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New challenges in microalgae biotechnology

Federico Valverde[a,*], Francisco J. Romero-Campero[a,b], Rosa León[c], Miguel G. Guerrero[a] and Aurelio Serrano[a,*]

[a]Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y CSIC, Av. Américo Vespucio 49, 41092 Sevilla, Spain; [b]Department of Computer Science and Artificial Intelligence, ETSII, Universidad de Sevilla, Av. Reina Mercedes s/n, 41012 Sevilla, Spain; [c]Área de Bioquímica - Departamento de Química y Ciencia de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, Av. de las Fuerzas Armadas, 21071 Huelva, Spain.

*Corresponding authors:

E-mail addresses: federico.valverde@ibvf.csic.es (F. Valverde) and aurelio@ibvf.csic.es (A. Serrano)

Abstract

Photosynthetic protists, also called microalgae, have been systematically studied for more than a century. However, only recently broad biotechnological applications have fostered a novel wave of research on their potentialities as sustainable resources of renewable energy as well as valuable industrial and agro-food products. At the recent VII European Congress of Protistology held in Seville, three outstanding examples of different research strategies on microalgae with biotechnological implications were presented, which suggested that integrative approaches will produce very significant advances in this field in the next future. In any case, intense research and the application of systems biology and genetic engineering techniques are absolutely essential to reach the full potential of microalgae as cell-factories of bio-based products and, therefore, could contribute significantly to solve the problems of biosustainability and energy shortage.

Keywords*:* Algae biotechnology; Biofuels; Genetic engineering; Microalgae; Photosynthetic protists; Systems biology

Introduction

Energy and its sustainable production is one of the most important resources for mankind. Sunlight is by far the most important input of energy to Earth, and photosynthesis is the main biological process channeling solar energy into the biosphere. Eukaryotic microalgae are a taxonomically broad and heterogeneous group of phototrophic protists of increasing biotechnological interest due to their higher photosynthetic efficiencies relative to land plants (microalgae contribute up to 25% of global photosynthetic productivity), elevated growth rates and vast metabolic capabilities (Raven and Falkowski 1999). In particular, the green microalgae (*Chlorophyta*) share the same photosynthetic machinery as the higher plants, according to their close phylogenetic relationships.

Microalgae such as *Chlorophyceae* and *Bacillariophyceae* use sunlight energy and a simple set of abundant, cheap resources (carbon dioxide, water and minerals) to generate a potential large number of valuable products of technological interest. These products can be applied, either directly or after transformation, in industrial, pharmaceutical and agro-food processes; examples are carotenoids, oils, polysaccharides, pigments, bioethanol, hydrogen, microalgal biomass (León et al. 2008; Finazzi et al. 2010; Cadoret et al. 2012). Indeed, some microalgal species accumulate important amounts of these compounds under specific environmental conditions, so this biotechnologically relevant phenotypic feature is amenable to optimization by genetic engineering approaches (León et al. 2008, Cadoret et al. 2012).

This review summarizes the contributions presented by three microalgal biotechnologists at the symposium *New Challenges in Microalgae Biotechnology* held during the VII European Congress of Protistology, which was organized for the first time as a joint meeting in partnership with the International Society of Protistologists (VII ECOP – ISOP Joint Meeting) in Seville, Spain, 5-10 September 2015. The use of microalgae as sustainable oil sources for biofuels will be evaluated and discussed in the first section, given that some of these phototrophic protists intrinsically accumulate high oil levels (up to more than 80% of the dry weight). Significant recent advances in the development of genetic manipulation tools, aimed to improve biotechnological features of microalgae as sources of renewable resources, are presented in the second contribution. Finally, the application of computational modeling as a systems biology strategy to better understand microalgal metabolic and cell signaling networks will doubtless contribute to discover novel

properties with relevant biotechnological implications, as is presented in the third contribution. In any case, it is clear that intense research and the application of genetic engineering are absolutely essential to reach the full potential of microalgae as cell factories and, therefore, will significantly contribute to solve the problems of biosustainability and energy shortage.

