand the use of algae systems resulting in new products approved for use in target populations.

Key Perspectives for the Field of Producing BFs in Microalgae

Optimizing Nuclear Expression

Algae species have shown a broad biosynthetic capacity, being able to produce several BFs that differ in complexity and application. Chapter 2 has presented a detailed compilation on the genetic engineering and expression approaches for algae species. In fact, chloroplast-based expression has allowed attaining attractive yields of up to 3.28 mg/L of culture medium at pilot greenhouse scale (Gimpel et al. 2015a, b). However, limitations in terms of yields under nuclear expression strategies have been found likely due to epigenetic silencing (Cerutti et al. 1997; Rasala et al. 2010). Therefore, optimizing nuclear expression is a need since some important processes, such as protein secretion and glycosylation, do not occur in chloroplasts; and thus nuclear expression should be followed when such kind of processing is required. Interestingly, the sustained research activity on the optimization of expression strategies revealed improvements that will be critical to advance in increasing the productivity of the algae-based platforms.

For instance, it has been proven that codon optimization is critical since codon usage determines translational efficiency and mRNA stability. In fact, unfavorable GC content disturbs gene expression by the induction of heterochromatinization (Barahimipour et al. 2015). In addition recent advances also add interesting prospects to the scenario, which include the use of codon-optimized selectable markers as well as specific strains improved by UV light-induced mutations. These strains allow high-level transgene expression due, at least in part, to a lower susceptibility to epigenetic transgene suppression and the lack of repressive chromatin structure in the insertion site (Barahimipour et al. 2015; Neupert et al. 2009). When

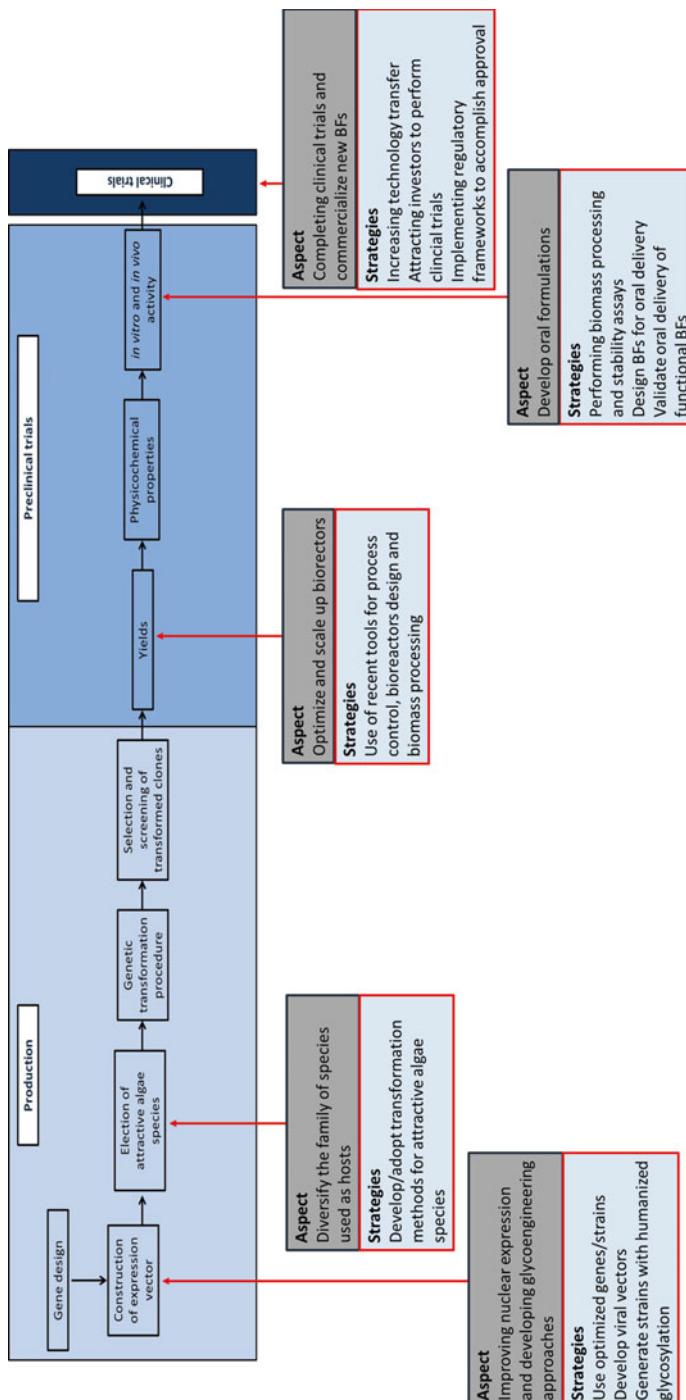


Fig. 9.2 Aspects in which substantial research and development are expected in the following years for the field of algaе-made BF_s

combined with a codon-optimized version of the *nptII* gene, the transformation efficiency also improved (Barahimipour et al. 2016). In addition, a key future prospect in this field comprises the implementation of transient expression approaches mediated by viral vectors; which have led to a great success in plant-based platforms (Gleba et al. 2014; Hahn et al. 2015). These systems enable massive protein accumulation in a short period, however no stocks are generated since they are based on transient expression.

Therefore, a number of new possibilities for maximizing productivity are identified and will lead undoubtedly to significant advances in the following years (see Chap. 2). Despite the yield limitations observed in certain cases, an overall evaluation of the advantages offered by algae-based systems such as better safety and low-cost production; indicates that these surpass the current limitations related with yields making the system viable and attractive, especially under the conception of using algal cells as a delivery vehicle, avoiding the need for purification processes.

