

**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; Chepurinov *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  $\beta$ -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.*, 2010a; 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.*, 2010a; 2010b) and ubiquitin- $\Omega$  (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  $\beta$ -galactosidase (lacZ),  $\beta$ -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 *psbA* 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 *FokI* 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 *FokI*. 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 *FokI* 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 FokI nucleases were utilized to achieve higher specificity (> fourfold) compared to paired nickases (Guilinger *et al.*, 2014). It is known that dimerization of FokI nuclease is necessary for DNA cleavage (Bitinaite *et al.*, 1998), hence, in this approach, dead Cas9 (dCas9) or inactive Cas9 was fused to FokI 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 (Miyaoaka *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 (Miyaoaka *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  $\beta$ 3-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), *Pseudochoircystis 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).

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

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

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

Polyethylene glycol/  
 dimethyl sulfoxide



*Agrobacterium*-  
mediated  
transformation

*P. yezoensis* (Cheney *et al.*, 2001)

*Cyanidioschyzon merolae* (Ohnuma *et al.*, 2008)

*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)

*C. reinhardtii* (Kim *et al.*, 2014)

Positively charged  
nanoparticles

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