Biofuel from microalgae?

Microalgae are a polyphyletic group and a huge pool of biological diversity. Properties typical of higher plants are combined in microalgae with biotechnologically amenable attributes of microbial cells. These and other properties of microalgae (such as their metabolic plasticity, tolerance to extreme environmental conditions and amenability to genetic engineering), are valuable for bioindustry. These photosynthetic microorganisms are a source of compounds with commercial value, such as carotenoids, phycobiliproteins, polyunsaturated fatty acids, polysaccharides and an array of bioactive compounds for agriculture and food, feed, pharmaceutical, cosmetic, and chemical industries. Microalgae can also be of use in the recovery of wastewater and in abatement of carbon dioxide.

Microalgae have been proposed as an alternative source for renewable biofuel, capable of meeting the global demand for transport fuels. Although the “microalgae to biofuel” concept was first suggested in the 1940s, it has recently received new attraction and support. A seminal article by Yusuf Chisti published in 2007 (Chisti 2007) has been particularly effective in drawing the attention of researchers and investors. In this way, many research groups were attracted to the field, together with commercial ventures established thereafter. According to data available in Thomson Reuters’ Web of Science™, the number of published items per year on “biofuel from microalgae” has grown exponentially, from less than 5 before 2007 to over 390 in 2014.

Attention on microalgae for biotechnological reasons has also benefited from the fact that mass production of liquid biofuels from plant biomass is being increasingly questioned. The “food versus fuel” dilemma and the limitations in available fertile land for a world’s growing population are reasons to reconsider the biofuel production from crop plants (Searchinger et al. 2015). Microalgae represent an

alternative to land plants, since cultures could be developed in non-arable land, employing brackish, saline or even waste water, as well as carbon dioxide from flue gases as carbon source. Values for expected fuel productivity around 20,000 L per hectare and year seem reasonable for outdoor culture of microalgae (Moody et al. 2014), although some substantially higher projections are frequently argued in the literature. However, most of the projected values originate from gross extrapolations, both in area and time, from short-term trials in small size facilities, if not directly from laboratory experiments. Analogous considerations apply to published life cycle assessments and to production prices appraisals for either biomass or biofuel from microalgae. The escalation of these processes offers a very challenging subject for applied research.

Up to now, scarcity of scientific and technical knowledge, as well as limited practical experience, determines a high price for microalgal biomass and the biofuel thereof. The lowest production cost in commercial algae production seems to be about US$ 4–5 per kg algal biomass. Significant R&D efforts are currently being addressed to the development of viable processes able to massively generate microalgal biofuels at prices that can compete with those of established fuels (Sing et al. 2013). The production step has to be considerably improved, but also harvesting, biomass drying and extraction of biofuel precursor and its conversion into the final product still need substantial optimization.

Selection of the most appropriate microalgal strains is a key issue (Figure 1). Not just the content of the biofuel precursor (either fermentable sugars or fatty acids) should be considered, but rather the production capacity, looking for the optimal combination of product level and biomass productivity. The continuous culture approach is the most appropriate methodology for the screening of microalgae for the purpose of biofuel production, as it allows the determination of real productivity for a particular biofuel precursor (Del Río et al. 2015). Also crucial in the selection of the strain is the ability to develop outdoors as a monoalgal culture throughout the year. Many expectations are placed on the potential of genetic engineering for the generation of strains with superior productivity of either fatty acids or fermentable carbohydrates, but further development of novel techniques for efficient manipulation of microalgae is still needed.

Production of biofuels is largely policy-driven and its profitability has been questioned, even at oil prices above US$ 100/barrel (bbl). Current average price for crude oil is around US$ 50/bbl, and it is expected to increase up to about US$ 90/bbl by 2025 (World Bank Group 2015). Within this framework it

does not seem that conventional biofuels have an easy way to develop in the near future, worst still when considering those from microalgae. In such a scenario and in order to compete with oil at current prices, the production price for microalgal biomass containing 25% oil should be around US$ 0.1/kg. At such prices, protein-rich microalgal biomass would compete favorably with other protein sources, such as soybean (current price, US$ 0.4/kg).

