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Advances in editing microalgae genomes

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With 2 figures

Abstract: There have been significant advances in microalgal genomics over the last decade. Nevertheless, there are still insufficient tools for the manipulation of microalgae genomes and the development of microalgae as industrial biofactories. Several research groups have recently contributed to progress by demonstrating that particular nucleases can be used for targeted and stable modifications of the genomes of some microalgae species. The nucleases include Meganucleases, Zinc Finger nucleases, TALE nucleases and, as shown most recently, the CRISPR/Cas9 system. These molecular editing tools allow gene knock-out and gene knock-in manipulations and the introduction of enzyme variants; they will facilitate the study of microalgae and in particular gene function and, thereby favoring exploitation of the metabolic potential of microalgae.

Keywords: Microalgae, biotechnology, targeted genome modifications, engineered nucleases, TALENs, CRISPR/CAS9 system

Microalgae, the rough diamond of biotechnology

Microalgae have enormous potential as cell factories: like plants properties, they can produce complex molecules using CO₂ as a substrate; like microorganisms, they grow rapidly; and they produce a wide variety of bioproducts (proteins, carbohydrates, lipids and pigments). Consequently, they are the rough diamonds of biotechnology (Barra et al. 2014). Microalgae also have potential as expression systems for medically useful proteins and as edible vaccines (Specht et al. 2010). Against the background of the exhaustion of fossil fuels and efforts to decrease greenhouse gas emissions, microalgae have emerged as promising biocatalysts for the sustainable production of chemicals and biofuels, because they can accumulate and store energy-rich compounds, such as triacylglycerol and carbohydrates (Alonso et al. 2000). Nevertheless, a number of barriers need to be overcome before profitable commercialisation of microalgae in the chemical and biofuel markets.

Traditional techniques to improve performance and yield

For several years, there have been efforts to develop the metabolic potential of microalgae. Various techniques are available for enhancing cell growth and/or the accumulation of bioproducts. Oceanographic campaigns have been conducted to

explore biodiversity and discover new species with particular properties. In parallel, there has been work to optimise cultivation conditions to enrich microalgae in specific compounds. Nutrient deprivation has been successfully used to boost lipid content, although generally at the expense of biomass productivity (Hu et al. 2008, Breuer et al. 2012). Physical and chemical mutagens have been used to modify the genetic heritage, and efficient, rapid and robust high-throughput screening- methodologies have been employed with the aims of circumventing dependence on stress and to decrease cultivation-related costs (Huesemann et al. 2009, Beacham et al. 2015). This strategy is attractive, but the control of genome modifications is still limited impeding research into microalgae metabolism, a prerequisite for metabolic engineering.

From classical genetic tools to targeted genome editing

The development of new and affordable methods for whole-genome sequencing, and the availability of techniques allowing transformation of more than 50 microalgae species facilitate analysis of gene function. Techniques now used are based on random insertional mutagenesis followed by phenotypic selection (Galván et al. 2007) or on modulation of gene expression by overexpression or silencing targeted genes using RNA interference (Schroda et al. 1999, Schroda 2006, Doron et al. 2016) or microRNA (Molnár et al. 2007, Zhao et al. 2009, Cerutti et al. 2011, Doron et al. 2016).

These methodologies have provided knowledge about the metabolism and physiology of microalgae (Cerutti et al. 2011, Doron et al. 2016). They are nevertheless limited for the creation of industrially useful strains: RNA interference often only incompletely repressed gene expression; the long-term stability of the epigenetic repression is uncertain; and the deletion or introduction of mutations in relevant target genes is unlikely with random processes.

In contrast, the targeted insertion of a DNA sequence via homologous recombination (HR) circumvents these limitations to a large extent. This process is very efficient and common in cyanobacteria and the chloroplast genome of some microalgae (Matsuoka et al. 2001, Marín-Navarro et al. 2007), but is much less effective for modifying the nuclear genome in microalgae. Targeted nuclear genome modification based on HR is currently restricted to three microalgae species: *Cyanidioschizon merolae* (Minoda et al. 2004), *Nannochloropsis* sp. (Kilian et al. 2011) and *Ostreococcus tauri* (Lozano et al. 2014). For all other species, including the model species *P. tricornutum* and *C. reinhardtii*, the efficiency of HR is extremely low ($< 10^{-6}$).

