Random mutagenesis and precise gene editing technologies: applications in algal crop improvement and functional genomics

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ABSTRACT

The establishment of a system for gene modification is crucial for the generation of new improved algal strains and elucidation of functional genome organization to enhance our understanding of algal biology. Several gene transfer methods have been developed for stable introduction of transgenes into algae allowing expression of desired foreign proteins. Site-specific gene integration and gene knockdown were achieved through homologous recombination and RNA interference approaches. The nuclease-associated gene editing technologies such as CRISPR-associated RNA-guided endonuclease Cas9 **(**CRISPR-Cas9**)** could efficiently generate stable targeted gene editing in algae. Although gene modification technologies have been established for algae, there are still practical difficulties that need to be addressed prior to commercialization such as transgene stability, potential risks and public acceptance. Genetic mitigation and containment strategies should be considered for commercial-scale production of transgenic algae.

KEYWORDS CRISPR-Cas9; gene knockdown; genetic transformation; insertional mutagenesis; reverse genetics; transgenic algae

Abbreviations

ALE, adaptive laboratory evolution (ALE); AmiRNA, artificial microRNA; BER, base excision repair; CaMV35S, Cauliflower Mosaic Virus 35S; Cas9: CRISPR-associated protein 9; Cas9n: Cas9 mutant nickase; CRISPR: clustered, regularly interspaced, short palindromic repeats; crRNA: CRISPR RNA; dCAS9: nuclease-deficient Cas9, dead Cas9 or inactive Cas9; DSB: double-stranded break; GM: genetically modified; HDR: homology-directed repair; HR: homologous recombination; miRNA: microRNA; mRNA: messenger RNA; NHEJ: non-homologous end joining; NiR: nitrite reductase; NR: nitrate reductase; PAM: Protospacer-Adjacent Motif; RNAi: RNA interference; sgRNA: single guide RNA; siRNA: small interfering RNA; sRNA: small RNA; SV40: Simian vacuolating virus 40 or Simian virus 40; TALEN: transcription-activator like effector nuclease; tracrRNA: transactivating CRISPR RNA; UTR: Untranslated region; ZFN: zinc-finger nuclease

Introduction

Improved varieties of many crops have been generated through conventional plant breeding methods (Batchvarov, 1993; Chiang *et al*., 1993; Crisp & Tapsell, 1993; Breseghello & Coelho, 2013). For algae, new varieties have been obtained through several generations of inbreeding, strain selection and cross breeding of different male and female gametophytes (Wu & Lin, 1987; Patwary & van der Meer, 1992; Chepurnov *et al*., 2011). Other non-recombinant approaches utilized spontaneous or induced variation in cultured algal cells, tissues or callus as well as intra- and inter-specific protoplast fusion (Scowcroff & Larkin, 1988; Fujita & Saito, 1990; Mizukami *et al.*, 1995; Kito *et al*., 1998; Meneses & Santelices, 1999; Gupta *et al*., 2015; Abomohra *et al*., 2016). Charrier *et al.* (2015) outline the technological developments of algal culture systems over the past three decades that have been designed to generate morphological or genetic variants through somatic embryogenesis, somatic hybridization, intrageneric hybridization, intergeneric hybridization, transdivisional hybridization and parthenogenesis.

Alternatively, adaptive laboratory evolution (ALE) can be utilized to select mutations over multiple generations in strains that are adapting to defined conditions or stresses (Dragosits & Mattanovich, 2013). As mutations arise, those beneficial to performance under the selection pressure are fixed over time in the population. In ALE, microorganisms are exposed to defined perturbed conditions for a prolonged period in batch cultures or chemostats to allow selection of strains with improved phenotypes (Tenaillon *et al*., 2012; Dragosits & Mattanovich, 2013). For example, after exposure to continuous light for 1,880 generations, acetate-associated fast growing

Chlamydomonas reinhardtii cells were obtained with up-regulated growth-related genes and down-regulated genes for DNA repair and photosynthesis pathways (Perrineau *et al*., 2014). Apart from allowing studies on the underlying genetic factors involved in stress adaptation, the ALE approach has been applied in algae to enhance carotenoid and lipid biosynthesis (Fu *et al*., 2013; Yu *et al*., 2013; Velmurugan *et al*., 2014; Yi *et al*., 2015) as well as to obtain stress-tolerant algal strains (Tillich *et al*., 2014; Wang *et al*., 2014; Li *et al*., 2015; Uchiyama *et al*., 2015). In addition, stable new variants with improved traits have been obtained through random mutagenesis caused by either a physical mutagen such as UV light and gamma ray irradiation which induced a high frequency of mutations (Sandesh Kamath *et al*., 2008; Choi *et al*., 2014; Liu *et al*., 2015; Sharma *et al*., 2015) or a chemical mutagen such as N-methyl-N'-nitro-nitrosoguanidine (MNNG), N′-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS) (Sandesh Kamath *et al*., 2008; Cordero *et al*., 2011; Harper & Lee, 2012; Lee *et al*., 2014, Liang *et al*., 2016). Various mutagen-mediated algal mutations including natural mutations were discussed by Charrier *et al*. (2015).

The introduction of desired foreign genes into algae was made possible with the development of recombinant DNA technology. For example, increased levels of carotenoids were obtained in genetically engineered *Chlamydomonas reinhardtii* expressing either the β-carotene ketolase gene isolated from *Haematococcus pluvialis* or zeaxanthin epoxidase from *Chlorella zofingiensis* (León *et al*., 2007; Couso *et al*., 2012). Besides allowing selective gene improvement, recent advances in genome editing technologies are driving studies on functional genomics (Kilian *et al*., 2011; Deng *et al*., 2013; Levitan *et al*., 2014; Lozano *et al*., 2014) and enabling custom genome design of biological systems for specific applications such as

biofuel production (Radakovits *et al*., 2011; Georgianna & Mayfield, 2012; Ortiz-Marquez *et al*., 2013) and drug development (Dauvillée *et al.*, 2010; Dreesen *et al*., 2010; Rasala *et al.*, 2010; Patra *et al*., 2015). This review describes applications of various gene editing approaches in algal biotechnology with an emphasis on the clustered, regularly interspaced, short palindromic repeat (CRISPR) technology while highlighting challenges and future directions for algal biotechnology.

Conventional gene manipulation methods applied in algal research

Conventional gene modification methods have been used to achieve transient and stable transformation in algal nuclear genomes and chloroplasts. A variety of methods have been successful at introducing transgenes into algal cells (Table 1). Among them, particle bombardment is a common DNA introductory method with reported stable transformation in algae (Jiang *et al*., 2003; Jakobiak *et al*., 2004; Lerche & Hallmann, 2009; Li *et al*., 2009; Wang *et al*., 2010*a*; Kong *et al*., 2017). Alternatively, stable transformation can be achieved via electroporation (Brown *et al.*, 1991; Geng *et al*., 2003, Guo *et al*., 2013), *Agrobacterium* mediation (Cheney *et al*., 2001; Kathiresan *et al.*, 2009; Anila *et al*., 2011; Cheng *et al*., 2012; Pratheesh *et al*., 2014) and glass bead agitation (Wang *et al*., 2010b; Economou *et al*., 2014). Recently, gene introductory methods such as bombardment and electroporation have been applied to deliver CRISPR associated protein 9 (Cas9) gene/protein and single guide RNA (sgRNA) into algae for precise genome modification (Jiang *et al*., 2014; Hopes *et al*., 2016; Nymark *et al*., 2016; Shin *et al*., 2016; Wang *et al*., 2016).

In addition to a highly efficient DNA delivery method for stable transgene integration, the development of an efficient transformation system also involves the construction of an expression cassette (part of a transformation vector which consists of promoter sequence, open reading frame and untranslated region) for high transgene expression and selection of transformants. Although exogenous promoters such as CaMV35S (Brown *et al*., 1991; Cheney *et al.*, 2001; Kathiresan *et al*., 2009), SV40 (Gan *et al*., 2003; Wang *et al*., 2010*a*; 2010*b*) and ubiquitin-Ω (Chen *et al*., 2001; Geng *et al*., 2003) have been incorporated into expression cassettes to drive transgene expression in transgenic algae, higher levels of expression were reported with the use of endogenous promoters (Fukuda *et al.*, 2008; Li *et al*., 2009). Moreover, construct design strategies such as codon optimization, inclusion of specific untranslated regions (UTR) and endogenous intron-enhanced transgene expression are important for achieving higher transformation efficiencies (Lumbreras *et al.*, 1998; Nickelsen, 1999; Jakobiak *et al.*, 2004; Fukuda *et al*., 2008).

Reporter genes such as β-galactosidase (lacZ), β-glucuronidase (GUS), green fluorescent protein (GFP) and luciferase (Luc) are normally included in the vector construct for selection of transformed algae (Falciatore *et al.*, 1999; Gan *et al*., 2003; Jiang *et al*., 2003; Tolonen *et al.*, 2006; Kathiresan *et al.*, 2009; Cheng *et al*., 2012; Guo *et al.*, 2013). Alternatively, selectable marker genes conferring resistance to the antibiotics chloramphenicol, G418, hydromycin, kanamycin, paromomycin, phleomycin and spectinomycin or the herbicides sulfometuron methyl, Basta and Norflurazon can be used (Bateman & Purton, 2000; Kim *et al*., 2002; Lapidot *et al.*, 2002; Mayfield *et al*., 2003; Jakobiak *et al*., 2004; Jiang *et al*., 2005; Steinbrenner & Sandmann, 2006; Kathiresan *et al*., 2009; Niu *et al*., 2011; Sizova *et al*., 2011; Takahashi *et al*., 2011;

Cheng *et al*., 2012; Guo *et al*., 2013; Feng *et al*., 2014). Khawaja *et al.* (2016) provided an overview of the various types of reporter genes and selectable markers used for algal gene manipulation while Day & Goldschmidt-Clermont (2011) focussed on selectable markers applied to chloroplast transformation and discussed marker excision with the use of direct-repeat-mediated excision via homologous recombination (HR) and Cre site-specific recombinases. To date, there have been several reviews on the advances of genetic manipulation in macroalgae (Qin *et al*., 2005; Lin & Qin, 2014; Mikami, 2014) and microalgae (Jinkerson & Jonikas, 2015; Doron *et al*., 2016; Scaife & Smith, 2016).

Although conventional gene modification methods mostly result in random insertional mutagenesis, targeted gene knockout and gene replacement can also be achieved using HR approach. Disruption of the nitrate reductase gene was achieved through the HR approach in *Volvox* and *Chlamydomonas* cells (Sodeinde & Kindle, 1993; Hallmann *et al*., 1997). In 2011, Kilian *et al*. reported the design of gene knockdown constructs (KO) which consisted of a NT7 selection marker cassette (with zeocin resistance gene) flanked on both sides by ~1 kb regions to target the nitrate reductase (NR) and nitrite reductase (NiR) genes. These constructs were introduced into wild type *Nannochloropsis* cells using electroporation to achieve successful knockdown of NR and NiR genes. It is important to note that the effectiveness of the HR approach is affected by the variability of HR frequencies in different species. Due to low HR frequencies, large numbers of transformants and highly selective screening procedures are required for detection of the events (Sodeinde & Kindle, 1993; Hallmann *et al*., 1997). In *Chlamydomonas*, the frequency of homologous-to-random integration events achieved via bombardment and glass bead methods were 1:24 and 1:1000, respectively (Sodeinde & Kindle, 1993). Although the HR approach is well-established

in microalgae, its application to macroalgae is limited to the report on plastid transformation of *Pyropia yezoensis* utilizing the 5′ and 3′ UTRs of *psb*A from *P. yezoensis* (Kong *et al*., 2017).