The question therefore is, does it make sense to use microalgal biomass for fuel or rather as food/feed?

Development of new molecular tools for genetic engineering of eukaryotic microalgae

In the last years, there has been an increasing interest on the genetic engineering of microalgae, as a potential tool for economically feasible production of bulk materials and to enhance productivity of high-added compounds (Wijffels et al. 2010). However, routine genetic manipulation has been limited to a few species until recently. The lack of suitable promoters and other regulatory sequences are, besides low efficiency and instability of transgenes expression, the main difficulties preventing nuclear transformation of new microalgal strains (León et al. 2007). Since low expression of exogenous genes is hampering the efficient engineering of metabolic pathways and the use of microalgae as platforms for the production of recombinant proteins, it is necessary to develop new tools, which ensure stability and high expression levels of the transgenes.

Here, we propose a new method to express transgenes in microalgae: co-transformation with two naked promoter-less genes, a selectable antibiotic-resistant gene and the gene of our interest (Figure 2). These genes are randomly inserted into the nuclear genome so that their transcription relies on their adequate insertion in a region adjacent to an endogenous genomic promoter or in frame with a native gene. This approach is especially appropriate to transform microalgal species for which no endogenous promoters or specific expression plasmids have been designed. The fact that the transgenes are expressed under the control of endogenous promoters reduces the risk of silencing events and their integration into the genomic environment of the promoter guarantees the presence of enhancers, transcription factors or other regulatory

regions essential for the adequate expression of the transgene. A promoter-less co-transformation approach has been successfully used to express yeast flocculins in *Chlamydomonas reinhardtii* in order to obtain transgenic microalgal strains with higher self-flocculation ability than the control of untransformed ones (Díaz-Santos et al., 2015). Flocculation is very important from the engineering point of view if we consider that microalgal harvesting can make up 30% of the total cost of algal biomass production (Salim et al. 2012). However, cloning of flocculins has been limited by their toxicity to bacteria caused by their excessive length and large number of tandem repeats in their central domain. Promoter-less co-transformation avoids the need of cloning because large amounts of DNA from the desired gene can be directly obtained by amplification or by artificial synthesis and inserted in the genome of the host strain.

We have designed also a plasmid for the translational fusion of the gene of interest with a selectable antibiotic-resistant gene, where the protein of interest and the protein conferring resistance to the antibiotic, fused by a self-cleaving peptide (De Felipe et al. 2006), are processed from the same polyprotein. Screening transformants with increasing amounts of the selective antibiotic provides a simple method for selecting clones with the highest expression level of the selectable marker gene and, consequently, of the gene of interest; furthermore, maintaining the transformants under selective conditions improves the stability of the transgenes.

Although much work is still necessary, these new molecular tools will allow the improvement of transgene expression in microalgal nuclei and the genetic modification of new species of industrial interest.

A study on the basic helix-loop-helix transcription factor family in *Chlamydomonas reinhardtii* using the gene co-expression network ChlamyNET

The photosynthetic protist *Chlamydomonas reinhardtii* is the most important model organism for unicellular green algae (Harris, 2001; Hanikenne, 2003; Matsuo and Ishiura, 2011; Slaveykova et al., 2016). Recently, *Chlamydomonas* has attracted attention due to its potential biotechnological applications (Kruse and Hankamer 2010; Sivakumar et al. 2010). In order to characterize different *Chlamydomonas* strains and their response to different conditions, a massive amount of 'omics' data has been produced (Castruita et al. 2011;

Gonzalez-Ballester et al. 2010; Miller et al. 2010; Urzica et al. 2012). In order to integrate these data and generate systemic and global characterizations, a first approach based on molecular systems biology has been taken (Dal'Molin et al. 2011; Lopez et al. 2011; Romero-Campero et al. 2013; Zheng et al. 2014). In this study, we used ChlamyNET (Romero-Campero et al. 2015), a gene co-expression network that integrates RNA-seq data, to analyze the basic helix-loop-helix (bHLH) transcription factor (TF) family in *Chlamydomonas*. Specifically, using ChlamyNET, we have determined the biological processes potentially regulated by bHLH TFs and the DNA sequences recognized by them.