Implementing Glycoengineering Approaches

Oligosaccharides linked to proteins used as BFs may affect their functionality, half-life, folding, assembly, solubility, and charge (Roth et al. 2010; Solá and Griebenow 2010; Varki 1993). In addition, differential glycosylation may convert the biopharmaceutical (BF) into an immunogenic molecule that leads to undesired events such as the production of BF-blocking antibodies or hypersensitivity reactions (Jin et al. 2008; Van Ree et al. 2000). Therefore when a BF is produced recombinantly, variations in glycosylation could negatively modify the properties of the molecule. However, in some cases differential glycosylation may have positive effects and thus it should be considered that it is not always an inconvenient event. For instance, the glucocerebrosidase currently used in humans for replacement therapy possesses a glycosylation pattern that comprises 100 % exposed paucimannose structures, including an (α 1-3)-linked fucose attached to the reducing N-acetylglucosamine (GlcNAc) and a xylose and thus does not require an *in vitro* modification to be efficiently internalized by the target cells (Shaaltiel et al. 2007). In the case of vaccines, glycans linked to the antigen may modulate the antigen uptake and thus favor the vaccine immunogenicity and efficacy (Bosch and Schots 2010). Therefore glycosylation is an aspect that may influence the activity, safety, and efficacy of BFs and should be evaluated case by case.

To exploit algae species for the production of BFs with proper glycosylation patterns, it will be fundamental to know in detail the endogenous machinery and the glycans produced by the host species. Typically, *N*-glycan processing initiates with a $\text{Man}_5\text{GlcNAc}_2\text{-PP-dolichololigosaccharide}$ intermediate that is first synthesized onto a dolichol pyrophosphate on the cytosolic ER surface. After a translocation of the intermediate to the ER lumen, it is modified to form the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-dolichol}$ *N*-glycan precursor; which is subsequently linked to the asparagine residues of the consensus Asn-X-Ser/Thr sequences present in the target proteins (Welti

2013). The precursor linked to the protein is modified in several steps that lead to removal and addition of monosaccharides, processes that play an important role in proper protein folding (Tannous et al. 2015). At this level, the process is highly conserved in eukaryotes and generates a limited number of oligomannoside *N*-glycans. On the other hand, a complex *N*-glycan modification occurs in the Golgi apparatus, leading to a large diversity of oligosaccharides structures. First, processing in Golgi apparatus comprises the action of type I mannosidases that degrade the oligosaccharides into oligomannoside *N*-glycans; which range from $\text{Man}_9\text{GlcNAc}_2$ (Man-9) to $\text{Man}_5\text{GlcNAc}_2$ (Man-5). Second, *N*-acetylglucosaminyltransferase I (GnT I) links the GlcNAc residue on the $\alpha(1,3)$ -mannose arm of Man-5; which leads to the synthesis of polyantennary complex-type *N*-glycans (Parodi 2000).

The description of the glycosylation that occurs in algae is rather limited and the most advanced studies have been performed for green microalgae (Chlorophyta) (Balshüsemann and Jaenicke 1990; Becker and Melkonian 1992; Becker et al. 1995, 1996; Gödel et al. 2000; Mamedov and Yusibov 2011; Mathieu-Rivet et al. 2013; Weerapana and Imperiali 2006). The typical glycans found in the eukaryotic organisms have been identified in algae, comprising oligomannosides or mature *N*-glycans with a xylose core residue. However, two reports concerning the investigation of *N*-glycosylation on the green microalga *C. reinhardtii* have resulted in different findings. In a study by Mathieu-Rivet et al. (2013), *N*-glycans of the oligomannose type were mainly found in soluble and membrane protein fractions. A minor fraction of *N*-linked glycans containing mannose, methylated mannose, and xylose residues was reported. In contrast the presence of sialylated *N*-linked oligosaccharides, which are characteristics of mammalian oligosaccharides, has been reported when total protein extracts were analyzed (Mamedov and Yusibov 2011).

In the case of *Phaeodactylum tricornutum*, it is known that high mannose type *N*-glycans are prominently present in glycoproteins (Man-5 to Man-9); while pauci-mannose type glycans are present in a minor proportion. In addition, minor glycans Man-3 and Man-4 carrying a 1,3-linked fucose were detected (Baïet et al. 2011). Moreover, oligosaccharides with 6-O-MeMan and xylose were found in the red microalgae *Porphyridium* sp. (Levy-Ontman et al. 2011, 2014).

In terms of the glycosylation patterns observed in BFs produced in algae, very limited information is available. Interestingly, the characterization of the influenza vaccine constituted by the HA antigen expressed in *Schizochytrium* sp included the determination of the glycosylation profiles (Bayne et al. 2013). The study revealed typical high mass fragmentation patterns for each of the glycans, with predominant fragmentation at the bisecting GlcNAc residues. Hyper-mannosylation was also detected but at very low levels. Interestingly, no fucose or xylose residues were detected among any glycoforms; which is in contrast with the patterns of plant and green algal glycans (Bosch and Schots 2010). Sialic acid residues were not detected in this analysis and a survey of the *Schizochytrium* genome database found no genes related to sialic acid biosynthesis.

Regarding the implications of this pattern on efficacy or safety of the vaccine, the detected high-mannose structures, such as $(\text{GlcNAc})_2(\text{Man})_{5-6}$; are assembled in part by α -1,3 and α -1,6 linkages which have been demonstrated to be immunogenic

in mammals (Geijtenbeek and Gringhuis 2009). The glycan (GlcNAc)₂(Man)₆ is also found at Asn 65 of the HA1 subunit from mammalian cells infected with influenza (Mir-Shekari et al. 1997), however it is unclear to what extent these α -1,3 mannoses may promote immunity to viral infection when associated with vaccine antigens.

Although glycoengineering approaches in algae species have not been explored thus far, the previous experiences in plants and moss constitute a valuable reference for the implementation of their counterparts in microalgae species (Decker et al. 2014; Loos and Steinkellner 2014). It is of remarkable interest that in plants and moss, glycoengineering approaches have allowed the introduction of specific glycosylases producing human-like glycosylation patterns. Asparagine (Asn/N)-linked protein glycosylation in plants shares a common core structure with mammalian N-glycosylation, i.e. di-antennary glycans of the complex type. However, some differences comprise the lack of terminal β -1,4 galactose and sialic acid residues in plants; and complex-type N-glycans are modified by the action of β -1,2-xylosyltransferase and α -1,3-fucosyltransferase (Bosch and Schots 2010; Koprivova et al. 2003; Lerouge et al. 1998; Vitale and Chrispeels 1984).