Another reverse genetics approach for analysing gene function utilizes the RNA interference (RNAi) pathways which target messenger RNA (mRNA) molecules based on sequence complementarity (Fire *et al.*, 1998; Ketting, 2011). An RNAi approach via constructs containing either anti-sense or inverted repeat sequences of target genes successfully reduced levels of target mRNA in algae indicating possible transcriptional repression and/or RNA degradation (Schroda *et al*., 1999; Ishikawa *et al*., 2008; De Riso *et al*., 2009; Jia *et al*., 2009; Sakaguchi *et al*., 2011; Wei *et al*., 2017). In addition to these silencing mechanisms, translational repression was also observed in *Phaeodactylum tricornutum* resulting in a reduction of cognate protein with unaltered transcript levels (De Riso *et al*., 2009). Moreover, inducible RNAi in *C. reinhardtii* under ammonium starvation was achieved with the *NIT1* promoter driving the expression of an inverted repeat corresponding to the target gene (Koblenz & Lechtreck, 2005). Obstacles limiting the application of inverted repeat constructs in algae include unstable or inconsistent levels of gene silencing (Yamasaki *et al.*, 2008) and off-target effects (Kulkarni *et al*., 2006; Xu *et al*., 2006). Strategies have been developed to reduce off-target effects, for example, the use of artificial microRNA (amiRNA) molecules which are more stable than double-stranded RNA (dsRNA), with reduced off-target effects (Eamens *et al*., 2014; Tiwari *et al*., 2014). Endogenous small RNA (sRNA) molecules such as microRNA (miRNA) and small interfering RNA (siRNA) play important roles in gene regulation by inducing gene silencing via mRNA degradation, translation inhibition and/or transcriptional repression (Carthew &

Sontheimer, 2009; Ghildiyal & Zamore, 2009; Castel & Martienssen, 2013). These noncoding sRNA molecules have been reported in the algae *Chlamydomonas* (Molnár *et al*., 2007; Zhao *et al*., 2007), *Pyropia yezoensis* (Liang *et al*., 2010), *Phaeodactylum tricornutum* (Huang *et al*., 2011) and *Ectocarpus siliculosus* (Cock *et al*., 2010). Use of the amiRNA strategy for specific gene targeting was reported for *Chlamydomonas* (Molnár *et al*., 2009; Zhao *et al*., 2009) with inducible silencing via the use of the *NIT1* promoter (Schmollinger *et al.*, 2010).

CRISPR technology for sequence-specific genome editing

The utilization of nucleases such as zinc-finger (ZFN) and transcription activator-like effector (TALEN) for targeted genome editing in living cells has moved gene engineering research up to a higher level and established a powerful tool for functional genomics and gene therapy (Pabo *et al.*, 2001; Joung & Sander, 2013). The ZFN architecture consists of a fusion between the non-specific nuclease domain of the restriction enzyme *Fok*I with zinc-finger (ZF) proteins that recognize and bind DNA at a specific 3-bp site (Durai *et al*., 2005). Site-specific DNA cleavage occurs when two adjacent ZFNs are present in the correct orientation to allow dimerization of *Fok*I. The selection of ZF proteins is critical for the design of sequence-specific ZFNs and more ZF proteins could be incorporated into a ZFN system for specific recognition of larger target DNA sequences (Urnov *et al*., 2005). Several strategies such as phage display, bacterial two-hybrid system, bacterial one-hybrid system and OPEN system were reported for the selection of ZF proteins with high specificity and affinity (Durai *et al*.,

2005; Urnov *et al*., 2010). However, these methods require certain expertise (Durai *et al*., 2005). Other limitations include complexity in design, high construction cost and possible off-target effects (Abdallah *et al*., 2015).

Similar to ZFNs, TALENs also require DNA binding motifs and, in this case, the highly conserved repeats derived from transcription activator-like effectors (TALEs) to direct the non-specific *Fok*I nuclease domain for target specific cleavage (Joung & Sander, 2013). However, they are less complex than ZFNs because each TALE domain recognizes a single nucleotide. In TALEN, constructing monomers with specific binding to target DNA and incorporating 20 or more monomers into a single construct is challenging, but much easier than ZFN (Abdallah *et al*., 2015). Web-based tools such as E-TALEN have been introduced for target prediction and for designing TALENS with either a single target or for targeting a large number of genes (Heigwer *et al*., 2013) while TALENoffer provides genome-wide off-target prediction (Grau *et al*., 2013).

Recently, the clustered, regularly interspaced, short palindromic repeat (CRISPR) technology utilizing a bacterial CRISPR-associated protein-9 nuclease (CRISPR-Cas9) has been a breakthrough in molecular biology, functioning either as an active molecular scissors or a broken scissors to edit gene and regulate gene expression (Ledford, 2016). In contrast to the protein-guided ZFN and TALEN technologies, the sequence-guided CRISPR-Cas9 technology offers a simple, easy-to-design, efficient and less expensive method which can simultaneously induce disruption of multiple genes (Li *et al*., 2013; Walsh & Hochedlinger, 2013; Wang *et al*., 2013).

In the CRISPR-Cas9 system, the Cas9 nuclease cleaves DNA at a specific site directed by a single guide RNA (sgRNA; with a variable region of 20 nucleotides) according to simple base-pairing rules. The chimaeric sgRNA consists of two joined

RNA molecules, the target-specific CRISPR RNA (crRNA) and the structural transactivating CRISPR RNA (tracrRNA) with a 3-nt NGG sequence or PAM (Protospacer Adjacent Motif) after the 3′ end of the guide RNA (Jinek *et al*., 2012). The seed sequence of sgRNA of ca. 10-12 base pairs adjacent to the PAM was reported to play a significant role in determining Cas9 specificity although an initial PAM recognition is essential for the identification of potential Cas9 target sites as well as for the regulation of Cas9 cleaving activity (Jinek *et al.*, 2012; Cong *et al*., 2013; Sternberg *et al*., 2014). Cas9 consists of two distinct nuclease domains, HNH and RuvC which induce site-specific cleavage of the complementary DNA strand and noncomplementary strand, respectively (Gasiunas *et al*., 2012; Jinek *et al*., 2012).

CRISPR-Cas9 targeted genome editing could be utilized to introduce targeted and highly efficient genome alterations in a wide range of cells or organisms. However, high frequencies of undesired off-target mutagenesis are associated with CRISPR-Cas9 technology due to mismatches between the guide RNA and its target DNA, which might reduce its value as an efficient precise genome editing tool, especially in clinical applications such as gene therapy for the treatment of human genetic disorders (Fu, Foden *et al*., 2013). Although DNA methylation does not affect Cas9 RNA guided nuclease cleavage, the tolerance of the Cas9 system towards mismatches is sequence- and locus-dependent and can be affected by the quantity, position and distribution of mismatches (Hsu *et al*., 2013). Several mitigating approaches reported to reduce the frequency of off-target mutagenesis include use of suitable dosages of the Cas9 nuclease and sgRNAs as well as the availability of web tools to guide the selection of sgRNAs or genomic target sites and to predict off-target loci (Hsu *et al*., 2013; Bae *et al*., 2014; Cradick *et al*., 2014; Lei *et al*., 2014; Xie *et al*., 2014; Liu *et al*., 2015).

In addition, selecting unique target sequences and modifying the Cas9-sgRNA system could be used to minimize off-target effects (Cho *et al*., 2014). Although extending the sequence of sgRNA might not enhance the targeting specificity of the Cas9 system, a double nicking approach was shown to facilitate homology-directed repair with significant reduction of the off-target mutagenesis. In this approach, a Cas9 system was engineered to consist of a pair of sgRNAs guiding Cas9 mutant nickases (Cas9n) which induce double nicking (Cong *et al*., 2013; Ran *et al*., 2013; Cho *et al*., 2014). A partial mutagenesis approach was used to induce an aspartate to alanine mutation in Cas9 to inactivate the RuvC nuclease domain resulting in the production of the Cas9 nickase mutant, D10A, which caused a single-strand cleavage rather than a blunt double-stranded DNA cleavage (Gasiunas *et al*., 2012; Jinek *et al.*, 2012; Cong *et al*., 2013). The Cas9n system with a pair of offset sgRNA complementary to opposite strands of the target induces double-stranded DNA breaks (DSBs) by synergistic interaction of the paired Cas9n-sgRNA. In addition to monomeric Cas9n, dimeric RNA-guided Fokl nucleases were utilized to achieve higher specificity (> fourfold) compared to paired nickases (Guilinger *et al*., 2014). It is known that dimerization of Fokl nuclease is necessary for DNA cleavage (Bitinaite *et al*., 1998), hence, in this approach, dead Cas9 (dCas9) or inactive Cas9 was fused to Fokl monomers for the design of sgRNA that induced double nicking when two monomers of FokI-dCas9 sgRNA simultaneously bound adjacent regions (Guilinger *et al*., 2014; Tsai *et al*., 2014).

In fact, the CRISPR-Cas9 system causes targeted DSBs in the genome of host cells leading to subsequent cellular repair mechanisms via error-prone nonhomologous end-joining (NHEJ) or precise homology-directed repair (HDR) pathways to introduce

desired mutations (Urnov *et al*., 2010; Carroll, 2011; Cho *et al*., 2013; Cong *et al*., 2013). In HDR, homologous donor DNA is involved in repairing DNA damage while in NHEJ, insertions or deletions (indels) are generated when the cleaved DNA ends are joined together. Hence, strategies to increase the HDR: NHEJ ratio would reduce the off-target effects. However, the HDR: NHEJ ratios are affected by gene locus, type of nucleases and host cells. In some cases, it was found that the native Cas9 favoured the induction of NHEJ when compared to the double nicking Cas9n, although both induced similar levels of HDR (Miyaoka *et al*., 2016). HDR levels recorded for double nicking sgRNA pairs were comparable to the native Cas9 but much higher than for the single-guide Cas9n (Ran *et al*., 2013). In addition, single-stranded nicks associated with the use of Cas9n could induce DNA repair via the high-fidelity base excision repair (BER) pathway (Dianov & Hubscher, 2013). On the other hand, FokI-dCas9 induced mostly the NHEJ mechanism (Miyaoka *et al*., 2016). However, the levels of HDR in this system were significantly increased by the HDR enhancer RS-1 (Pan *et al*., 2016).

Methods of delivery also affect the Cas9 targeting specificity such as reduction of off-target mutagenesis and undesired immune effects associated with plasmid-mediated transfection by the use of purified recombinant Cas9 protein and sgRNA (Kim *et al*., 2014; Ramakrishna *et al*., 2014). Moreover, small molecules such as L755507 (a β3-adrenergic receptor agonist) and Brefeldin A could be utilized to enhance the efficiency of genome editing via their effects on the NHEJ or HDR repair mechanisms (Yu *et al*., 2015).

Applications of random mutagenesis and targeted gene editing systems in algal research

In algal research, random mutagenesis and insertional mutagenesis have been applied to generate new mutants with specific traits (Adam *et al*., 1993; Davies *et al*., 1994; Prieto *et al*., 1996). Genetic manipulation has been used to enhance biofuel production in algae (Dexter & Fu, 2009; Radakovits *et al*., 2011; Ortiz-Marquez *et al*., 2013). Radakovits *et al*. (2010) provided an overview on research involved in developing gene manipulation tools, and highlighted various strategies used in transgenic microalgae for biofuel production. Alternatively, transgenic algae can be used as bioreactors for the production of pharmaceuticals (Hawkins & Nakamura, 1999; Geng *et al*., 2003; Mayfield *et al*., 2003; Sun *et al*., 2003). For example, *C. reinhardtii* chloroplasts have been established as factories for the production of recombinant proteins (Manuell *et al*., 2007; Mayfield *et al.*, 2007; Muto *et al*., 2009; Rasala *et al.*, 2010) and offer efficient systems for high yield production of complex proteins which were properly folded into functional molecules such as recombinant antibodies (Mayfield *et al*., 2003; Tran *et al*., 2009) and vaccines (Surzycki *et al*., 2009).