The bHLH transcription factor family of plants is characterized by the presence of a conserved protein domain consisting of two α helices connected by a loop and identified in the Protein family (Pfam) database with the id PF00010. Recent genomic analyses have identified eight bHLH TFs in the *Chlamydomonas* genome (Pérez-Rodríguez et al. 2010). Seven of these transcriptions factors exhibit significant gene co-expression patterns and can be identified in ChlamyNET using its search utility and the bHLH Pfam id PF00010, as shown in Figure 3A. These TFs constitute three different clusters (Figure 3B). The biggest cluster includes the bHLH genes Cre14.g620850, Cre01.g011150, g4643 and g4645 and it is located at the core of ChlamyNET. *bHLHs* are co-expressed with a significantly high number of genes, which makes them hub genes in the network playing key roles in the transcriptome robustness and information processing.

In order to determine the potential biological processes regulated by bHLH TFs in *Chlamydomonas*, we performed a Gene Ontology (GO) term enrichment analysis over the genes co-expressed with them using ChlamyNET. We identified transmembrane transport and carbohydrate metabolism as the two most significant biological processes potentially regulated by the bHLH TFs in *Chlamydomonas* (Figure 3C). For instance, the genes Cre02.g110800 and Cre13.g569850 that codify for nitrate and ammonium transporters respectively are highly co-expressed with the bHLH TF Cre01.g011150. This bHLH TF is also highly co-expressed with genes codifying for proteins involved in nitrogen metabolism such as the nitrite and nitrate reductases, Cre09.g410750 and Cre09.g410950, respectively. At the same time, the genes Cre08.g384750 and g3160 that codify for an alpha-amylase and an isoamylase respectively are highly co-expressed with the bHLH TF Cre14.g620850.

In order to determine DNA sequences potentially recognized by the bHLH TFs in *Chlamydomonas* we analyzed, using ChlamyNET, the promoter sequences of the genes co-expressed with them. This analysis revealed that the E-box sequence and one of its variant recognized by the PIF5 TF in *Arabidopsis thaliana* actually appeared in the promoters of many genes highly co-expressed with bHLH TFs (Figure 3D). These DNA sequences are present, for instance, in the promoters of the genes Cre02.g110800 and Cre13.g569850 that codify for nitrate and ammonium transporters respectively and in the promoter of the gene Cre08.g384750 that codify for an alpha-amylase.

Summarizing, the analysis using ChlamyNET suggests that the family of bHLH TFs plays a key role in the regulation of relevant biological processes in *Chlamydomonas* physiology such as transmembrane transport and nitrogen/carbon metabolism. This regulation seems to be exerted through recognition of E-boxes and similar DNA sequences located in the promoter of potential target genes. These *in silico* predictions should be taken as a hypothesis that needs *in vivo* and *in vitro* validation.

Prospects

In order to cope with the high expectations created in the microalgal biotechnological field, a number of challenges will need to be addressed in the near future. In this review we have shown a small sample of these solutions; other important aspects related to the genetic manipulation of algae and upscaling of the laboratory experimental biomass production trials to large, industrial installations, will be needed.

As new ecological niches are prospected and new massive big-data techniques are employed, the number of accounted photosynthetic protists is rising. Today we know some 50,000 microalgal species, but estimates suggest more than 500,000 species are spread over the suitable habitats on Earth (Cadoret et al. 2012). In order to raise the number of isolates and increase the chance of getting interesting new microalgal biotypes with novel enhanced characteristics for biofuel production or with rare metabolic trades, the research into phylogenomics will certainly grow in the following years. This area of study is bound to bring surprises and open up new fields of applied research, as the mists that cover the huge ecological importance of this heterogeneous group of protists will be unveiled.