To achieve a humanized glycosylation in the host, genetic engineering approaches can be applied to delete genes coding for glycosyltransferases responsible for the undesired glycans (Koprivova et al. 2004; Parsons et al. 2012; Schähs et al. 2007). Silencing those genes by RNAi is another possibility (Cox et al. 2006; Sourrouille et al. 2008; Strasser et al. 2008). Finally, the introduction of genes coding for the glycosyltransferases conferring the patterns that are absent in the host is also a possibility (Bakker et al. 2001; Huether et al. 2005; Palacpac et al. 1999). Therefore, in plants, the production of proteins with humanized glycan patterns implies the following modifications: knocking-out or knocking-down the plant-specific β -1,2-xylosyltransferase and core fucosyltransferase; introducing α -1,3-mannosyl- β -1,4-N-acetylglucosaminyltransferase (GnTIV) and α -1,6-mannosyl- β -1,6-N-acetylglucosaminyltransferase (GnTV) responsible for branching; introducing β -1,4-galactosyltransferase; introducing sialyltransferase as well as the biosynthetic pathway to produce activated sialic acid.

Algae species with efficient homologous recombination machinery at the nuclear level will be ideal targets to achieve gene-targeting approaches in an effort to implement glycoengineering approaches. Unfortunately *C. reinhardtii* lacks of an efficient homologous recombination at the nuclear genome, which imposes a major limitation for efficient gene-targeting (Sodeinde and Kindle 1993; Zorin et al. 2009). However, the discovery of efficient homologous recombination in other species is opening interesting perspectives in this field. For instance, the alga *Nannochloropsis* sp. was described as an organism in which mutants can be generated by homologous recombination at the nuclear genome level. Thus, precise gene targeting can be easily achieved to delete specific glycosylases and insert at specific sites those desired glycosylases that are absent in the host without the risk of interrupting endogenous genes or inserting the transgene in a locus with low transcriptional activity.

Since fucose and xylose residues are not typical in algae and are, in fact, absent in some species; and considering the evidence of 1,4-Gal associated with a sialylated

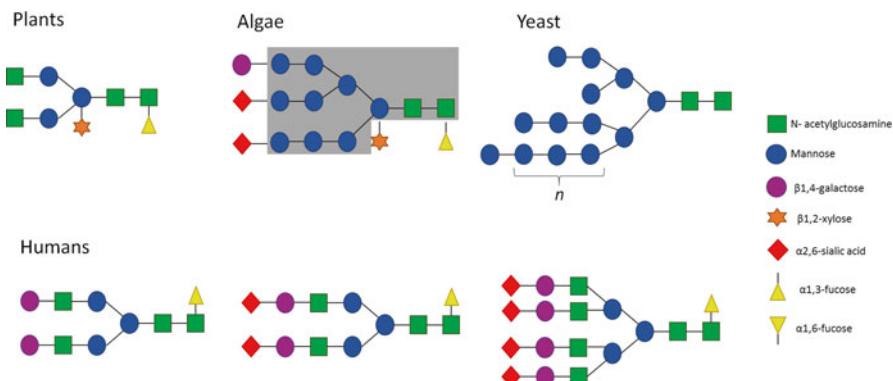


Fig. 9.3 Graphic description of the typical glycosylation patterns from distinct organisms used as hosts for BFs production and possibilities for glycoengineering in algae species. Algae possess predominantly high mannose glycans (*shaded structure*), however in some species hybrid and complex types of N-linked glycans are also reported (*residues outside the shadow*) comprising the following residues: fucose, xylose, sialic acid (Baïet et al. 2011). Since no yeast-like hypermannosylation has been described in algae, glycosylation could be engineered more easily. Since algae species have no fucose and xylose as typical residues and they possess 1,4-Gal associated to sialylated complex glycan structures, these hosts are attractive for implementing glycoengineering approaches to obtain strains with humanized glycosylation patterns. It should be noted that glycoengineering would be especially necessary in cases where BFs are used for passive immunity-based or replacement therapies in which the generation of BF-blocking antibodies occurs as consequence of differential glycosylation. In contrast for the case of vaccines, differential glycosylation might account for the vaccine immunogenicity. Therefore, the need for glycoengineering approaches is determined case by case

complex glycan structure, algae are attractive hosts for implementing glycoengineering approaches for the production of strains with humanized glycosylation patterns under easier methodologies than those required for yeasts and plants. However, the systematic knowledge of glycobiology in algae is still considered an almost virgin field; therefore further research is required to expand the knowledge on the glycosylation machinery and thus identify the approaches required to achieve the development of strains offering specific glycosylation patterns convenient for a particular purpose (e.g. humanized glycosylation patterns; Fig. 9.3).

Exploring New Approaches for Oral Delivery

The ambitious goal of delivering BFs orally by using as vehicle the same cell that have served as the biofactory of the therapeutic molecule supposes great savings in terms of downstream processing since purification is avoided. In addition, the administration costs are also reduced since no sterile devices and specialized personnel are required. Moreover, in terms of patients comfort oral administration is a painless procedure. The cold chain could be also avoided by the use of formulations based in

freeze-dried algae biomass, which allows for long term storage at room temperature with the additional advantage of concentration of the BF (Kohli et al. 2014).

The development of systems for orally delivery of BFs deals with a number of obstacles related to degradation and dilution of the BF as well as poor uptake rates at the intestinal level for most of the proteins. Therefore, several objectives focused on validating the efficacy of oral delivery of algae-made BFs by using the algae cell as the delivery vehicle are still pending. There is some evidence on the delivery of intact antibodies in mice (Barrera et al. 2015) as well as the induction of protective immunoprotective responses against pathogens induced by orally administered vaccines (Dreesen et al. 2010; Gregory et al. 2013), which indicates a promising potential that should be investigated in detail by exploring other candidates and characterizing the efficacy of the BFs that become available by the oral route.