Moreover, the development of efficient target genome editing methods to specifically knock down or knock out genes could be exploited for functional genomic analyses to enhance understanding of algal biology. The HR approach was successfully used for targeted gene disruption in *Nannochloropsis* (Kilian *et al*., 2011) and *Ostreococcus* (Lozano *et al*., 2014). Several algal genes were identified and characterized by gene silencing via RNA interference (RNAi) (Iseki *et al*., 2002; Ishikawa *et al*., 2008; Sun *et al*., 2008; Cerutti *et al*., 2011; Sakaguchi *et al*., 2011; Deng

et al., 2013). Levitan *et al.* (2014) demonstrated the use of RNAi in understanding how the remodelling of intermediate metabolism under nitrogen stress could affect lipid biosynthesis and reported an increase in cellular lipid content by knocking down the nitrate reductase gene in *Phaeodactylum tricornutum*.

Applications of recent emerging genome editing technologies such as ZFN and TALEN have been reported in *Chlamydomonas* (Sizova *et al*., 2013), *Pseudochoricystis ellipsoidea* (Kasai *et al*., 2015) and *Phaeodactylum tricornutum* (Daboussi *et al*., 2014; Weyman *et al*., 2015). The CRISPR-Cas9 technology has also been adapted for targeted gene disruption in algae. Vector-based transient expression of the codon-optimized Cas9 and sgRNA genes, driven respectively by the cauliflower mosaic virus (CaMV) 35S and the *Arabidopsis* U6 gene promoters, resulted in precise gene targeting in *C. reinhardtii.* However, no viable stable transformants were recorded indicating that continuous expression of Cas9 might be toxic to the cells (Jiang *et al*., 2014). A similar approach was applied in *P. tricornutum* (Nymark *et al*., 2016), *Thalassiosira pseudonana* (Hopes *et al*., 2016) and *Nannochloropsis* spp. (Wang *et al*., 2016) using endogenous promoters. Moreover, to knock out the urease gene in *T. pseudonana*, Hopes *et al.* (2016) assembled two sgRNA genes into a single construct using the Golden Gate cloning method (Weber *et al*., 2011; Sakuma *et al*., 2014; Xing *et al*., 2014) to induce large deletions at multiple target sites.

Vectors constructed with CRISPR-Cas9 have been introduced by electroporation into *C. reinhardtii* (Jiang *et al*., 2014) and *Nannochloropsis* spp. (Wang *et al*., 2016) while bombardment was applied to *T. pseudonana* (Hopes *et al*., 2016) and *P. tricornutum* (Nymark *et al*., 2016). A higher proportion of mutations (31%) was recorded with the use of bombardment (Nymark *et al*., 2016) than the approximately

1/1000 to 1/100 observed with electroporation (Wang *et al*., 2016). Nevertheless, the use of bombardment might lead to fragmentation that would affect the efficiency of the CRISPR-Cas9 system because intact Cas9 and sgRNA are required for mutagenesis (Jacobs *et al*., 2015; Hopes *et al*., 2016). Although *Agrobacterium*-mediated CRISPR-Cas9 transformation has not been used in algae, it was reported to generate high mutation frequencies in plant genetic engineering (Jacobs *et al*., 2015, Char *et al*., 2016).

Recently, in order to reduce accumulation of toxic Cas9 associated with vector-based expression (Jiang *et al*., 2014), direct delivery of the Cas9 ribonucleoprotein (RNP) and synthetic sgRNA was used to achieve higher targeting efficiency in *C. reinhardtii* (Shin *et al*., 2016). Finally, developing and effectively utilizing the CRISPR-Cas9 technology in various algal strains is very demanding.

Future directions: biosafety, risk assessment and practicality

Marine algae could potentially be genetically modified for biofuel production and as cell factories for efficient production of recombinant proteins. More generally, gene modification technology has been applied to crop improvement and several genetically modified (GM) crops such as maize, cotton and soya are available on the global market. Although the acceptance of GM crops has increased over the years, especially in developing countries, there are debates over social and ethical concerns. Hurdles include biosafety issues such as unpredictable long term effects of GM products on ecosystems and human health (Dale, 1999; Key *et al*., 2008; Maghari & Ardekani,

2011) as well as risks associated with the use of antibiotic resistance marker genes even though the resistance risk is much lower than for inappropriate antibiotic prescription practices (Gay & Gillespie, 2005; Ramessar *et al*., 2007). The adoption of GM algae faces similar challenges (Hallmann, 2007). Edible green algae as expression systems might be relatively safe as there is no contamination by human viral DNA or prions (Mayfield *et al*., 2007). In fact, the US FDA (Food and Drug Administration) has granted GRAS (Generally recognized as safe) status to several microalgae such as *Spirulina*, *Chlorella* and *Dunaliella* (Costa & de Morais, 2013; Fu *et al*., 2016), which could thus be a possible means for oral delivery of therapeutic proteins.

Major ecological concerns are the impacts of escaping transgenic algae on gene flow, biodiversity and ecosystems especially when cultivated in the open sea. The design of vectors completely derived from algae, using an endogenous promoter and replacing the antibiotic resistance gene with a reverse mutation approach, as well as the removal of marker genes after transformation, especially those that confer antibiotic resistance, could minimize the effects of transgene escapes (Qin *et al*., 2012; Day & Goldschmidt-Clermont, 2011). Other strategies involve system design to reduce the fitness of GM organisms in the wild and to minimize the chances of gene exchange with wild types via horizontal gene transfer (Henley *et al*., 2013). The paradox of the plankton (Hutchinson, 1961) raises the need for risk analyses when cultivating the corresponding non-native wild type (Gressel *et al*., 2013; 2014), and indicates the importance of gene mitigation strategies such as the disruption of a gene which is not required in culture but is essential for survival in the natural environment (Gressel, 1999; Al-Ahmad *et al*., 2006). For transgenic microalgae, transgene escape could be minimized by containment in enclosed bioreactors (Pulz, 2001). However, the design

of a cost-effective photobioreactor system is critical for large-scale cultivation of GM algae (Qin *et al*., 2012).

Generally, comprehensive multidisciplinary research including algal genetics, biology and ecology is needed to provide evidence-based biosafety and risk assessments of GM and to avoid unnecessary hurdles to commercialization. Collaborations among industry, academia and government are required for the progress and application of gene modification technology (Glass, 2015). Recently, promising results were observed in the first US Environmental Protection Agency (EPA) approved outdoor cultivation experiment of transformed *Acutodesmus dimorphus*. Transgenes and respective traits were stable in mutated strain throughout a culture period of 50 days in 800L outdoor pond and the GE strain has similar ecological impacts as its wild counterpart (Szyjka *et al*., 2017). Moreover, both wild and GE strains did not outcompete native algae from five lakes (Miramar, Murray, Poway, Lindo and Santee).

With regard to algal research, gene editing technology enables precise manipulation of specific gene elements, and facilitates the functional elucidation of target genes in algal biology leading to greater insight into molecular mechanisms. Despite the promises of CRISPR-Cas9 technology, there are limited reports of its application in algae. In addition, further studies are needed to improve the technology and address issues such as off-target mutations, PAM dependence, sgRNA production and delivery methods of CRISPR-Cas9 (Zhang *et al*., 2014). Data obtained from algal genomics could contribute to the selection of target sites and the design of highly specific sgRNA.

With recent advances in genomics and gene editing technology, exploitation of transgenic algae could expand. However, greater research effort would be needed to increase the competitiveness of GM algae against other systems in terms of cost-

effectiveness, safety, feasibility and scalability. Strategies to reduce unintended environmental consequences should be considered to smooth the approval process by regulatory agencies for commercial scale production. Apart from commercial applications, gene editing technologies in conjunction with high-throughput quantitative -omics technologies will lead to a more comprehensive understanding of algal biology and functional genomics, which will subsequently realize the potential of algal synthetic biology aiming to construct an entirely new biological system for new purposes (Georgianna & Mayfield, 2012; Wang *et al*., 2012; Church *et al*., 2014; Scaife & Smith, 2016).

References

Abdallah, N.A., Prakash, C.S. & McHughen, A.G. (2015). Genome editing for crop improvement: Challenges and opportunities. *GM Crops & Food*, **6**: 183-205.

Abomohra, A.E-F., El-Sheekh, M. & Hanelt, D. (2016). Protoplast fusion and genetic recombination between *Ochromonas danica* (Chrysophyta) and *Haematococcus pluvialis* (Chlorophyta). *Phycologia,* **55**: 65-71.

Adam, M., Lentz, K.E. & Loppes, R. (1993). Insertional mutagenesis to isolate acetate-requiring mutants in *Chlamydomonas reinhardtii*. *FEMS Microbiology Letters*, **110**: 265-268.

Al-Ahmad, H., Dwyer, J., Moloney, M. & Gressel, J. (2006). Mitigation of establishment of *Brassica napus* transgenes in volunteers using a tandem construct containing a selectively unfit gene. *Plant Biotechnology Journal*, **4:** 7-21.

Anila, N., Chandrashekar, A., Ravishankar, G.A. & Sarada, R. (2011). Establishment of *Agrobacterium tumefaciens*-mediated genetic transformation in *Dunaliella bardawil*. *European Journal of Phycology*, **46**: 36-44.

Apt, K.E., Kroth-Pancic, P.G. & Grossman, A.R. (1996). Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Molecular & General Genetics*, **252**: 572-579.

Bae, S., Park, J. & Kim, J.S. (2014). Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics*, **30**: 1473-1475.

Batchvarov, S. (1993). Garlic *Allium sativum* L. In *Genetic improvement of vegetable crops* (Kalloo, G. & Bergh, B.O., editors), 15-27. Pergamon Press, New York.

Bateman, J.M. & Purton, S. (2000). Tools for chloroplast transformation in *Chlamydomonas*: expression vectors and a new dominant selectable marker. *Molecular & General Genetics*, **263**: 404-410.

Bitinaite, J., Wah, D.A., Aggarwal, A.K. & Schildkraut, I. (1998). FokI dimerization is required for DNA cleavage. *Proceedings of the National Academy of Sciences of the United States of America*, **95**: 10570-10575.

Brahamsha, B.A. (1996). Genetic manipulation system for oceanic cyanobacteria of the genus *Synechococcus*. *Applied & Environmental Microbiology*, **62**:1747-1751.

Breseghello, F. & Coelho, A.S.G. (2013). Traditional and modern plant breeding methods with examples in rice (*Oryza sativa* L.). *Journal of Agricultural and Food Chemistry*, **61**: 8277-8286.

Brown, L.E., Sprecher, S.L. & Keller, L.R. (1991). Introduction of exogenous DNA into *Chlamydomonas reinhardtii* by electroporation. *Molecular & Cellular Biology*, **11**: 2328-2332.

Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics*, **188**: 773-782.

Carthew, R.W. & Sontheimer, E.J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell*, **136**: 642-655.

Castel, S.E. & Martienssen, R.A. (2013). RNA interference (RNAi) in the nucleus: roles for small RNA in transcription, epigenetics and beyond. *Nature Reviews Genetics*, **14**: 100-112.

Cerutti, H., Ma, X., Msanne, J., & Repas, T. (2011). RNA-mediated silencing in algae: biological roles and tools for analysis of gene function. *Eukaryotic Cell*, **10**: 1164-1172.