As described for plants (Puchta et al. 1993), yeast (Plessis et al. 1992, Choulika et al. 1995) and mammalian

cells (Rouet et al. 1994), the introduction of a double-strand break (DSB), by the homing endonuclease I-SceI, significantly increases HR-frequencies 1000-fold. Consequently, enzymes able to recognize and introduce a DSB in a site-specific sequence have been designed. There are two main mechanisms for repairing DSB (Fig. 1): non-homologous end joining (NHEJ), in which the broken chromosomes are re-joined more or less accurately, often with small deletions and insertions (Takata et al. 1998); and homologous recombination (HR), in which DNA templates bearing sequence similarity to the break site are used resulting in sequence changes to the target locus. NHEJ seems to be the main DSB repair mechanism in plants, mammalian cells and probably in microalgae (with the exception of *Cyanidioschizon merolae*, *Nannochloropsis* sp and *Ostreococcus tauri*). Very little is known about these processes in microalgae due to the difficulties of assigning a function to a gene. Most current knowledge is from the analysis of a few *C. reinhardtii* DSB-repair mutants obtained by physical and/or chemical mutagenesis (Vlcek et al. 2008, Plecenikova et al. 2014) and not even the key genes have been identified. Even so, a race is underway to develop molecular scissors able to modify the genome of microalgae species.

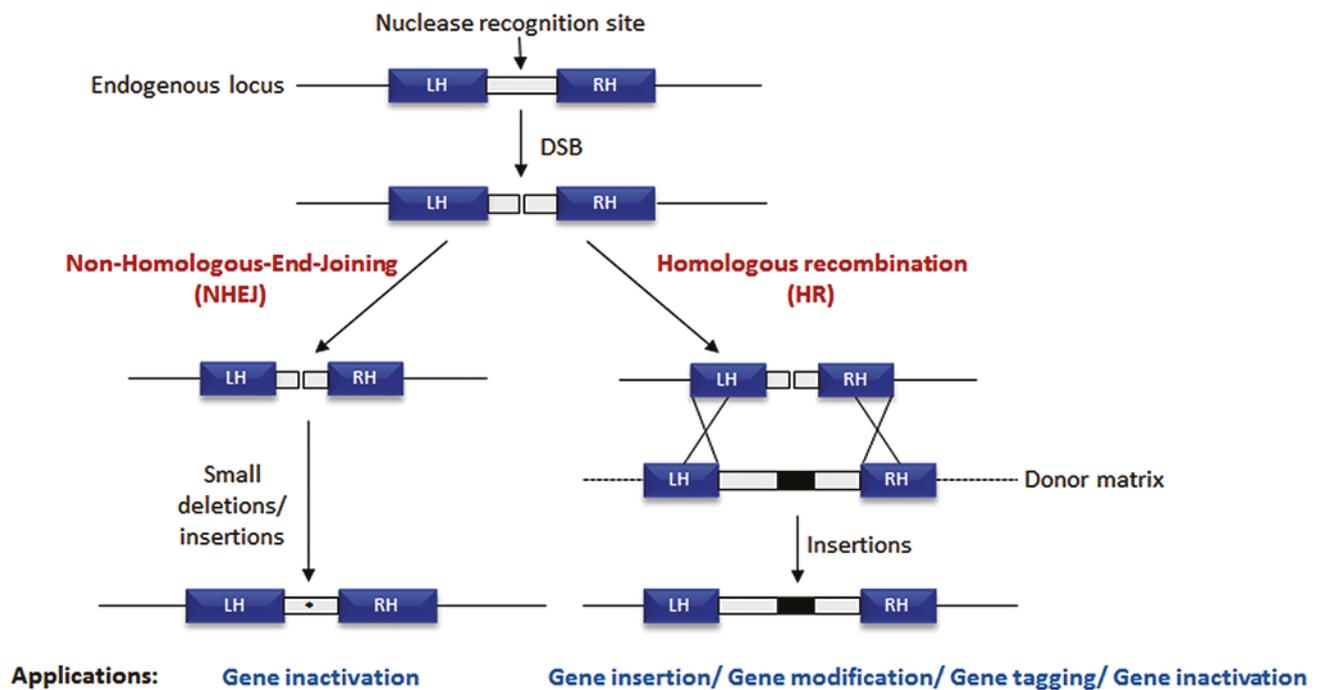


Fig. 1. Overview of in-vivo double-strand break repair mechanisms by non-homologous-end-joining (NHEJ) and homologous recombination (HR) and how they can be exploited to generate genetic modifications; the result in these examples is disruption of the nuclease target site and repair either by targeted mutagenesis (*) or targeted gene insertion (black rectangle). For targeted gene insertion, the donor matrix consists of two homologous regions (LH, left homology; RH, right homology) flanking a sequence to be inserted. This process enables the integrated DNA to (i) express one or several genes, (ii) inactivate specific genes, (iii) produce a protein marker (i.e. fluorescent protein) fused to a protein of interest (gene tagging) and (iv) replace a gene in a targeted manner or introduce single-nucleotide substitutions (for gene correction or to generate enzyme variants).