The case of plant cells used as delivery vehicles of BFs deserves a special mention due to their similarities with algae species and the substantial advances on their characterization as oral delivery vehicle. Plant cells have been proposed as delivery vehicles based on the fact that bioencapsulated BFs are protected in the stomach and gut from acids and enzymes and may be absorbed into the circulatory system following the plant cell wall rupture or partial digestion (Kwon et al. 2013). To date this principle has been proven for a diverse group of molecules, providing not only evidence on the availability but also on the therapeutic effects (Kwon and Daniell 2015; for review see Rosales-Mendoza et al. 2016). A remarkable example is the glucocerebrosidase (GCD) made in carrot cells, which is approved for parenteral administrations in humans with Gaucher's disease (GD). However, the intravenous administration implies discomfort or pain and might cause local and systemic infections. Then, the development of orally administered products will avoid these problems and thus this enzyme is now under evaluation in oral delivery schemes using carrot cells as the vehicle. Feeding experiments with rat and pig as model animals using carrot cells containing GCD led to the detection of the active recombinant GCD in the digestive tract and blood system reaching the liver and the spleen, which are the target organs in GD (Shaaltiel et al. 2015; Fig. 9.4). Therefore, the oral administration of proteins encapsulated in plant cells is feasible and thus proposed as an alternative to intravenous administration.

Based on the few studies with promising results on the delivery of BFs by algae, it is expected that the extension of these studies and the generation of new models for the delivery of BFs by algae biomass will complete this scenario and will allow evaluating the potential to achieve an efficient delivery that guarantees the desired therapeutic effects. Another important factor to consider is biomass productivity, in which bioreactor technologies are fundamental to maximize yields.

Optimizing Large-Scale Production in Bioreactors

Algae species are generally grown in a wide range of conditions at low cost. The simplest approach for algae cultivation consists in using open ponds. Although this method offers very low costs, the cultures can be easily contaminated and a

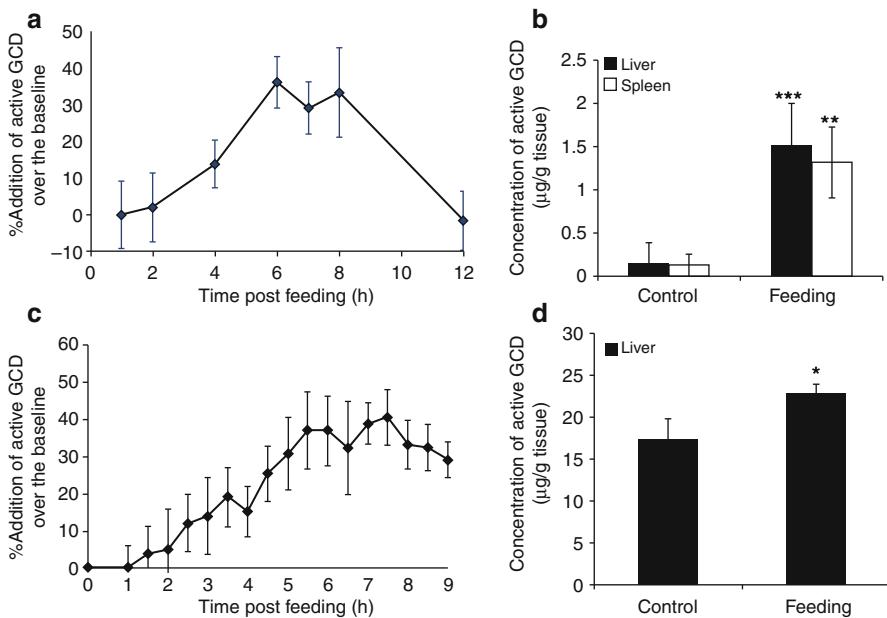


Fig. 9.4 Oral delivery of a recombinant enzyme, β -glucocerebrosidase, in rats and pigs indicates a great potential for using the biopharmaceutical expression host as a next generation delivery vehicle. In this pioneering approach, carrot cells successfully delivered the recombinant enzyme. In fact, the carrot-made β -glucocerebrosidase is currently commercialized in the form of an injectable product and was the first plant-made BF approved for human use. Groups of test pigs or rats were fed with freeze-dried carrot biomass and enzyme activity measured in distinct samples. The detection of the enzyme activity in plasma of rats (a) or pigs (c) as well as in the liver and spleen of rats (b) and the liver of pigs (d) indicates the delivery of the functional enzyme and opens key perspectives in the development of new pharmaceutical forms. Such kind of approaches can be applied in the algae-made BFs field. * $P < 0.05$; ** $P < 0.01$. *** $P < 0.001$ (Figure taken from Shaaltiel et al. (2015), permit number 3804940254503)

poor control of growth parameters exists (Ketheesan and Nirmalakhandan 2011; Rhodes 2012).

Process optimization and implementation of good manufacturing practices (GMPs) is an important goal for the purpose of producing BFs since the standards are different from those of biofuel or feed production. Since bioreactors are generally closed systems, these devices are the most appropriate for algae-based BFs production. In this regard, the bioreactor design and operation are critical aspects in the production process. The cultivation of algae in bioreactors offers several advantages such as the possibility of full containment, which enhances biosafety; moreover, an adequate control of the growth parameters to optimize the production is possible. These advantages can easily meet the regulatory legislations.

The microalgae growth is influenced by the factors coupling photosynthesis, hydrodynamics, and irradiance distribution in the reactor (Luo et al. 2003). Therefore, the production yields have high dependency on flow and light regimes of the system. For instance mixing keeps cells in suspension, avoids thermal

stratification, provides adequate nutrient distribution, and favors gas–liquid mass transfer preventing oxygen accumulation as well as homogeneous light capture by the cells (Hu and Richmond 1996; Ugwu et al. 2008). In addition, a concern in a process involving genetically modified organisms is containment to avoid their undesired spread into the environment. The cultivation of algae for BFs production should meet the requirements for GMP-compliant processes, requiring closed bioreactor systems for biomass growth, avoiding contamination.