Cha, T.S., Chen, C.F., Yee, W., Aziz, A. & Loh, S.H. (2011). Cinnamic acid, coumarin and vanillin: alternative phenolic compounds for efficient *Agrobacterium*-mediated transformation of the unicellular green alga, *Nannochloropsis* sp. *Journal of Microbiological Methods*, **84**: 430-434.

Char, S.N., Neelakandan, A.K., Nahampun, H., Frame, B., Main, M., Spalding, M.H., Becraft, P.W., Meyers, B.C., Walbot, V., Wang, K. & Yang, B. (2017). An *Agrobacterium*-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. *Plant Biotechnology Journal*, **15**: 257-268.

Charrier, B., Rolland, E., Gupta, V. & Reddy, C.R.K. (2015). Production of genetically and developmentally modified seaweeds: exploiting the potential of artificial selection techniques. *Frontiers in Plant Science*, **6**: 127.

Chen, Y., Wang, Y., Sun, Y., Zhang, L & Li, W. (2001). Highly efficient expression of rabbit neutrophil peptide-1 gene in *Chlorella ellipsoidea* cells. *Current Genetics*, **39**: 365-370.

Cheney, D., Metz, B. & Stiller, J. (2001). *Agrobacterium*-mediated genetic transformation in the macroscopic marine red alga *Porphyra yezoensis*. *Journal of Phycology*, **37**: 11.

Cheng, R., Ma, R., Li, K., Rong, H., Lin, X., Wang, Z., Yang, S. & Ma, Y. (2012). *Agrobacterium tumefaciens* mediated transformation of marine microalgae *Schizochytrium*. *Microbiological Research*, **167**: 179-186.

Chepurnov, V.A., Chaerle, P., Roef, L., Meirhaeghe, A. & Vanhoutte, K. (2011). Classical breeding in diatoms: scientific background and practical perspectives. In *The Diatom World* (Seckbach, J. & Kociolek, J.P., editors), 167-194. Springer, Netherlands.

Chiang, M.S., Chong, C., Landry, B.S. & Crete, R. (1993). Cabbage *Brassica oleracea* subsp. *capitata* L. In *Genetic improvement of vegetable crops* (Kalloo, G. & Bergh, B.O., editors), 113-155. Pergamon Press, New York.

Cho, S.W., Kim, S., Kim, J.M. & Kim, J.S. (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotechnology*, **31**: 230-232.

Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S. & Kim, J.S. (2014). Analysis of off-target effects of CRISPR/ Cas-derived RNA-guided endonucleases and nickases. *Genome Research*, **24**: 132-141.

Choi, J.I., Yoon, M., Joe, M., Park, H., Lee, S.G., Han, S.J. & Lee, P.C. (2014). Development of microalga *Scenedesmus dimorphus* mutant with higher lipid

content by radiation breeding. *Bioprocess & Biosystems Engineering*, **37**: 2437-2444.

Chow, K.C. & Tung, W.L. (1999). Electrotransformation of *Chlorella vulgaris*. *Plant Cell Reports*, **18**: 778-780.

Church, G.M., Elowitz, M.B., Smolke, C.D., Voigt, C.A. & Weiss, R. (2014). Realizing the potential of synthetic biology. *Nature Reviews Molecular Cell Biology*, **15**: 289-294.

Cock, J.M. *et al*. (2010). The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. *Nature*, **465**: 617-621.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**: 819-823.

Cordero, B.F., Obraztsova, I., Couso, I., Leon, R., Vargas, M.A. & Rodriguez, H. (2011). Enhancement of lutein production in *Chlorella sorokiniana* (Chorophyta) by improvement of culture conditions and random mutagenesis. *Marine Drugs*, **9**: 1607-1624.

Costa, J.A.V. & de Morais, M.G. (2013). *Microalgae for food production*. In *Fermentation Processes Engineering in the Food Industry* (Soccol, C.R., Pandey, A. & Larroche, C., editors), 405. CRC Press, Taylor & Francis Group, USA.

Couso, I., Cordero, B.F., Vargas, M.Á. & Rodríguez, H. (2012). Efficient heterologous transformation of *Chlamydomonas reinhardtii* npq2 mutant with the zeaxanthin epoxidase gene isolated and characterized from *Chlorella zofingiensis*. *Marine Drugs*, **10**: 1955-1976.

Cradick, T.J., Qiu, P., Lee, C.M., Fine, E.J. & Bao, G. (2014). COSMID: A web-based tool for identifying and validating CRISPR/Cas off-target sites. *Molecular Therapy Nucleic Acids*, **3**: e214.

Crisp, P. & Tapsell, C.R. (1993). Cauliflower *Brassica oleracea* L. In *Genetic Improvement of Vegetable Crops* (Kalloo, G. & Bergh, B.O., editors), 157-178. Pergamon Press, New York.

Daboussi, F., Leduc, S., Maréchal, A., Dubois, G., Guyot, V., Perez-Michaut, C., Amato, A., Falciatore, A., Juillerat, A., Beurdeley, M., Voytas, D.F., Cararec, L. & Duchateau, P. (2014). Genome engineering empowers the diatom *Phaeodactylum tricornutum* for biotechnology. *Nature Communications*, **5:** 3831.

Dale, P.J. (1999). Public reactions and scientific responses to transgenic crops. *Current Opinion in Biotechnology*, **10**: 203-208.

Dauvillée, D., Delhaye, S., Gruyer, S., Slomianny, C., Moretz, S.E., d'Hulst, C., Long, C.A., Ball, S.G. & Tomavo, S. (2010). Engineering the chloroplast targeted malarial vaccine antigens in *Chlamydomonas* starch granules. *PLoS One*, **5**: e15424.

Davies, J.P., Yildiz, F. & Grossman, A.R. (1994). Mutants of *Chlamydomonas* with aberrant responses to sulphur deprivation. *The Plant Cell*, **6**: 53-63.

Day, A. & Goldschmidt-Clermont, M. (2011). The chloroplast transformation toolbox: selectable markers and marker removal. *Plant Biotechnology Journal*, **9**: 540-553.

Deng, X., Cai, J. & Fei, X. (2013). Effect of the expression and knockdown of citrate synthase gene on carbon flux during triacylglycerol biosynthesis by green algae *Chlamydomonas reinhardtii*. *BMC Biochemistry*, **14**: 38.

De Riso, V., Raniello, R., Maumus, F., Rogato, A., Bowler, C. & Falciatore, A. (2009). Gene silencing in the marine diatom *Phaeodactylum tricornutum*. *Nucleic Acids Research*, **37**: e96.

Dexter, J. & Fu, P. (2009). Metabolic engineering of cyanobacteria for ethanol production. *Energy & Environmental Science*, **2**: 857-864.

Dianov, G.L. & Hubscher, U. (2013). Mammalian base excision repair: the forgotten archangel. *Nucleic Acids Research*, **41**: 3483-3490.

Doetsch, N.A., Favreau, M.R., Kuscuoglu, N., Thompson, M.D. & Hallick, R.B. (2001). Chloroplast transformation in *Euglena gracilis*: splicing of a group III twintron transcribed from a transgenic *psbK* operon. *Current Genetics*, **39**: 49-60.

Doron, L., Segal, N. & Shapira, M. (2016). Transgene expression in microalgae-from tools to applications. *Frontiers in Plant Science*, **7:** 505.

Dragosits, M. & Mattanovich, D. (2013). Adaptive laboratory evolution – principles and applications for biotechnology. *Microbial Cell Factories*, **12**: 64.

Dreesen, I.A.J., Hamri, G.C.E. & Fussenegger, M. (2010). Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection. *Journal of Biotechnology*, **145**: 273-280.

Dunahay, T.G. (1993). Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers. *Biotechniques*, **15**: 452-455, 457-458, 460.

Dunahay, T.G., Jarvis, E.E. & Roessler, P.G. (1995). Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. *Journal of Phycology*, **31**: 1004-1012.

Durai, S., Mani, M., Kandavelou, K., Wu, J., Porteus, M.H. & Chandrasegaran, S. (2005). Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Research*, **33**: 5978-5990.

Eamens, A.L., McHale, M. & Waterhouse, P.M. (2014). The use of artificial microRNA technology to control gene expression in *Arabidopsis thaliana*. *Methods in Molecular Biology*, **1062**: 211-224.

Economou, C., Wannathong, T., Szaub, J. & Purton, S. (2014). A simple, low-cost method for chloroplast transformation of the green alga *Chlamydomonas reinhardtii*. *Methods in Molecular Biology*, **1132**: 401-411.

Falciatore, A., Casotti, R., Leblanc, C., Abrescia, C. & Bowler, C. (1999). Transformation of nonselectable reporter genes in marine diatoms. *Marine Biotechnology*, **1**: 239-251.

Feng, S., Feng, W., Zhao, L., Gu, H., Li, Q., Shi, K., Guo, S. & Zhang, N. (2014). Preparation of transgenic *Dunaliella salina* for immunization against white spot syndrome virus in crayfish. *Archives of Virology*, **159**: 519-525.

Feng, S.Y., Xue, L.X., Liu, H.T. & Lu, P.J. (2009). Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. *Molecular Biology Reports*, **36**: 1433-1439.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. & Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature*, **391**: 806-811.

Franklin, S., Ngo, B., Efuet, E. & Mayfield, S.P. (2002). Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *The Plant Journal*, **30**: 733-744.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. & Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology*, **31**: 822-826.

Fu, W., Chaiboonchoe, A., Khraiwesh, B., Nelson, D.R., Al-Khairy, D., Mystikou, A., Alzahmi, A. & Salehi-Ashtiani, K. (2016). Algal cell factories: approaches, applications, and potentials. *Marine Drugs*, **14:** 225.

Fu, W., Guðmundsson, O., Paglia, G., Herjólfsson, G., Andrésson, O.S., Palsson, B.O. & Brynjólfsson, S. (2013). Enhancement of carotenoid biosynthesis in the green microalga *Dunaliella salina* with light-emitting diodes and adaptive laboratory evolution. *Applied Microbiology & Biotechnology*, **97**: 2395-2403.

Fujita, Y. & Saito, M. (1990). Protoplast isolation and fusion in *Porphyra* (Bangiales, Rhodophyta). *Developments in Hydrobiology*, **58**: 161-166.

Fukuda, S., Mikami, K., Uji, T., Park, E.J., Ohba, T., Asada, K., Kitade, Y., Endo, H., Kato, I. & Saga, N. (2008). Factors influencing efficiency of transient gene expression in the red macrophyte *Porphyra yezoensis. Plant Science*, **174**: 329-339.

Gan, S.Y., Qin, S., Othman, R.Y., Yu, D. & Phang, S.M. (2003). Transient expression of *lacZ* in particle bombarded *Gracilaria changii* (Gracilariales, Rhodophyta). *Journal of Applied Phycology*, **15**: 345-349.

Gan, S.Y., Lim, P.E. & Phang, S.M. (2016). Genetic and metabolic engineering of microalgae. In *Algae Biotechnology, Green Energy and Technology* (Bux, F. & Chisti, Y., editors), 317-344. Springer International Publishing, Switzerland.

Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity

in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, **109**: 2579-2586.

Gay, P.B. & Gillespie, S.H. (2005). Antibiotic resistance markers in genetically modified plants: a risk to human health? *Lancet Infectious Diseases*, **5**: 637-646.

Geng, D., Wang, Y., Wang, P. Li, W. & Sun, Y. (2003). Stable expression of hepatitis B surface antigen gene in *Dunaliella salina* (Chlorophyta). *Journal of Applied Phycology*, **15**: 451-456.

Georgianna, D.R. & Mayfield, S.P. (2012). Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature*, **488**: 329-335.

Ghildiyal, M. & Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nature Reviews Genetics*, **10**: 94-108.