The recent efforts on the massive sequencing of microalgal genomes have opened new possibilities of targeted manipulation and edition of biotechnologically interesting genetic traits. Cutting-edge genome editing techniques in green microalgae and diatoms such as meganucleases, TALE nucleases and CRISPR/Cas9 systems (Daboussi et al. 2013; Jinkerson and Jonicas 2015) will allow the optimization of specific characteristics, but will also implement relevant, novel applied traits into existing microalgal systems to fulfill unresolved industrial necessities.

To satisfy the great expectations created around microalgal biotechnology, important new investments are needed. A great effort will be required to implement the important questions posed above, but also to create a number of dedicated academic and industrial actors able to meet the emerging challenges of the microalgae-based industry and research of the future.

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FIGURE LEGENDS

Figure 1. *Pseudokirchneriella subcapitata* (*Raphidocelis subcapitata*; *Chlorophyta*, *Chlorophyceae*, *Sphaeropleales*, *Selenastraceae*) and *Chlorococcum olefaciens* (*Chlorophyta*, *Chlorophyceae*, *Chlamydomonadales*, *Chlorococcaceae*), two strains with a great potential as oil producing microalgae (Del Río et al. 2015). Microphotographs kindly supplied by Dr. Esperanza Del Río.

Figure 2. Co-transformation of the chlorophycean microalga *Chlamydomonas* with two naked promoter-less genes. The aminoglycoside 3`phosphotransferase encoding gene from *Streptomyces rimosus* (SrAPHVIII), which confers resistance to paromomycin, and the BLE gene, which encodes the bleomycin binding protein from *Streptoalloteichus hindustanus* (ShBLE) and confers resistance to the antibiotic bleomycin, have been chosen as selectable gene and gene of interest, respectively, to check the efficiency of this co-transformation approach. Interestingly, in a high percentage of the obtained transformants, both genes are not only adequately incorporated in the nuclear genome, but also efficiently transcribed and translated.

Figure 3. (A) Localization of the bHLH TFs in the gene co-expression network ChlamyNET. Note that Cre14.g620850, Cre01.g011150, g4645 and g4643 are gene hubs located at the core of the network suggesting important roles of these genes in the regulation of *Chlamydomonas* transcriptome. (B) Heatmap representing the co-expression level between bHLH TFs. Red colour represents high co-expression whereas blue represents low co-expression. Observe that bHLH TFs are organized into three different clusters. (C) Gene Ontology term enrichment over the genes co-expressed with bHLH TFs. Transmembrane transport and carbohydrate metabolism are the two most likely biological processes regulated by bHLH TFs. (D) Identification of significant DNA sequences in the promoters of genes co-expressed with bHLH TFs. The E-box and one of its variant recognized in *Arabidopsis* by the protein PIF5 are likely to constitute transcription factor binding sites for bHLH in *Chlamydomonas*.