The current advances in cultivation and harvesting achieved by the industrial algal production for biofuel and other commercial products will greatly favor the implementation of systems for BFs production. In terms of types of bioreactors (BR), non-photosynthetic algae can be grown in conventional fermenters used for bacteria or yeast. *Schizochytrium* sp. is a representative case of this kind of algae species. The industrial use of *Schizochytrium* has been implemented to produce up to ten tons per annum of triglyceride oil from cultures of volumes larger than 150,000 L (Bailey et al. 2010; Ratledge 2004). Interestingly, this organism efficiently consumes nutrients at low dissolved oxygen levels; with yields of up to 200 g/L of dry biomass in a short time (4 days) (Barclay et al. 2005). For the production of an influenza vaccine, this species was efficiently grown in a 10 L fermentor with the following parameters: airflow set to 8 L/min, 20 % of dissolved oxygen, 357–900 rpm, 22.5 °C, and pH set to 6.75 (Bayne et al. 2013). Although such type of cultivation implies the use of reduced carbon in the form of glucose, which is not needed for photosynthetic species; *Schizochytrium* sp offers substantial savings in terms of materials, energy, and labor costs per unit of mass produced in fermenters when compared with many eukaryotic hosts. However, it should be recognized that the current industrial use is implemented for oil production and the adoption of this process for BFs production will require modifications to meet the standards required by the applicable regulations.

In the case of photosynthetic algae, strains can be cultivated in photobioreactors (PBRs) with the advantage of not requiring a reduced carbon source. PBRs are typically made of glass or plastic and sun light drives the generation of biomass and bioproducts using minimal, low cost media. In some cases the light source is artificial, such as light-emitting diodes (Darko et al. 2014). The type of PBRs comprise annular PBRs consisting of large columns, flat panel PBRs, and tubular PBRs (de Vree et al. 2015). The strategies for cultivating algae at the industrial scale comprise circulating ponds as well as flat panel and horizontal or vertical BRs (Fig. 9.5). The use of bags maintained in greenhouses has been assessed for the case of *Chlamydomonas* expressing a recombinant protein with nutraceutical activity (Gimpel et al. 2015a, b).

PBRs offer a tight control on growth parameters as well as full containment and axenic growth, which greatly facilitate the implementation of GMP-compliant processes. Although PBRs imply higher cost due to infrastructure, the total cost is lesser than that of the bioreactors used for mammalian cell lines since with PBRs fewer variables are controlled, there is no oxygen requirement (CO₂ instead), and low cost media are used.

The production of BFs using plant cells in culture is a closed reference for the algal systems and thus advances in this area deserve a mention. Disposable

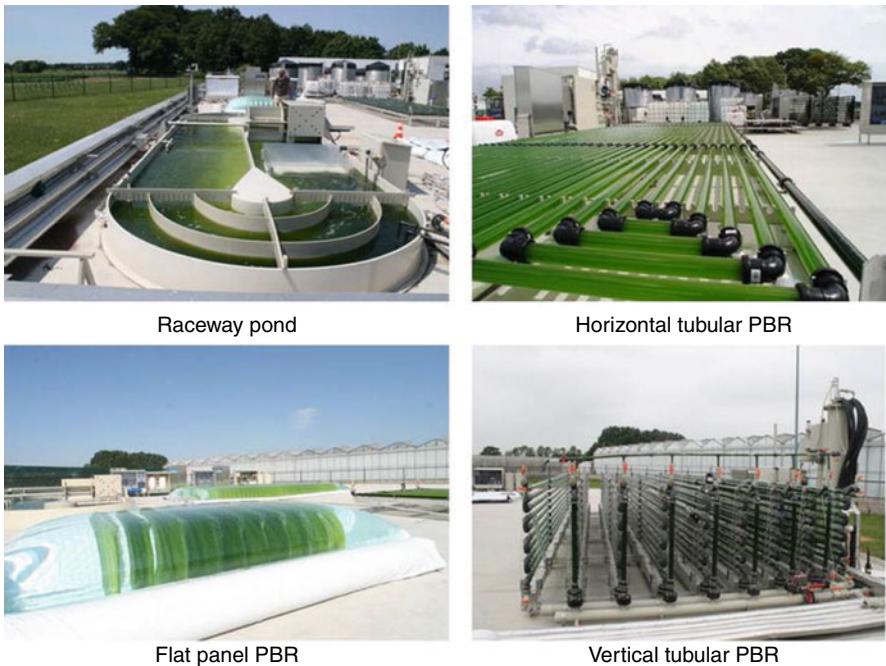


Fig. 9.5 Examples of algae growth systems currently used by the industry for algae production at a large scale, including raceway ponds and photobioreactors in flat panel, and vertical or horizontal tubular configurations (Figure taken from de Vree et al. (2015))

bag-based reactors of the wave-type are under use for the cultivation of tobacco BY-2 cells as an alternative to conventional stainless steel stirred-tank reactors. The claimed advantages for this approach include the simple bag geometry, scalability, and predictable process settings. A scale-up study in which 200 L orbitally-shaken bioreactor based on disposable bags allowed for the growth of BY-2 cells with yields of 300–387 g/L fresh weight biomass and 20 mg/L of the BF. Interestingly these yields were comparable to those obtained by standard shake flask cultivation (Raven et al. 2015). Greenovation, a German company, is currently using bag-based reactors of the wave-type for the production of BFs in moss at large-scale in a GMP facility (www.greenovation.com). This technology could be also used for algae-made BFs production.