Glass, D.J. (2015). Government regulation of the uses of genetically modified algae and other microorganisms in biofuel and bio-based chemical production. In *Algal Biorefineries* (Prokop, A., Bajpai, R.K. & Zappi, M.E., editors), 23-60. Springer International Publishing, Switzerland.

Gong, Q., Yu, W., Dai, J., Liu, H., Xu, R., Guan, H. & Pan, K. (2007). Efficient *gusA* transient expression in *Porphyra yezoensis* protoplasts mediated by endogenous beta-tubulin flanking sequences. *Journal of Ocean University of China*, **6**: 21-25.

Grau, J., Boch, J. & Posch, S. (2013). TALENoffer: genome-wide TALEN off-target prediction. *Bioinformatics*, **29**: 2931-2932.

Gressel, J. (1999). Tandem constructs: preventing the rise of superweeds. *Trends in Biotechnology*, **17**: 361-366.

Gressel, J., van der Vlugt, C.J.B. & Bergmans, E.N. (2013). Environmental risks of large scale cultivation of microalgae: Mitigation of spills. *Algal Research,* **2**: 286-298.

Gressel, J., van der Vlugt, C.J.B. & Bergmans, E.N. (2014). Cultivated microalgae spills: hard to predict/ easier to mitigate risks. *Trends in Biotechnology*, **32**: 65-69.

Guilinger, J.P., Thompson, D.B. & Liu, D.R. (2014). Fusion of catalytically inactive Cas9 to *Fok*I nuclease improves the specificity of genome modification. *Nature Biotechnology*, **32**: 577-582.

Guo, S., Zhao, X., Tang, Y., Wan, C., Alam, M.A., Ho, S., Bai, F. & Chang, J. (2013). Establishment of an efficient genetic transformation system in *Scenedesmus obliquus*. *Journal of Biotechnology*, **163**: 61-68.

Gupta, V., Kumari, P. & Reddy, C. (2015). Development and characterization of somatic hybrids of *Ulva reticulata* Forsskål (×) *Monostroma oxyspermum* (Kutz.) Doty. *Frontiers in Plant Science*, **6:** 3.

Hado, M., Okauchhi, M., Murase, N. & Mizukami, Y. (2003). Transient expression of GUS gene using Rubisco gene promoter in the protoplasts of *Porphyra yezoensis*. *Aquaculture Science,* **51**: 355-360.

Hallmann, A. (2007). Algal transgenics and biotechnology. *Transgenic Plant Journal,* **1**: 81-98.

Hallmann, A., Rappel, A. & Sumper, M. (1997). Gene replacement by homologous recombination in the multicellular green alga *Volvox carteri. Proceedings of the National Academy of Sciences of the United States of America*, **94**: 7469-7474.

Harper, M. & Lee, C.J. (2012). Genome-wide analysis of mutagenesis bias and context sensitivity of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). *Mutation Research*, **731**: 64-67.

Hawkins, R.L. & Nakamura, M. (1999). Expression of human growth hormone by the eukaryotic alga, *Chlorella*. *Current Microbiology*, **38**: 335-341.

He, P., Yao, Q., Chen, Q., Guo, M., Xiong, A., Wu, W. & Ma, J. (2001). Transferring and expression of glucose oxidase gene in *Porphyra yezoensis*. *Journal of Phycology*, **37**: 23.

Heigwer, F., Kerr, G., Walther, N., Glaeser, K., Pelz, O., Breinig, M. & Boutros, M. (2013). E-TALEN: a web tool to design TALENs for genome engineering. *Nucleic Acids Research*, **41**: e190.

Henley, W.J., Litaker, R.W., Novoveská, L., Duke, C.S., Quemada, H.D., Sayre, R.T. (2013). Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. *Algal Research*, **2**: 66-77.

Hirata, R., Takahashi, M., Saga, N. & Mikami, K. (2011). Transient gene expression system established in *Porphyra yezoensis* is widely applicable in Bangiophycean algae. *Marine Biotechnology*, **13**: 1038-1047.

Hopes, A., Nekrasov, V., Kamoun, S. & Mock, T. (2016). Editing of the urease gene by CRISPR-Cas in the diatom *Thalassiosira pseudonana*. *Plant Methods*, **12**: 49.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G. & Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, **31**: 827-832.

Huang, A., He, L. & Wang, G. (2011). Identification and characterization of microRNAs from *Phaeodactylum tricornutum* by high-throughput sequencing and bioinformatics analysis. *BMC Genomics*, **12**: 337.

Huang, X., Weber, J.C., Hinson, T.K., Mathieson, A.C. & Minocha, S.C. (1996). Transient expression of the GUS reporter gene in the protoplasts and partially digested cells of *Ulva lactuca* L (Chlorophyta). *Botanica Marina*, **39**: 467-474.

Huddy, S.M., Meyers, A.E. & Coyne, V.E. (2012). Transformation of *lacZ* using different promoters in the commercially important red alga, *Gracilaria gracilis*. *African Journal of Biotechnology*, **11**: 1879-1885.

Hutchinson, G.E. (1961). The paradox of the plankton. *American Naturalist*, **95**: 137-145.

Iseki, M., Matsunaga, S., Murakami, A., Ohno, K., Shiga, K., Yoshida, K., Sugai, M., Takahashi, T., Hori, T. & Watanabe, M. (2002). A blue-light-activated adenylyl cyclase mediates photoavoidance in *Euglena gracilis*. *Nature*, **415**: 1047-1051.

Ishikawa, T., Nishikawa, H., Gao, Y., Sawa, Y., Shibata, H., Yabuta, Y., Maruta, T. & Shigeoka, S. (2008). The pathway via D-galacturonate/L-galactonate is significant for ascorbate biosynthesis in *Euglena gracilis*: identification and functional characterization of aldonolactonase. *The Journal of Biological Chemistry*, **283**: 31133-31141.

Jacobs, T.B., LaFayette, P.R., Schmitz, R.J. & Parrott, W.A. (2015). Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology*, **15:** 16.

Jakobiak, T., Mages, W., Scharf, B., Babinger, P., Stark, K. & Schmitt, R. (2004). The bacterial paromomycin resistance gene, *aphH*, as a dominant selectable marker in *Volvox carteri*. *Protist*, **155**: 381-393.

Jia, Y., Xue, L., Liu, H. & Li, J. (2009). Characterization of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the halotolerant alga *Dunaliella salina* and inhibition of its expression by RNAi. *Current Microbiology*, **58**: 426-431.

Jiang, G.Z., Lu, Y.M., Niu, X.L. & Xue, L.X. (2005). The actin gene promoter-derived bar as dominant selectable marker for nuclear transformation of *Dunaliella salina*. *Yi Chuan Xue Bao*, **32**: 424-433.

Jiang, P., Qin, S. & Tseng, C.K. (2002). Expression of hepatitis B surface antigen gene (*HBsAg*) in *Laminaria japonica* (Laminariales, Phaeophyta). *Chinese Science Bulletin*, **47**: 1438-1440.

Jiang, P., Qin, S. & Tseng, C.K. (2003). Expression of the *lacZ* reporter gene in sporophytes of the seaweed *Laminaria japonica* (Phaeophyceae) by gametophyte-targeted transformation. *Plant Cell Reports*, **21**: 1211-1216.

Jiang, W., Brueggeman, A.J., Horken, K.M., Plucinak, T.M. & Weeks, D.P. (2014). Successful transient expression of Cas9 and single guide RNA genes in *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, **13**: 1465-1469.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**: 816-821.

Jinkerson, R.E. & Jonikas, M.C. (2015). Molecular techniques to interrogate and edit the *Chlamydomonas* nuclear genome. *The Plant Journal*, **82**: 393-412.

Joung, J.K. & Sander, J.D. (2013). TALENs: a widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology*, **14**: 49-55.

Kakinuma, M., Ikeda, M., Coury, D.A., Tominaga, H., Kobayashi, I. & Amano, H. (2009). Isolation and characterization of the *rbcS* genes from a sterile mutant of *Ulva pertusa* (Ulvales, Chlorophytea) and transient gene expression using the *rbcS* gene promoter. *Fisheries Science*, **75**: 1015-1028.

Kasai, Y., Oshima, K., Ikeda, F., Abe, J., Yoshimitsu, Y. & Harayama, S. (2015). Construction of a self-cloning system in the unicellular green alga *Pseudochoricystis ellipsoidea*. *Biotechnology for Biofuels*, **8**: 94.

Kathiresan, S., Chandrashekar, A., Ravishankar, G.A. & Sarada, R. (2009). *Agrobacterium*-mediated transformation in the green alga *Haematococcus pluvialis* (Chlorophyceae, Volvocales). *Journal of Phycology*, **45:** 642-649.

Ketting, R.F. (2011). The many faces of RNAi. *Developmental Cell*, **20**: 148-161.

Key, S., Ma, J.K.-C. & Drake, P.M. (2008). Genetically modified plants and human health. *Journal of the Royal Society of Medicine*, **101**: 290-298.

Khawaja, I.B., Kim, M., Stahl, U. & Cho, M. (2016). Microalgae engineering toolbox: selectable and screenable markers. *Biotechnology and Bioprocess Engineering*, **21**: 224-235.

Kilian, O., Benemann, C.S.E., Niyogi, K.K. & Vick, B. (2011). High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proceedings of the National Academy of Sciences of the United States of America*, **108**: 21265-21269.

Kim, D., Kim, Y.T., Cho, J.J., Bae, J.H., Hur, S.B., Hwang, I. & Choi, T.J. (2002). Stable integration and functional expression of flounder growth hormone gene in transformed microalgae *Chlorella ellipsoidea*. *Marine Biotechnology*, **4**: 63-73.

Kim, S., Kim, D., Cho, S.W., Kim, J. & Kim, J.S. (2014). Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research*, **24**: 1012-1019.

Kim, S., Lee, Y.C., Cho, D.H., Lee, H.U., Huh, Y.S., Kim, G.J. & Kim, H.S. (2014). A simple and non-invasive method for nuclear transformation of intact-walled *Chlamydomonas reinhardtii*. *PLoS One*, **9**: e101018.

Kindle, K.L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, **87**: 1228-1232.

Kito, H., Kunimoto, M., Kamanishi, Y. & Mizukami, Y. (1998). Protoplast fusion between *Monostroma nitidum* and *Porphyra yezoensis* and subsequent growth of hybrid plants. *Journal of Applied Phycology*, **10**: 15-21.

Koblenz, B. & Lechtreck, K.F. (2005). The *NIT1* promoter allows inducible and reversible silencing of centrin in *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, **4**: 1959-1962.

Kong, F., Zhao, H., Liu, W., Li, N. & Mao, Y. (2017). Construction of plastid expression vector and development of genetic transformation system for the seaweed *Pyropia yezoensis*. *Marine Biotechnology*, **19**: 147-156.

Kuang, M., Wang, S.J., Li, Y., Shen, D.L. & Zeng, C.K. (1998). Transient expression of exogenous GUS gene in *Porphyra yezoensis* (Rhodophyta). *Chinese Journal of Oceanology & Limnology*, **16**: 56-61.

Kübler, J.E., Minocha, S.C. & Mathieson, A.C. (1994). Transient expression of the GUS reporter gene in protoplasts of *Porphyra miniata* (Rhodophyta). *Journal of Marine Biotechnology*, **1**: 165-169.

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N. & Mathey-Prevot, B. (2006). Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. *Nature Methods*, **3**: 833-838.

Kumar, S.V., Misquitta, R.W., Reddy, V.S., Rao, B.J. & Rajam, M.V. (2004). Genetic transformation of the green alga - *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. *Plant Science*, **166**: 731-738.

Kurtzman, A.M. & Cheney, D.P. (1991). Direct gene transfer and transient expression in a marine red alga using the biolistic method. *Journal of Phycology*, **27**(Supplement): 42.