Figure 1

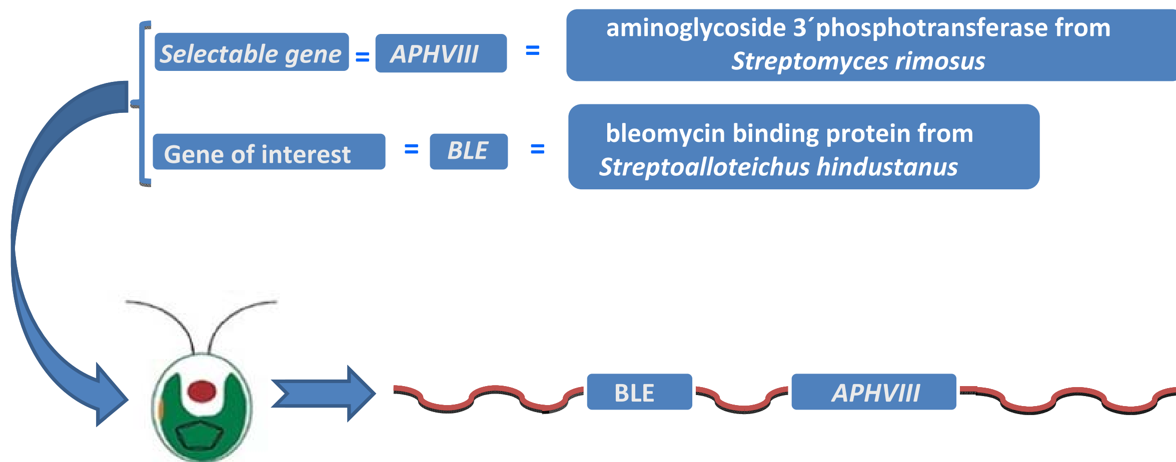

Figure 2

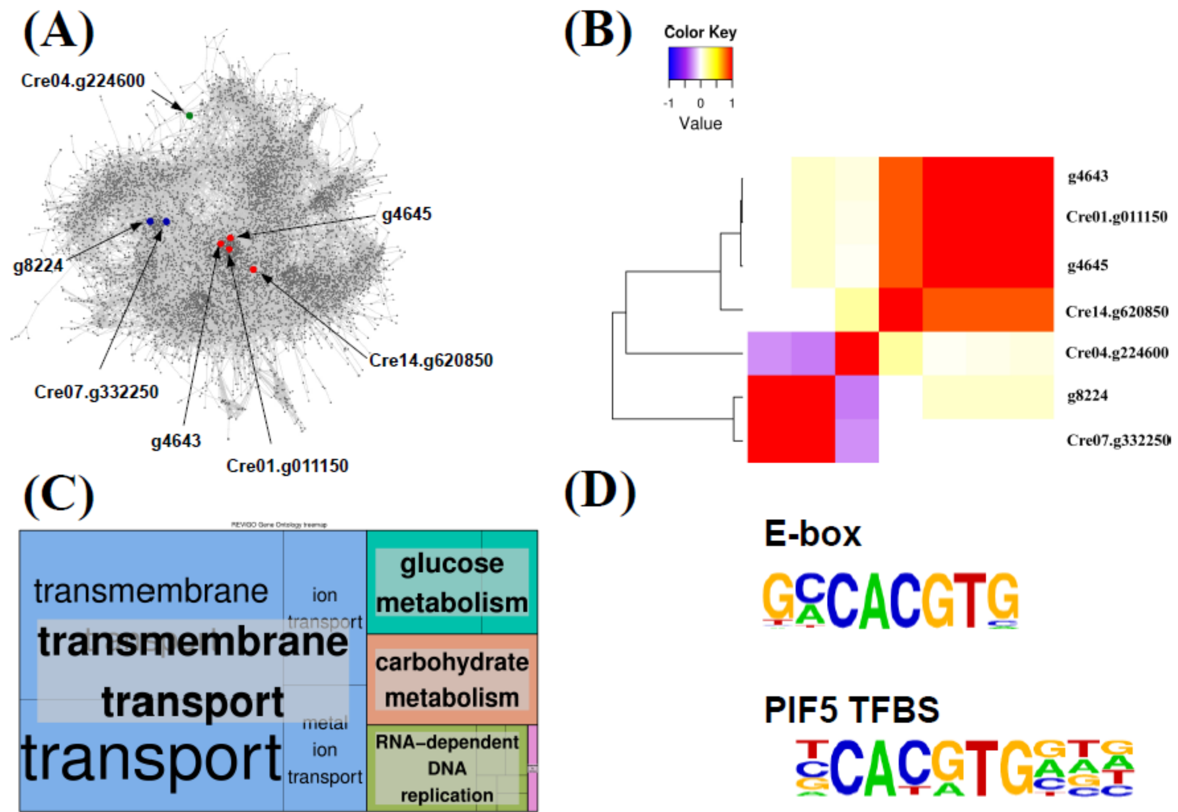

Figure 3