Designed nucleases, powerful tools to study gene function and to generate novel crops

Custom molecular scissors would allow modification of the genome in a site-specific manner, and thus facilitate basic and applied research. Using such tools, it would be possible to knock out and knock in targeted genes for gene inactivation, gene insertion, and gene replacement or to introduce specific mutations into genes. Gene function could then be studied, and foreign genes introduced into cells (Fig. 1). Here, we will focus on the four classes of sequence-specific nucleases (MNs, ZFNs, TALENs, CRISPR/Cas9) (Fig. 2) successfully used to modify the genome of microalgae species. All of them were derived from natural molecules, whose functions have been adapted to create powerful tools for genome engineering.

Meganucleases are designed sequence-specific endonucleases that recognise and cleave long DNA sequences (18–30 base pairs) generating DSBs (Silva et al. 2011). They are derived from homing endonucleases, which are involved in the lateral transfer of introns or inteins (Bell-Pedersen et al. 1990, Jacquier & Dujon 1985, Kostriken et al. 1983). Unlike other classes of nucleases, the meganuclease template con-

tains both the recognition and cleavage functions in the same scaffold. By introducing a small number of variations into the amino acid sequence in the DNA-binding domain of those proteins and then selecting functional proteins with variations of the natural recognition site, it is possible to generate customised meganucleases with a great range of target sites (Chevalier et al. 2002, Epinat et al. 2003, Silva et al. 2006, Daboussi et al. 2015). Further scaffold optimisations have improved nuclease activity and specificity (Smith et al. 2006, Grizot et al. 2009, Daboussi et al. 2012, Delacôte et al. 2013, Boissel et al. 2014). The first proof of concept of targeted genome editing in microalgae was by my group in the Collectis Company (Duchateau et al. 2013, Daboussi et al. 2014, Duchateau et al. 2014). High frequencies of both targeted mutagenesis and targeted gene insertion have been achieved in diatoms using two engineered meganucleases. However, the production of such molecular scissors is too time-consuming and not sufficiently flexible compared to TALEN and CRISPR/Cas9 systems, to envision a significant role in microalgae genome engineering.

Zinc Finger Nucleases are chimeric proteins composed of zinc finger DNA-binding domains fused to the non-specific type II restriction enzyme FokI. Each DNA-binding domain