Lapidot, M., Raveh, D., Sivan, A., Arad, S.M. & Shapira, M. (2002). Stable chloroplast transformation of the unicellular red alga *Porphyridium* species. *Plant Physiology*, **129**: 7-12.

Ledford, H. (2016). CRISPR: gene editing is just the beginning. *Nature*, **531**: 156-159.

Lee, B., Choi, G.G., Choi, Y.E., Sung, M., Park, M.S., Yang, J.W. (2014). Enhancement of lipid productivity by ethyl methane sulfonate-mediated random mutagenesis and proteomic analysis in *Chlamydomonas reinhardtii*. *Korean Journal of Chemical Engineering*, **31**: 1036-1042.

Lei, Y., Lu, L., Liu, H.Y., Li, S., Xing, F. & Chen, L.L. (2014). CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. *Molecular Plant*, **7**: 1494-1496.

León, R., Couso, I. & Fernández, E. (2007). Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, **130**: 143-152.

Lerche, K. & Hallmann A. (2009). Stable nuclear transformation of *Gonium pectorale*. *BMC Biotechnology*, **9**: 64.

Levitan, O., Dinamarca, J., Zelzion, E., Lun, D.S., Guerra, L.T., Kim, M.K., Kim, J., Van Mooy, B.A.S., Bhattacharya, D. & Falkowski, P.G. (2014). Remodeling of intermediate metabolism in the diatom *Phaeodactylum tricornutum* under nitrogen stress. *Proceedings of the National Academy of Sciences of the United States of America*, **112**: 412-417.

Li, F., Qin, S., Jiang, P., Wu, Y. & Zhang, W. (2009). The integrative expression of GUS gene driven by *FCP* promoter in the seaweed *Laminaria japonica* (Phaeophyta). *Journal of Applied Phycology*, **21**: 287-293.

Li, D., Wang, L., Zhao, Q., Wei, W. & Sun, Y. (2015). Improving high carbon dioxide tolerance and carbon dioxide fixation capability of *Chlorella* sp. by adaptive laboratory evolution. *Bioresource Technology*, **185**: 269-275.

Li, J.F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M. and Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology*, **31**: 688-691.

Liang, C., Zhang, X., Zou, J., Xu, D., Su, F. & Ye, N. (2010). Identification of miRNA from *Porphyra yezoensis* by high-throughput sequencing and bioinformatics analysis. *PLoS One*, **5**: e10698.

Liang, S., Guo, L., Lin, G., Zhang, Z., Ding, H., Wang, Y. & Yang, G. (2016). Improvement of *Nannochloropsis oceanica* growth performance through chemical mutation and characterization of fast growth physiology by transcriptome profiling. *Chinese Journal of Oceanology and Limnology,* 1-11.

Lin, H., & Qin, S. (2014). Tipping points in seaweed genetic engineering: scaling up opportunities in the next decade. *Marine Drugs,* **12**: 3025-3045.

Liu, J., Sun, Z., Gerken, H., Huang, J., Jiang, Y. & Chen, F. (2014). Genetic engineering of the green alga *Chlorella zofingiensis*: a modified norflurazon-resistant phytoene desaturase gene as a dominant selectable marker. *Applied Microbiology & Biotechnology*, **98**: 5069-5079.

Liu, L., Wang, Y., Zhang, Y., Chen, X., Zhang, P. & Ma, S. (2013). Development of a new method for genetic transformation of the green alga *Chlorella ellipsoidea*. *Molecular Biotechnology,* **54**: 211-219.

Liu, H., Wei, Z., Dominguez, A., Li, Y., Wang, X. & Qi, L.S. (2015). CRISPR-ERA: a comprehensive design tool for CRISPR-mediated gene editing, repression and activation. *Bioinformatics,* **31**: 3676-3678.

Liu, H.Q., Yu, W.G., Dai, J.X., Gong, Q.H., Yang, K.F. & Zhang, Y.P. (2003). Increasing the transient expression of *GUS* gene in *Porphyra yezoensis* by 18S rDNA targeted homologous recombination. *Journal of Applied Phycology*, **15**: 371-377.

Liu, S., Zhao, Y., Liu, L., Ao, X., Ma, L., Wu, M. & Ma, F. (2015). Improving cell growth and lipid accumulation in green microalgae *Chlorella* sp. via UV irradiation. *Applied Biochemistry & Biotechnology*, **175**: 3507-3518.

Lozano, J.C., Schatt, P., Botebol, H., Verge, V., Lesuisse, E., Blain, S., Carre, I.A. & Bouget, F.Y. (2014). Efficient gene targeting and removal of foreign DNA by homologous recombination in the picoeukaryote *Ostreococcus*. *Plant Journal*, **78:** 1073-1083.

Lumbreras, V., Stevens, D.R. & Purton, S. (1998). Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *The Plant Journal*, **14**: 441-447.

Maghari, B.M. & Ardekani, A.M. (2011). Genetically modified foods and social concerns. *Avicenna Journal of Medical Biotechnology*, **3**: 109-117.

Manuell, A.L., Beligni, M.V., Elder, J.H., Siefker, D.T., Tran, M., Weber, A., McDonald, T.L. & Mayfield, S.P. (2007). Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. *Plant Biotechnology Journal*, **5**: 402-412.

Matsunaga, T., Takeyama, H. & Nakamura, N. (1990). Characterization of cryptic plasmids from marine cyanobacteria and construction of a hybrid plasmid potentially capable of transformation of marine cyanobacterium, *Synechococcus* sp. and its transformation. *Applied Biochemistry & Biotechnology,* **24/25**: 151-160.

Mayfield, S.P., Franklin, S.E. & Lerner, R.A. (2003). Expression and assembly of a fully active antibody in algae. *Proceedings of the National Academy of Sciences of the United States of America,* **100:** 438-442.

Mayfield, S.P., Manuell, A.L., Chen, S., Wu, J., Tran, M., Siefker, D., Muto, M. & Marin-Navarro, J. (2007). *Chlamydomonas reinhardtii* chloroplasts as protein factories. *Current Opinion in Biotechnology*, **18**: 126-133.

Maruyama, M., Horáková, I., Honda, H., Xing, X., Shiragami, N. & Unno, H. (1994). Introduction of foreign DNA into *Chlorella saccharophila* by electroporation. *Biotechnology Techniques*, **8**: 821-826.

Meneses, I. & Santelices, B. (1999). Strain selection and genetic variation in *Gracilaria chilensis* (Gracilariales, Rhodophyta). *Journal of Applied Phycology,* **11**: 241-246.

Mikami, K. (2014). A technical breakthrough close at hand: feasible approaches toward establishing a gene-targeting genetic transformation system in seaweeds. *Frontiers in Plant Science*, **5**: 498.

Mikami, K., Uji, T., Li, L., Takahashi, M., Yasui, H. & Saga, N. (2009). Visualization of phosphoinositides via the development of the transient expression system of a cyan fluorescent protein in the red alga *Porphyra yezoensis*. *Marine Biotechnology*, **11**: 563-569.

Miyagawa, A., Okami, T., Kira, N., Yamaguchi, H., Ohnishi, K. & Adachi, M. (2009). Research note: high efficiency transformation of the diatom *Phaeodactylum tricornutum* with a promoter from the diatom *Cylindrotheca fusiformis*. *Phycological Research*, **57**: 142-146.

Miyagawa, A., Okami, T., Kira, N., Yamaguchi, H., Ohnishi, K. & Adachi, M. (2011). Stable nuclear transformation of the diatom *Chaetoceros* sp. *Phycological Research*, **59**: 113-119.

Miyaoka, Y., Berman, J.R., Cooper, S.B., Mayerl, S.J., Chan, A.H., Zhang, B., Karlin-Neumann, G.A. & Conklin, B.R. (2016). Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Scientific Reports*, **6**: 23549.

Mizukami, Y., Hado, M., Kito, H., Kunimoto, M. & Murase, N. (2004). Reporter gene introduction and transient expression in protoplasts of *Porphyra yezoensis. Journal of Applied Phycology*, **16**: 23-29.

Mizukami, Y., Okauchi, M., Kito, H., Ishimoto, S., Ishida, T. & Fuseya, M. (1995). Culture and development of electrically fused protoplasts from red marine algae, *Porphyra yezoensis* and *P. suborbiculata*. *Aquaculture*, **132**: 361-367.

Molnár, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D. & Baulcombe, D. (2009). Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii. The Plant Journal*, **58**: 165-174.

Molnár, A., Schwach, F., Studholme, D.J., Thuenemann, E.C. & Baulcombe, D.C. (2007). miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. *Nature*, **447**: 1126-1129.

Muto, M., Henry, R.E. & Mayfield, S.P. (2009). Accumulation and processing of a recombinant protein designed as a cleavable fusion to the endogenous Rubisco LSU protein in *Chlamydomonas* chloroplast. *BMC Biotechnology,* **9**: 26.

Neupert, J., Shao, N., Lu, Y. & Bock, R. (2012). Genetic transformation of the model green alga *Chlamydomonas reinhardtii*. *Methods in Molecular Biology*, **847**: 35-47.

Nickelsen, J. (1999). Transcripts containing the 5' untranslated regions of the plastid genes psbA and psbB from higher plants are unstable in *Chlamydomonas reinhardtii* chloroplasts. *Molecular & General Genetics*, **262**: 768-771.

Niu, Y.F., Zhang, M.H., Xie, W.H., Li, J.N., Gao, Y.F., Yang, W.D., Liu, J.S. & Li, H.Y. (2011). A new inducible expression system in a transformed green alga, *Chlorella vulgaris. Genetics & Molecular Research*, **10**: 3427-3434.

Nymark, M., Sharma, A.K., Sparstad, T., Bones, A.M. & Winge, P. (2016). A CRISPR/Cas9 system adapted for gene editing in marine algae. *Scientific Reports*, **6**: 24951.

Ohnuma, M., Yokoyama, T., Inouye, T., Sekine, Y. & Tanaka, K. (2008). Polyethylene glycol (PEG)-mediated transient gene expression in a red alga, *Cyanidioschyzon merolae* 10D. *Plant & Cell Physiology*, **49**: 117-120.

Ohresser, M., Matagne, R.F. & Loppes, R. (1997). Expression of the arylsulphatase reporter gene under the control of the *nit1* promoter in *Chlamydomonas reinhardtii*. *Current Genetics*, **31**: 264-271.

Ortiz-Marquez, J.C.F., Do Nascimento, M., Zehr, J.P. & Curatti, L. (2013). Genetic engineering of multispecies microbial cell factories as an alternative for bioenergy production. *Trends in Biotechnology*, **31**: 521-529.

Pabo, C.O., Peisach, E. & Grant, R.A. (2001). Design and selection of novel Cys2His2 zinc finger proteins. *Annual Reviews of Biochemistry*, **70**: 313-340.

Pan, Y., Shen, N., Jung-Klawitter, S., Betzen, C., Hoffmann, G.F., Hoheisel, J.D. & Blau, N. (2016). CRISPR RNA-guided FokI nucleases repair a PAH variant in a phenylketonuria model. *Scientific Reports*, **6**: 35794.

Patra, K.P., Li, F., Carter, D., Gregory, J.A., Baga, S., Reed, S.G., Mayfield, S.P. & Vinetz, J.M. (2015). Alga-produced malaria transmission-blocking vaccine candidate Pfs25 formulated with a human use-compatible potent adjuvant induces high-affinity antibodies that block *Plasmodium falciparum* infection of mosquitoes. *Infection and Immunity*, **83**: 1799-1808.

Patwary, M.U. & van der Meer, J.P. (1992). Genetics and breeding of cultivated seaweeds. *Algae*, **7**: 281-318.

