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 (Scowcroft & 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., 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-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 sulfonyluron 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).
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 (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
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


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


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

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<th>Methods</th>
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<td>carbon whiskers</td>
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<td>C. reinhardtii (Dunahay, 1993)</td>
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<td>Electroporation</td>
<td>Pyropia yezoensis (Kuang et al., 1998; He et</td>
<td>C. reinhardtii (Brown et al., 1991; Shimogawara et al.,</td>
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al., 2001; Hado et al., 2003; Liu et al., 2003; 1998)
Mizukami et al., 2004; Gong et al., 2007) Chlorella saccharophila (Maruyama et al., 1994)
P. haitanensis (Zuo et al., 2007) C. vulgaris (Chow & Tung, 1999; Niu et al., 2011)
P. miniata (Kübler et al., 1994) C. zofingiensis (Liu et al., 2014)
Ulva lactuca (Huang et al., 1996) 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) Chaetoceros sp. (Miyagawa et al., 2011)
Gracilaria changii (Gan et al., 2003) C. reinhardtii (Bateman & Purton, 2000; Franklin et al.,
G. gracilis (Huddy et al., 2012) 2002; Mayfield et al., 2003; Sun et al., 2003; Neupert et
Kappaphycus alvarezii (Kurtzman & Cheney, al., 2012)
1991; Wang et al., 2010α) C. zofingiensis (Liu et al., 2014)
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<td><em>Laminaria japonica</em></td>
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<td>Poulsen &amp; Kröger, 2005</td>
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