Tubular PBRs for specific algae species have been implemented for several species (Ortiz Montoya et al. 2014; Zhang et al. 2013) and the improvement of PBRs is an active field with recent innovations. For instance, the use of partially covered glass tubes has allowed for the generation of controllable light/dark cycle that favors biomass generation (Fig. 9.6a). By using this approach in *Chlorella pyrenoidosa* cultures, the biomass productivity increased by $21.6 \pm 2.1\%$ when the frequency of the light/dark cycle was set at 100 Hz (Liao et al. 2014). This design will be important for attaining high light-to-biomass conversion in large-scale outdoor applications. However, no systematic research on using tubular PBRs for the production of algae expressing BFs exists constituting a key prospect. Innovations in the PBRs field also comprise the use of flat-plate

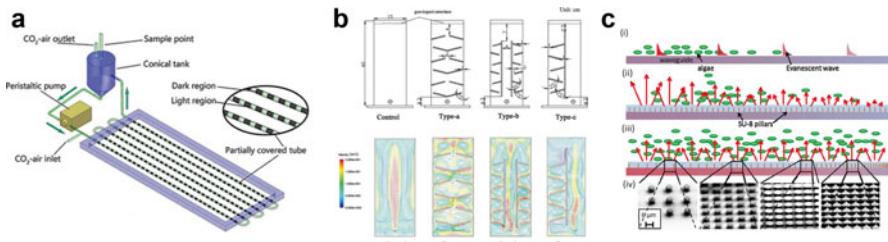


Fig. 9.6 Recent innovations in photobioreactors aimed at increasing productivity by: (a) the use of tubular reactors with intermittent light/dark cycle. (b) The evaluation of distinct stirring strategies in flat panel bioreactors, where the type-a design allowed to achieve a 42.9 % increase in biomass yield with respect to the control reactor. (c) Internal wave-guiding structures incorporating engineered light scattering schemes. (i) Algae can be excited via evanescent waves where growth is confined closer to the surface of the waveguide; (ii) Uniform distribution of scatterers leads to a non-uniform illumination across the reactor; (iii) A differential distribution of pillars along the reactor leads to a more uniform illumination; (iv) SEM images of the pillars showing the differential density along the PBR (Figures taken from Liao et al. (2014) (a), Huang et al. (2014) (b) and Ahsan et al. (2014) (c). Permits number 3805541041159 and 3805541103253; respectively)

designs. Special mixers have been recently evaluated to increase the biomass yield as consequence of increasing the mixing degree along the light gradient (Fig. 9.6b).

Given that the light distribution is a critical issue in PBRs, which is typically poor due to shading effects, improving its distribution is an important goal in bioreactor design. Several approaches to improve this aspect have been implemented including designs that incorporate larger surface areas (Ugwu et al. 2008), evanescent excitation (Jung et al. 2012), air-lift driven raceway reactors (Zimmerman et al. 2011), surface-plasmon-based light backscattering (Torkamani et al. 2010), and internal wave-guiding structures incorporating engineered light scattering schemes (Ahsan et al. 2014; Fig. 9.6c).

Besides reactor design improvements, the simulation of control strategies is under development and constitutes a valuable tool for implementing the production of algae-made BFs at the industrial scale (Dormido et al. 2014). Although no detailed analysis of BFs accumulation kinetics in response to growth parameters has been performed, some studies on the optimization of growth are ongoing. For instance, the hydrodynamic and light regime characteristic of the novel PBRs were investigated through computational fluid dynamics. Compared with the control reactor without mixer, the novel reactors can effectively increase liquid velocity along the light gradient, the frequency of light/dark (L/D) cycles, and the algal growth rates of *Chlorella pyrenoidosa*. Biomass yields have been incremented up to 42.9 % by this method. The correlation analysis of algal growth rate with the characteristics of mixing and light regime shows that the key factors affecting algal photoautotrophic growth are liquid velocity along the light gradient and L/D cycles rather than the macro-mixing degree (Huang et al. 2014). This kind of approaches will be important to optimize the growth of genetically engineered algae strains producing BFs. Since advanced control strategies are under design, these will be valuable resources to optimize the production of biomass and BFs protein accumulation at the pilot and large scales (Costache et al. 2013; Fernández et al. 2012).

Expanding the Group of Species Used as Hosts

Given the diversity of algae species, many species with attractive features exist and could be adopted to develop new platforms. For instance, the marine algae species deserve special consideration. The use of seawater to cultivate algae will avoid the use of fresh water and thus no interference with sources of drinking water or for agricultural use is generated.

An unexplored topic of a putative high impact factor is the case of extremophilic algae species that may provide robust production platforms, as is the case of *Nannochloropsis salina* (Varshney et al. 2015). On the other hand, *Hematococcus pluvialis* is an autotrophic fresh water microalga that has been applied for industrial production of pigments since several years ago (Fábregas et al. 2000; Olaizola 2000). *H. pluvialis* is able to grow in extreme environments and survive extreme fluctuations in light, temperature, and salt concentration (Saei et al. 2012). An important feature of *H. pluvialis* is its codon usage, which is highly similar to codon usage in humans as reported by Saei et al. (2012). This is an important trait since codon optimization is not considered necessary in the case of human origin BFs avoiding low productivity associated to codon usage issues. The development of genetic engineering tools for *H. pluvialis* has been recently reported. Gutiérrez et al. (2012) have developed a chloroplast transformation vector, designed using the endogenous 5'rbcL as promoter and 3'rbcL as terminator. These elements were used to express the aadA selection marker. The construct was introduced into the chloroplast through a biolistic method. The transgene insertion was stable through 40 sub-cultivation steps in three transgenic lines, although only one line became homoplastic after the selection rounds. Another important advantage of *H. pluvialis* is the availability of nuclear transformation protocols via agro-inoculation. In 2009 Kathiresan et al. used agrobacterium and elements from the pCAMBIA vector to express the reporter genes *gfp* and *uidA* (Kathiresan et al. 2009). The integration of the transgene was maintained after 2 years of sub-cultivation. These genetic engineering tools will be critical to explore the use of *H. pluvialis* as a new BFs production platform.

Nannochloropsis sp. is a microalga living in fresh and seawater that is related to diatoms and brown algae (Sukenik et al. 2009). The *Nannochloropsis* species have been used for several decades to produce nutraceuticals and feed supplements (Rodolfi et al. 2009). It is of interest to note that genetic engineering tools for nuclear transformation have been recently developed for this species. Kilian and coworkers, in 2011, established a protocol for nuclear transformation of *Nannochloropsis* via homologous recombination with high transformation efficiency (Kilian et al. 2011). These tools increase the possibilities for implementing robust BFs production platforms based in this species.

Thus these examples indicate that a number of algae species, to which culture and genetic modification tools are available, are an unexplored path that has the potential to originate attractive platforms. The following traits could be achieved in these systems: low contamination events due to cultivation in extreme culture conditions (e.g. high pH), higher protein yields, use of secretion machinery to facilitate downstream processing, and the use of seawater for cultivation instead of fresh

water. Important advances are expected in the following decades based on the use of new algae species for BFs production.