Perrineau, M.M., Gross, J., Zelzion, E., Price, D.C., Levitan, O., Boyd, J. & Bhattacharya, D. (2014). Using Natural Selection to Explore the Adaptive Potential of *Chlamydomonas reinhardtii*. *PLoS One*, **9**: e92533.

Poulsen, N., Chesley, P.M. & Kroger, N. (2006). Molecular genetic manipulation of the diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Journal of Phycology*, **42**: 1059-1065.

Poulsen, N. & Kröger, N. (2005). A new molecular tool for transgenic diatoms - Control of mRNA and protein biosynthesis by an inducible promoter-terminator cassette. *The FEBS Journal*, **272**: 3413-3423.

Pratheesh, P.T., Vineetha, M. & Kurup, G.M. (2014). An efficient protocol for the *Agrobacterium*-mediated genetic transformation of microalga *Chlamydomonas reinhardtii*. *Molecular Biotechnology*, **56**: 507-515.

Prieto, R., Dubus, A., Galván, A. & Fernáandez, E. (1996). Isolation and characterization of two new negative regulatory mutants for nitrate assimilation in *Chlamydomonas reinhardtii* obtained by insertional mutagenesis. *Molecular & General Genetics*, **251**: 461-471.

Pulz, O. (2001). Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology Biotechnology*, **57:** 287-293.

Purton, S. & Rochaix, J.D. (1995). Characterisation of the ARG7 gene of *Chlamydomonas reinhardtii* and its application to nuclear transformation. *European Journal of Phycology*, **30**: 141-148.

Qin, S., Lin, H. & Jiang, P. (2012). Advances in genetic engineering of marine algae. *Biotechnology Advances*, **30**: 1602-1613.

Qin, S., Jiang, P. & Tseng, C. (2005). Transforming kelp into a marine bioreactor. *Trends in Biotechnology*, **23**: 264-268.

Radakovits, R., Eduafo, P.M. & Posewitz, M.C. (2011). Genetic engineering of fatty acid chain length in *Phaeodactylum tricornutum*. *Metabolic Engineering*, **13**: 89-95.

Radakovits, R., Jinkerson, R.E., Darzins, A. & Posewitz, M.C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell*, **9**: 486-501.

Ramakrishna, S., Kwaku Dad, A.B., Beloor, J., Gopalappa, R., Lee, S.K. & Kim, H. (2014). Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Research*, **24**: 1020-1027.

Ramessar, K., Peremarti, A., Gómez-Galera, S., Naqvi, S., Moralejo, M., Muñoz, P., Capell, T. & Christou, P. (2007). Biosafety and risk assessment framework for selectable marker genes in transgenic crop plants: a case of the science not supporting the politics. *Transgenic Research*, **16**: 261-280.

Ran, F.A., Hsu, P.D., Lin, C.Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y. & Zhang, F. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*, **154**: 1380-1389.

Rasala, B.A., Muto, M., Lee, P.A., Jager, M., Cardoso, R.M.F., Behnke, C.A., Kirk, P., Hokanson, C.A., Crea, R., Mendez, M. & Mayfield, S.P. (2010). Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnology Journal*, **8**: 719-733.

Sakaguchi, T., Nakajima, K. & Matsuda, Y. (2011). Identification of the UMP synthase gene by establishment of uracil auxotrophic mutants and the phenotypic complementation system in the marine diatom *Phaeodactylum tricornutum. Plant Physiology*, **156**: 78-89.

Sakuma, T., Nishikawa, A., Kume, S., Chayama, K. & Yamamoto, T. (2014). Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. *Scientific Reports*, **4**: 5400.

Sandesh Kamath, B., Vidhyavathi, R., Sarada, R. & Ravishankar, G.A. (2008). Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants. *Bioresource Technology*, **99**: 8667-8673.

Scaife, M.A. & Smith, A.G. (2016). Towards developing algal synthetic biology. *Biochemical Society Transactions*, **44**: 716-722.

Schmollinger, S., Strenkert, D. & Schroda, M. (2010). An inducible artificial microRNA system for *Chlamydomonas reinhardtii* confirms a key role for heat shock factor 1 in regulating thermotolerance. *Current Genetics*, **56**: 383-389.

Schroda, M., Vallon, O., Wollman, F.A. & Beck, C.F. (1999). A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *Plant Cell*, **11**: 1165-1178.

Scowcroft, W.R. & Larkin, P.J. (1988). Somaclonal variation. In Applications of plant cell and tissue culture (Bock, G. & Marsh, J., editors), 21-35. John Wiley and Sons Ltd, UK.

Sharma, S.K., Nelson, D.R., Abdrabu, R., Khraiwesh, B., Jijakli, K., Arnoux, M., O'Connor, M.J., Bahmani, T., Cai, H., Khapli, S., Jagannathan, R. & Salehi-

Ashtiani, K. (2015). An integrative Raman microscopy-based workflow for rapid in situ analysis of microalgal lipid bodies. *Biotechnology for Biofuels*, **8**: 164.

Shimogawara, K., Fujiwara, S., Grossman, A. & Usuda, H. (1998). High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics*, **148**: 1821-1828.

Shin, S.E., Lim, J.M., Koh, H.G., Kim, E.K., Kang, N.K., Jeon, S., Kwon, S., Shin, W. S., Lee, B., Hwangbo, K., Kim, J., Ye, S.H., Yun, J.Y., Seo, H., Oh, H.M., Kim, K.J., Kim, J.S., Jeong, W.J., Chang, Y.K. & Jeong, B. (2016). CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. *Scientific Reports*, **6**: 27810.

Sizova, I., Fuhrmann, M. & Hegemann, P. (2011). A *Streptomyces rimosus aphVIII* gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. *Gene*, **277**: 221-229.

Sizova, I., Greiner, A., Awasthi, M., Kateriya, S. & Hegemann, P. (2013). Nuclear gene targeting in *Chlamydomonas* using engineered zinc-finger nucleases. *Plant Journal*, **73**: 873-882.

Sode, K., Tatara, M., Takeyama, H., Burgess, J.G. & Matsunaga, T. (1992). Conjugative gene-transfer in marine cyanobacteria - *Synechococcus* sp., *Synechocystis* sp. and *Pseudanabaena* sp. *Applied Microbiology & Biotechnology*, **37**: 369-373.

Sodeinde, O.A. & Kindle, K.L. (1993). Homologous recombination in the nuclear genome of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, **90**: 9199-9203.

Son, S.H., Ahn, J.W., Uji, T., Choi, D.W., Park, E.J., Hwang, M.S., Liu, J.R., Choi, D., Mikami, K. & Jeong, W.J. (2012). Development of an expression system using the heat shock protein 70 promoter in the red macroalga, *Porphyra tenera*. *Journal of Applied Phycology*, **24**: 79-87.

Srinivasan, R. & Gothandam, K.M. (2016). Synergistic action of D-glucose and acetosyringone on *Agrobacterium* strains for efficient *Dunaliella* transformation. *PLoS One*, **11**: e0158322.

Steinbrenner, J. & Sandmann, G. (2006). Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. *Applied & Environmental Microbiology*, **72**: 7477-7484.

Sternberg, S.H., Redding, S., Jinek, M., Greene, E.C. & Doudna, J.A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature*, **507**: 62-67.

Sun, M., Qian, K., Su, N., Chang, H., Liu, J. & Shen, G. (2003). Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. *Biotechnology Letters*, **25**: 1087-1092.

Sun, G.H., Zhang, X.C., Sui Z.H. & Mao, Y.X. (2008). Inhibition of *pds* gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). *Marine Biotechnology*, **10**: 219-226.

Surzycki, R., Greenham, K., Kitayama, K., Dibal, F., Wagner, R., Rochaix, J.D., Ajam, T. & Surzycki, S. (2009). Factors effecting expression of vaccines in microalgae. *Biologicals*, **37**: 133-138.

Szyjka, S.J., Mandal, S., Schoepp, N.G., Tyler, B.M., Yohn, C.B., Poon, Y.S., Villareal, S., Burkart, M.D., Shurin, J.B. & Mayfield, S.P. (2017). Evaluation of phenotype

stability and ecological risk of a genetically engineered alga in open pond production. *Algal Research*, **24**: 378-386.

Takahashi, M., Mikami, K., Mizuta, H. & Saga, N. (2011). Identification and efficient utilization of antibiotics for the development of a stable transformation system in *Porphyra yezoensis* (Bangiales, Rhodophyta). *Journal of Aquaculture Research & Development*, **S3**: 002.

Tan, C., Qin, S., Zhang, Q. Jiang, P. & Zhao, F. (2005). Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. *Journal of Microbiology*, **43**: 361-365.

Te, M.R., Lohuis & Miller, D.J. (1998). Genetic transformation of dinoflagellates (*Amphidinium* and *Symbiodinium*): expression of GUS in microalgae using heterologous promoter constructs. *The Plant Journal*, **13**: 427-435.

Tenaillon, O., Rodriguez-Verdugo, A., Gaut, R.L., McDonald, P., Bennett, A.F., Long, A.D. & Gaut, B.S. (2012). The molecular diversity of adaptive convergence. *Science*, **335**: 457-461.

Teng, C., Qin, S., Liu, J., Yu, D., Liang, C. & Tseng, C. (2002). Transient expression of *lacZ* in bombarded unicellular green alga *Haematococcus pluvialis*. *Journal of Applied Phycology*, **14**: 495-500.

Tillich, U.M., Wolter. N., Franke, P., Dühring, U. & Frohme, M. (2014). Screening and genetic characterization of thermo-tolerant *Synechocystis* sp. PCC6803 strains created by adaptive evolution. *BMC Biotechnology*, **14**: 66.

Tiwari, M., Sharma, D. & Trivedi, P.K. (2014). Artificial microRNA mediated gene silencing in plants: progress and perspectives. *Plant Molecular Biology*, **86**: 1-18.

Tolonen, A.C., Liszt, G.B. & Hess, W.R. (2006). Genetic manipulation of *Prochlorococcus* strain MIT9313: green fluorescent protein expression from an RSF1010 plasmid and Tn5 transposition. *Applied & Environmental Microbiology*, **72**: 7607-7613.

Toyomizu, M., Suzuki, K., Kawata, Y., Kojima, H. & Akiba, Y. (2001). Effective transformation of the cynobacterium *Spirulina platensis* using electroporation. *Journal of Applied Phycology*, **13**: 209-214.

Tran, M., Zhou, B., Pettersson, P.L., Gonzalez, M.J. & Mayfield, S.P. (2009). Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. *Biotechnology & Bioengineering*, **104**: 663-673.

Tsai, S.Q., Wyvekens, N., Khayter, C., Foden, J.A., Thapar, V., Reyon, D., Goodwin, M.J., Aryee, M.J. & Joung, J.K. (2014). Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nature Biotechnology*, **32**: 569-576.

Uchiyama, J., Kanesaki, Y., Iwata, N., Asakura, R., Funamizu, K., Tasaki, R., Agatsuma, M., Tahara, H., Matsuhashi, A., Yoshikawa, H., Ogawa, S. & Ohta, H. (2015). Genomic analysis of parallel-evolved cyanobacterium *Synechocystis* sp. PCC 6803 under acid stress. *Photosynthesis Research*, **125**: 243-254.

Uji, T., Hirata, R., Fukuda, S., Mizuta, H. & Saga, N. (2014). A codon-optimized bacterial antibiotic gene used as selection marker for stable nuclear transformation in the marine red alga *Pyropia yezoensis*. *Marine Biotechnology*, **16**: 251-255.