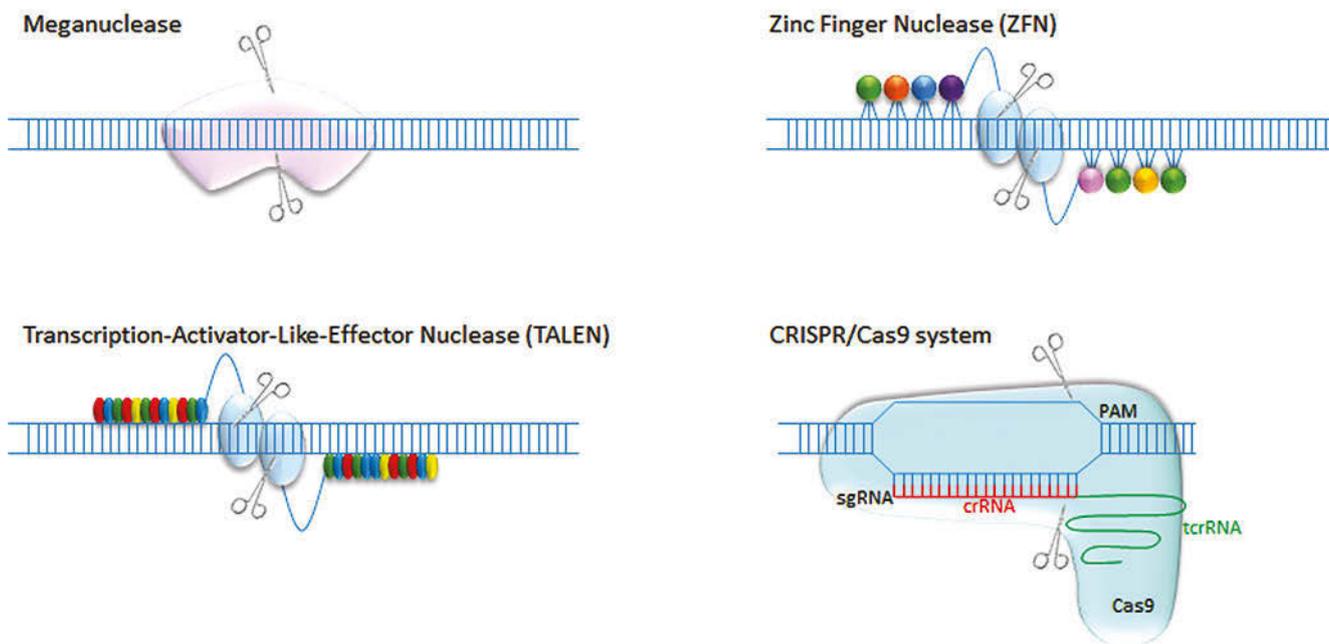


Fig. 2. Molecular scissors for causing double-strand break (a) Meganucleases derived from homing endonucleases such as dimeric meganuclease I-CreI recognise a 21-nucleotide binding site. The DNA-binding domain of I-CreI was modified to recognize specific target sequences, (b) Zinc finger nuclease composed of a DNA-binding domain of three to four zinc fingers, each zinc finger recognizing three nucleotides, resulting in recognition of a 9 to 12-nucleotide-long DNA sequence. Each DNA-binding domain is fused to the FokI nuclease domain. A heterodimeric enzyme. (c) Transcription activator-like effector nucleases (TALENs): the DNA-binding domain consists of 14–24 module repeats of 33 to 35 amino acids whose two polymorphic amino acids are responsible for the specific recognition of one nucleotide. (d) RNA-guided endonuclease CRISPR/Cas9, where a chimeric single-guide RNA (sgRNA) corresponding to 20 nucleotides of a protospacer-adjacent motif (PAM: NGGs responsible in the case of *S. pyogenes* Cas9) is responsible for the specificity of the Cas9 nuclease, containing two RuvC and HNH nuclease domains.

is composed of three to four binding arrays from transcription factors (each one recognising a three nucleotide DNA sequence). The DNA-binding domain of each monomer of the ZFN enzyme therefore recognises a 9–12 nucleotide sequence in genomic sequence. The FokI cleavage domain functions enzymatically as a dimer, thus two DNA binding domains are required to form a dimer for activity (Kim et al. 1996, Smith et al. 2000). Using ZFN, Sizova and collaborators modified the sequence of the *COP3* gene (encoding the light-activated ion channel channelrhodopsin-1) in *C. reinhardtii* via both targeted NHEJ-induced-mutagenesis and targeted mutagenesis induced by homologous recombination (Sizova et al. 2013). Despite efforts at optimisation, ZFN are still labour-intensive and lack specificity (Gabriel et al. 2011), leading scientists to favour the new methodologies described below.

TALENs have architecture similar to ZFNs because the DNA-binding domain is also fused to the non-specific catalytic head FokI (Christian et al. 2010). The DNA-binding domain of TALENs is derived from TALE (Transcriptional Activator-Like Effectors), proteins produced by a plant bacterial pathogen belonging to the genus *Xanthomonas*. These proteins activate the transcription of a specific plant gene to promote bacterial infection (Bogdanove et al. 2010). The DNA-binding domain comprises an array of typically 14–24 repeat units of approximately 33 to 35 amino acids, nearly identical to one another, except for two polymorphic amino acids called RVDs (repeat variable di-residue) located at positions 12 and 13, responsible for the specific recognition of a particular nucleotide. There is preferential pairing between A, C, G, T nucleotides and the repeat modules harbouring respectively NI, HD, NN, and NG RVDs (Boch et al. 2009, Moscou & Bogdanove 2009). Based on this specificity, it is straightforward to generate engineered TALE DNA-binding domains (like LEGO® blocks), and fuse them to nuclease (N) domains. The TALEN-mediated genome modification has been reported for the diatom *Phaeodactylum tricorutum* for both studying gene function (Weyman et al. 2014, Fortunato et al. 2016) and generating crops with industrial potential (Duchateau et al. 2013, Duchateau et al. 2014, Daboussi et al. 2014). The proof of concept of TALEN-induced genetic modification in microalgae was established in 2013 by demonstrating a high frequency of targeted mutagenesis resulting from targeting seven different endogenous loci. The potential of these molecular editing tools for metabolic engineering was illustrated by the creation of a high-lipid-producing strain by inactivating of a gene key for energy storage in the form of sugars (Daboussi et al. 2014). Several research groups have exploited this TALEN-genome editing strategy for studying gene function, in experiments involving the inactivation of the red/far-red light-sensing *phytochrome* (*DPH*) via NHEJ (Fortunato et al. 2016), and the disruption of the urease via HR (Weyman et al. 2014).

The CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) **system is based on** an RNA-guided DNA cleavage defence system, present in bacteria and archaea. This system confers an “acquired immunity” against bacteriophages by storing the foreign DNA in their genome memory at the CRISPR locus. The molecular mechanism governing the accurate positioning of the double-strand breaks in DNA in the natural CRISPR/Cas9 system has been described (Deltcheva et al. 2011), and then engineered to produce targeted DSBs at specific targeted sites (Jinek et al. 2012). The CRISPR/Cas9 system has been developed into a simple toolkit based on a custom single guide RNA (sgRNA) that contains a targeting sequence (crRNA sequence) and a cas9 nuclease-recruiting sequence (tracrRNA). The crRNA region includes nucleotides homologous to the genomic target to be modified. The binding specificity is based on the gRNA and a 3-nucleotide sequence downstream called the protospacer adjacent motif (PAM; NGG, in the case of *S. pyogenes* Cas9). The Cas9 nuclease carries two nuclease domains (HNH and RvuC) and cleaves both DNA strands generating DSBs at sites defined by the 20-nucleotide guide sequence. **There are three main reasons why CRISPR/Cas9 has contributed to making genome engineering straightforward:** first, it is simple, due to the complementarity of the RNA guide with the target DNA; second, the simultaneous use of several RNA guides, each targeting a different sequence, allows multiple genetic modifications simultaneously; and third, it is cheap, and a few dozen Euros are sufficient to obtain scissors that can be used by scientists around the world for genome editing (see for a review Hsu et al. 2014).

The first demonstration of targeted genome modification by CRISPR/Cas9 technology in microalgae was in *C. reinhardtii* but cellular toxicity hampered the generation of genome-modified strains (Jiang et al. 2014). More than two years were required to develop efficient Cas9 strategies that did not affect cell growth, in first *P. tricorutum* by inactivating the *CpSRP54* gene encoding the chloroplast signal recognition particle 54 (Nymark et al. 2016) and then in *C. reinhardtii*. Both targeted mutagenesis and targeted homologous recombination induced by the CRISPR/Cas9 system have been obtained in *C. reinhardtii* by targeting three genes: *MAA7* that encodes the beta subunit of tryptophan synthase (TSB); the antennal assembly gene *CpSRP43* that encodes chloroplast SRP43; and the chlorophyll biosynthetic gene *ChlM* that encodes Mg-protoporphyrin IX S-adenosyl methionine O-methyl transferase. A novel strategy was used to avoid cytotoxicity: Cas9 ribonucleoproteins were provided and the Cas9 protein and sgRNAs were combined (Baek et al. 2016, Shin et al. 2016). This technology will undoubtedly be adapted to other microalgae species as indicated by recent work with the diatom *Thalassiosira pseudonana* (Hopes et al. 2016) and the industrial oleaginous microalga *Nannochloropsis oceanica* (Wang et al. 2016).

Conclusion and perspectives

Genome engineering can be considered to have been subject to a revolution rather than having seen significant progress. Genome editing tools now available allow efficient and specific modification of the genome of microalgae species, facilitating diverse investigations. This is just the beginning and scientists are racing to develop new genome editing tools and adapt them to other microalgae species. The specificity of CRISPR/Cas9 system has been widely debated over the past year. Indeed, mismatches are tolerated in the 5'-end region of sgRNA, thereby reducing Cas9 specificity to less than 15 bp, which is much lower than that of meganucleases, ZFNs and TALENs. Although there has been extensive work on this issue in bacteria, mammals and plants, the extent of off-site activity is still not completely clear. To overcome this potential drawback, software tools have been created to identify potential off-target sites *in silico* and select the most specific target sites. In addition, new engineerable scaffolds have been developed to improve specificity (Ran et al. 2013, Guilinger et al. 2014, Tsai et al. 2014). Progress made in improving the specificity of these designed nuclease-based technologies and in optimising these tools should help with modifying the microalgae genome more predictably.

To create new tools for the control of gene expression, the TALE DNA-targeting domain and the catalytically inactive Cas9 have been fused to a variety of other functional domains. Several functional fusions have been reported with activator, repressor, DNA and histone methylase domains. These fusions can form hybrid proteins that function as site-specific transcription activators, transcription repressors or epigenetic modifiers (Gao, X. et al. 2014). The adaptation of these promising approaches to microalgae (as already done by Gao, H. et al. 2014 in *C. reinhardtii*) will provide powerful tools. This will in turn allow a better understanding of gene expression and both the manipulation of endogenous regulatory networks and creation synthetic gene circuits, which is the basis of synthetic biology.

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