Technology Transfer and Regulatory Approval

Following the successful proof-of-concept assessments, the goal of achieving commercialization of algae-made BFs in the benefit of population; especially in the developing world, implies several subsequent steps in which collaborations between various groups are required. Most of the ongoing research in this field is performed in academic institutions, thus the academia-industry relationship will be a key action to attract investors and accelerate intellectual property protection. Patents are essential incentives to foster innovation, in particular for the case of BFs since these require lengthy, costly, and risky processes to perform the research and development (R&D) procedures. This importance remains despite the many changes in the market and patent landscape (Cockburn and Long 2015). Since many BFs are facing patent expiration, the field of BFs has entered into the biosimilars (generic substitutes) production era; which offers great possibilities for producing already known therapeutic molecules using innovative hosts. The strong background on the efficacy and wide clinical experience with innovator products will make the development of biosimilars a smother path. However due to the high molecular complexity of BFs, the process of development and approval of biosimilars is complicated. The main challenge consists in the fact that variations in the biosimilar could be generated since no information on the originator's processes is available. For this reason, regulatory requirements are needed to ensure biosimilarity, comparability, and interchangeability with respect to efficacy and safety (Stevenson 2015). Both the EU and the US have prepared guidance documents for biosimilars that will result in biotherapeutics that are as safe and efficacious as the innovator product (Daller and Daller 2015).

Generating and complying a regulatory framework for algae-made BFs (biosimilars or innovator products) will be a key objective to accomplish approval by the Food and Drug Administration (FDA), the UE, or local regulatory agencies. Fortunately the case of plant-made BFs, which is a close related approach, has generated a key reference that will greatly favor the success in accomplishing the approvals of algae-made BFs. Since a plant-made BF consisting of an injectable carrot-made glucocerebrosidse was approved by the FDA in 2012, valuable experience in coping with establishing new guidelines and procedures for approval has been gained (Mor 2015). Although a number of circumstances favored the approval of this BF, it constitutes a key precedent that generates a smother avenue for new BFs produced in next generation platforms including algae-made BFs. Perhaps the greatest challenge will be validating those products based on oral delivery by using whole freeze-dried algae biomass. Again, plant-based platforms constitute a pioneering reference in this regard. For instance, the validation and production at industrial levels of plant-based vaccines is ongoing and near to completion.

A remarkable case is the current investigations on growing hydroponic lettuce and processing biomass by freeze-drying and milling to produce a tolerogenic vaccine at the industrial scale. By using the Fraunhofer cGMP hydroponic system, yields of up to 870 kg fresh or 43.5 kg dry weight per 1000 ft² per annum can be achieved, yielding 24,000–36,000 doses for 20-kg pediatric patients (Su et al. 2015). Therefore, this technology offers the possibility for producing the first commercial drug for oral tolerance induction in hemophilia B. On the other hand, the processing developments achieved thus far using systems based in transient expression mediated by viral vectors in Nicotiana species also represent a reference for establishing GMP compliant processes for algae-made BFs. In fact, automated systems have been developed and constitute a viable strategy to comply with GMP compliant processes (Wirz et al. 2012) and influenza vaccines produced in these systems are currently under clinical trial evaluation (Landry et al. 2014).

The EU Sixth Framework Programme Integrated Project ‘Pharma-Planta’ is another interesting case for the field since cooperative efforts between several organizations achieved an approved manufacturing process for an HIV-neutralizing monoclonal antibody (2G12). Since only draft regulations were initially available covering the production of recombinant proteins in transgenic tobacco plants, ‘plant-specific’ regulatory and technical challenges for the development of a process suitable for the acquisition of a manufacturing license for clinical phase I trials were established and validated. Upstream process development was established, comprising several steps from plant transformation to host plant uniformity; as well as product-specific aspects, such as product quantity. All these procedures were GMP/generally accepted compliance practice (GACP)-compliant. As an active pharmaceutical ingredient (API), the plant-made P2G12 antibody was subjected to (i) the guidelines for the manufacture of monoclonal antibodies in cell culture systems; (ii) the draft European Medicines Agency Points to Consider document on quality requirements for APIs produced in transgenic plants; and (iii) de novo guidelines developed with European national regulators.

These efforts allowed translating this process into a prototypic manufacturing process that was approved by the German regulatory agency and subsequently a first-in-human, double-blind, placebo-controlled, randomized, dose-escalation phase I safety study was performed (Ma et al. 2015; Sack et al. 2015). This remarkable achievement is a valuable example of the collaboration required to advance in the adoption of innovative platforms for BFs production addressing not only a proof of concept but achieving regulatory framework and approvals.

The current established processes for the production of some algae species at the industrial scale to produce biofuels or other compounds will be with no doubt useful in this field. Therefore, an integration in the experience gained for plant-made BFs and industrial production of algae for other applications will catalyze the commercialization of algae-made BFs.

A reduced number of companies can be identified in the algae-made BFs field. For instance Triton Algae Innovations Ltd., is a company that produces proteins, enzymes, and other biologics in Chlamydomonas. One of the products currently in the market is the Mammary Associated Amyloid, which is contained in the colostrum of mammals

and can be used to treat intestinal disease in livestock, companion animals, and humans. In addition, anti-bacterials, anti-oxidants, biosurfactants, DNA repair enzymes, antimicrobials, intestinal health proteins, growth factors, bone growth enhancers, and vaccines are in the pipeline. All these candidates will have implications in the development of pharmaceuticals, nutraceuticals, cosmetics, and human and animal health nutrition products; applications in agricultural and other retail markets are also envisioned. DSM, a company focused on nutritional products, is also involved in the production of influenza vaccines in *Schizochytrium* sp. (Bayne et al. 2013).