Uji, T., Takahashi, M., Saga, N. & Mikami, K. (2010). Visualization of nuclear localization of transcription factors with cyan and green fluorescent proteins in the red alga *Porphyra yezoensis*. *Marine Biotechnology*, **12**: 150-159.

Urnov, F.D., Miller, J.C., Lee, Y.L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D. & Holmes, M.C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, **435**: 646-651.

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S. & Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics*, **11**: 636-646.

Velmurugan, N., Sung, M., Yim, S.S., Park, M.S., Yang, J.W. & Jeong, K.J. (2014). Systematically programmed adaptive evolution reveals potential role of carbon and nitrogen pathways during lipid accumulation in *Chlamydomonas reinhardtii*. *Biotechnology for Biofuels*, **7**: 117.

Walsh, R.M. & Hochedlinger, K. (2013). A variant CRISPR-Cas9 system adds versatility to genome engineering. *Proceedings of the National Academy of Sciences of the United States of America*, **110**: 15514-15515.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F. & Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, **153**: 910-918.

Wang, J., Jiang, P., Cui, Y., Deng, X., Li, F., Liu, J. & Qin, S. (2010a). Genetic transformation in *Kappaphycus alvarezii* using micro-particle bombardment: a potential strategy for germplasm improvement. *Aquaculture International*, **18**: 1027-1034.

Wang, J., Jiang, P., Cui, Y., Guan, X. & Qin, S. (2010b). Gene transfer into conchospores of *Porphyra haitanensis* (Bangiales, Rhodophyta) by glass bead agitation. *Phycologia*, **49**: 355-360.

Wang, Q., Lu, Y., Xin, Y., Wei, L., Huang, S. & Xu, J. (2016). Genome editing of model oleaginous microalgae *Nannochloropsis* spp. by CRISPR/Cas9. *The Plant Journal*, **88:** 1071-1081.

Wang, Y., Shi, M., Niu, X., Zhang, X., Gao, L., Chen, L., Wang, J. & Zhang, W. (2014). Metabolomic basis of laboratory evolution of butanol tolerance in photosynthetic *Synechocystis* sp. PCC 6803. *Microbial Cell Factories*, **13**: 151.

Wang, B., Wang, J., Zhang, W. & Meldrum D.R. (2012). Application of synthetic biology in cyanobacteria and algae. *Frontiers in Microbiology*, **3:** 344.

Weber, E., Engler, C., Gruetzner, R., Werner, S. & Marillonnet, S. (2011). A modular cloning system for standardized assembly of multigene constructs. *PLoS One*, **6**: e16765.

Wei, L., Xin, Y., Wang, Q., Yang, J., Hu, H. & Xu, J. (2017). RNAi-based targeted gene knockdown in the model oleaginous microalgae *Nannochloropsis oceanica*. *The Plant Journal*, **89**: 1236-1250.

Weyman, P.D., Beeri, K., Lefebvre, S.C., Rivera, J., McCarthy, J.K., Heuberger, A.L., Peers, G., Allen, A.E. & Dupont, C.L. (2015). Inactivation of *Phaeodactylum tricornutum* urease gene using transcription activator-like effector nuclease-based targeted mutagenesis. *Plant Biotechnology Journal*, **13**: 460-470.

Wu, C.Y. & Lin, G.H. (1987). Progress in the genetics and breeding of economic seaweeds in China. *Hydrobiology*, **151/152**: 57-61.

Xie, S., Shen, B., Zhang, C., Huang, X. & Zhang, Y. (2014). sgRNAcas9: A software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. *PLoS One*, **9**: e100448.

Xing, H.L., Dong, L., Wang, Z.P., Zhang, H.Y., Han, C.Y., Liu, B., Wang, X.C. & Chen, Q.J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*, **14**: 327.

Xu, P., Zhang, Y., Kang, L., Roossinck, M.J. & Mysore, K.S. (2006). Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiology*, **142**: 429-440.

Yamasaki, T., Miyasaka, H. & Ohama, T. (2008). Unstable RNAi effects through epigenetic silencing of an inverted repeat transgene in *Chlamydomonas reinhardtii*. *Genetics*, **180**: 1927-1944.

Yi, Z., Xu, M., Magnusdottir, M., Zhang, Y., Brynjolfsson, S. & Fu, W. (2015). Photo-oxidative stress-driven mutagenesis and adaptive evolution on the marine diatom *Phaeodactylum tricornutum* for enhanced carotenoid accumulation. *Marine Drugs*, **13**: 6138-6151.

Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., La Russa, M., Xie, M., Ding, S. & Qi, L.S. (2015). Small molecules enhance CRISPR genome editing in pluripotent stem cells. *Cell Stem Cell*, **16**: 142-147.

Yu, D.Z., Qin S., Sun G.Q. & Tzeng C.K. (2002). Transient expression of *lacZ* reporter gene in the economic seaweed *Undaria pinnatifida*. *High Technology Letters*, **12**: 93-95.

Yu, S., Zhao, Q., Miao, X. & Shi, J. (2013). Enhancement of lipid production in low-starch mutants *Chlamydomonas reinhardtii* by adaptive laboratory evolution. *Bioresource Technology*, **147**: 499-507.

Zaslavskaia, L.A., Lippmeier, J.C., Kroth, P.G., Grossman, A.R. & Apt, K.E. (2000). Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with

a variety of selectable marker and reporter genes. *Journal of Phycology*, **36**: 379-386.

Zhang, Y., Jiang, P., Gao, J., Liao, J., Sun, S., Shen, Z. & Qin, S. (2008). Recombinant expression of rt-PA gene (encoding Reteplase) in gametophytes of the seaweed *Laminaria japonica* (Laminariales, Phaeophyta). *Science in China Series C-Life Sciences*, **51**: 1116-1120.

Zhang, F., Wen, Y. & Guo, X. (2014). CRISPR/Cas9 for genome editing: progress, implications and challenges. *Human Molecular Genetics*, **23**: R40-R46.

Zhao, T., Li, G., Mi, S., Li, S., Hannon, G.J., Wang, X.J. & Qi, Y. (2007). A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. *Genes & Development*, **21**: 1190-1203.

Zhao, T., Wang, W., Bai, X. & Qi, Y. (2009). Gene silencing by artificial microRNAs in *Chlamydomonas*. *The Plant Journal*, **58**: 157-164.

Zorin, B., Grundman, O., Khozin-Goldberg, I., Leu, S., Shapira, M., Kaye, Y., Tourasse, N., Vallon, O. & Boussiba, S. (2014). Development of a nuclear transformation system for oleaginous green alga *Lobosphaera (Parietochloris) incisa* and genetic complementation of a mutant strain, deficient in arachidonic acid biosynthesis. *PLoS One*, **9**: e105223.

Zuo, Z., Li, B., Wang, C., Cai, J. & Chen, Y. (2007). Increasing transient expression of *CAT* gene in *Porphyra haitanensis* by matrix attachment regions and 18S rDNA targeted homologous recombination. *Aquaculture Research*, **38**: 681-688.

Table 1. Transformation of algae through conventional gene introduction methods (adapted from Gan *et al*., 2016).

Methods	Macroalgae	Microalgae
Conjugation		*Prochlorococcus* (Tolonen *et al*., 2006) *Pseudanabaena* (Sode *et al*., 1992) *Synechococcus* (Sode *et al*., 1992; Brahamsha, 1996) *Synechocystis* (Sode *et al*., 1992)
Agitation with glass beads	*Pyropia haitanensis* (Wang *et al*., 2010b)	*Chlamydomonas reinhardtii* (Kindle, 1990; Purton & Rochaix, 1995; Ohresser *et al*., 1997; León *et al*., 2007; Neupert *et al*., 2012) *Dunaliella salina* (Feng *et al*., 2009)
Agitation with silicon carbon whiskers		*Amphidinium* sp. (Te *et al*., 1998) *C. reinhardtii* (Dunahay, 1993) *Symbiodinium microadriaticum* (Te *et al*., 1998)
Electroporation	*Pyropia yezoensis* (Kuang *et al*., 1998; He *et*	*C. reinhardtii* (Brown *et al*., 1991; Shimogawara *et al*.,

	al., 2001; Hado *et al*., 2003; Liu *et al*., 2003; Mizukami *et al*., 2004; Gong *et al*., 2007) *P. haitanensis* (Zuo *et al*., 2007) *P. miniata* (Kübler *et al*., 1994) *Ulva lactuca* (Huang *et al*., 1996)	1998) *Chlorella saccharophilia* (Maruyama *et al*., 1994) *C. vulgaris* (Chow & Tung, 1999; Niu *et al*., 2011) *C. zofingiensis* (Liu *et al*., 2014) *D. salina* (Geng *et al*., 2003) *Lobosphaera (Parietochloris) incisa* (Zorin *et al*., 2014) *Nannochloropsis* (Kilian *et al*., 2011) *Scenedesmus obliquus* (Guo *et al*., 2013) *Spirulina platensis* (Toyomizu *et al*., 2001) *Synechococcus* (Matsunaga *et al*., 1990)
Biolistic microparticle bombardment	Bangiophycean algae (Hirata *et al*., 2011) *Gracilaria changii* (Gan *et al*., 2003) *G. gracilis* (Huddy *et al*., 2012) *Kappaphycus alvarezii* (Kurtzman & Cheney, 1991; Wang *et al*., 2010*a*)	*Chaetoceros* sp. (Miyagawa *et al*., 2011) *C. reinhardtii* (Bateman & Purton, 2000; Franklin *et al*., 2002; Mayfield *et al*., 2003; Sun *et al*., 2003; Neupert *et al*., 2012) *C. zofingiensis* (Liu *et al*., 2014)

	Laminaria japonica (Jiang *et al*., 2002; 2003; Qin *et al*., 2005; Zhang *et al*., 2008; Li *et al*., 2009) *Pyropia tenera* (Son *et al*., 2012) *P. yezoensis* (Kuang *et al*., 1998, Fukuda *et al*., 2008; Mikami *et al*., 2009; Uji *et al*., 2010; 2014; Kong *et al*., 2017) *Undaria pinnatifida* (Yu *et al*., 2002) *Ulva pertusa* (Kakinuma *et al*., 2009)	*Cylindrotheca fusiformis* (Poulsen & Kröger, 2005) *D. salina* (Tan *et al*., 2005) *Euglena gracilis* (Doetsch *et al*., 2001) *Gonium pectorale* (Lerche & Hallmann, 2009). *Haematococcus pluvialis* (Teng *et al*., 2002) *Navicula saprophila* (Dunahay *et al*., 1995) *Phaeodactylum tricornutum* (Apt *et al*., 1996; Falciatore *et al*., 1999; Zaslavskaia *et al*., 2000; Miyagawa *et al*., 2009) *Porphyridium* sp. (Lapidot *et al*., 2002) *Thalassiosira pseudonana* (Poulsen *et al*., 2006) *Volvox carteri* (Hallmann *et al*., 1997; Jakobiak *et al*., 2004)
Polyethylene glycol/ dimethyl sulfoxide		*Chlorella* (Hawkins & Nakamura, 1999) *C. ellipsoidea* (Kim *et al*., 2002; Liu *et al*., 2013)

		Cyanidioschyzon merolae (Ohnuma *et al*., 2008)
Agrobacterium-mediated transformation	*P. yezoensis* (Cheney *et al*., 2001)	*C. reinhardtii* (Kumar *et al*., 2004; Pratheesh *et al*., 2014)
		Dunaliella bardawil (Anila *et al*., 2011)
		D. salina (Srinivasan & Gothandam, 2016)
		H. pluvialis (Kathiresan *et al*., 2009)
		Nannochloropsis sp. (Cha *et al*., 2011)
		Schizochytrium (Cheng *et al*., 2012)
Positively charged nanoparticles		*C. reinhardtii* (Kim *et al*., 2014)