Indeed, novel strategies for funding are also required to support the development of the new BFs with the potential to save lives in low income countries (Hefferon 2013). The selection of new molecules should be defined under the advice of the World Health Organization (WHO). In this field, the local production of quality-assured medicines is a key needed to ensure reliable access to affordable medicines. For instance, in the case of Africa, it is projected that the pharmaceutical market will reach 30 billion US dollars per year by 2016; which represents the world's second fastest growing market (Donga and Mirza 2016; WHO 2011). In this context algae-based production platforms for BFs could play a relevant role. A positive interaction can be established between firms from developed countries and non-governmental organizations and scientists and institutions from developing countries- for research and development, including clinical trials (Rybicki et al. 2013). An example of such interaction is the case of Fiocruz/Bio-Manguinhos (Brazil), which established cooperation with iBio Inc., (USA) to produce a Yellow fever virus vaccine (Rybicki et al. 2013). In developing countries the fight against neglected diseases, in which no big pharma companies will invest, establishes that governments and non-profit organizations will play a critical role on promoting the adoption of the technology by local pharma companies or even government agencies involved in the production of BFs for the public health system. Therefore, it is hoped that the projected industrial growth and modernization can contribute towards social development and catalyze knowledge-based economic growth, research and development.

In conclusion, the current limitations of algae-made BFs production platforms can be addressed by the use of several innovative tools and recently generated knowledge. Therefore, the technology has the potential for addressing prohibitively expensive purification, cold storage/transportation, and short shelf life of current BFs such that they become a benefit for global health; especially in developing countries.

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Index

A

- Agrobacterium tumefaciens*, 10, 15, 18, 20–21, 30
Alkaloids, 136–137
Anti-venom, 90
Antigen delivery, 42
ApoB100, 44
Astaxanthin, 7, 123, 131, 132, 134, 136

B

- β-carotene, 7, 10–11, 131–133
β-cryptoxanthin, 131
Bioactive peptides, 128–129
Biobetter, 5, 11, 90
Bioreactor design
 flat panel, 126, 127
 stirred-tank, 152
 tubular, 151, 152
Biosafety, 89
Biosimilar, 3–5, 11, 90, 114
Bovine milk amyloid A (MAA), 122–128

C

- Cancer, 49, 53, 78, 87–90, 98–100, 104, 114, 117, 121, 131, 143–177
Carotenoids, 10, 121, 123, 131–137
Chlorella pyrenoidosa, 58
Chloroplast expression, 35, 49, 123, 136
Cholera toxin B subunit (CTB), 43, 44, 49, 50, 54, 55, 57, 58, 69, 70
Chronic diseases, 128
Classical swine fever virus (CSFV), 66–67
Control strategies, 113, 114

D

- Developing countries, 1, 11, 49, 71, 73, 122
Docosahexaenoic acid (DHA), 8, 11, 129, 130
Dunaliella salina, 9, 10, 54, 67, 97, 101, 113–114

E

- Eicosapentaenoic acid (EPA), 123, 129, 130
Erythropoietin, 3, 114–115
Expression vector, 16–19, 22, 33, 96, 110, 113, 114, 116

F

- Fibronectin domains, 111–113
Foot and mouth disease virus, 69–70
Functional food, 121

G

- Glycoengineering, 2, 26, 84, 89, 103–104, 117–118
Glycosylation, 1, 4, 24, 51, 79, 83, 89, 103–104, 115, 117, 118
GMP, 59
Greenhouse, 8, 9, 123, 124, 126, 127

H

- Haematococcus pluvialis*, 131
Hemagglutinin, 44, 50, 51, 70, 71
High-mobility group box 1 protein (HMGB1), 97–101, 124
HMGB1. *See* High-mobility group box 1 protein (HMGB1)

Human glutamic acid decarboxylase
 65 (hGAD65), 44
Human papillomavirus, 6, 44, 49–50, 52
Human vascular endothelial growth factor
 (VEGF), 97–99, 101–103, 112, 124
Hypertension, 55–56, 136

I

Immunotoxin, 77–90
Influenza virus, 11, 50–53, 70
Insulin, 1, 3, 128
Interferon β 1 (IFN- β 1), 96–97
Interleukin, 1, 95, 104

L

Lactoferricin (LfB), 110–112
Large single chain antibody, 79–81
Livestock, 65, 66, 69

M

Marketing, 3–5, 11, 66, 72, 118

N

Nannochloropsis oculata, 110
Non-communicable disease (NCDs), 1, 55,
 121, 128, 135
Non-photosynthetic, 8, 124, 125
Nuclear expression, 18, 23–24, 28, 54, 57, 89,
 103, 115, 117, 123, 133, 134

O

One health, 65
Oral delivery, 3, 42, 80, 85, 86,
 104, 105, 111, 128
Oral immunization, 46, 47, 49, 56
Oral vaccine, 42, 45, 49, 51, 58, 59, 66, 70

P

Patent, 3, 54, 70, 101, 114
Peanut allergens, 56
Phaeodactylum tricornutum, 8–10, 58, 83–84,
 103, 130

Photoautotrophic growth, 102

Photobioreactor, 9, 11

Plasmodium falciparum, 43, 46–49

Polyunsaturated fatty acids (PUFA), 51, 129,
 130, 137

Porcine circovirus, 70

R

Rabbit neutrophil peptide-1 (NP1), 109–111
Recombination, 8, 17, 18, 22, 24, 26, 29, 104

S

Schizochytrium sp., 8–11, 44, 50,
 51, 53, 70, 130
Silencing, 23, 24, 28, 32, 33, 57, 134
Single chain variable fragments
 (scFvs), 78, 88
Soybean Kunitz trypsin inhibitor (SKTI),
 113–114
Staphylococcus aureus, 43, 49

T

Taenia solium, 68–69
Tolerance, 5, 90
TRAIL. *See* Tumor necrosis factor-related
 apoptosis-inducing ligand (TRAIL)
Tropical neglected diseases
 (TNDs), 68–69, 71
Tumor necrosis factor-related apoptosis-
 inducing ligand (TRAIL), 100–101
Type I diabetes, 44, 55

U

Unsafe injections, 1

W

White spot syndrome virus (WSSV), 67–68
WSSV. *See* White spot syndrome virus
 (WSSV)

Z

Zoonosis, 67, 71, 73