Appl Microbiol Biotechnol (2016) 100:10225–10235
DOI 10.1007/s00253-016-7948-z

MINI-REVIEW

Cisgenesis and intragenesis in microalgae: promising advancements towards sustainable metabolites production

Janeth I. Galarza[1,2] · **Ninoska Delgado**[1] · **Vitalia Henríquez**[1]

Received: 6 July 2016 / Revised: 12 October 2016 / Accepted: 18 October 2016 / Published online: 7 November 2016

Abstract Microalgae are an economically important source of biomolecules and metabolites that can be exploited as feed, nutraceuticals and, potentially, as biofuels, among other biotechnological applications. Microalgae biotechnology involves both culture and metabolic pathways manipulation to obtain high-value products, such as omega-3 fatty acids and carotenoids. However, the introduction of genes and/or foreign regulatory sequences has caused public concern about the effect of genetically modified microalgae to achieve greater secondary metabolite accumulations. To placate these worries, we have focused on two cutting-edge concepts, cisgenesis and intragenesis in order to sustainably produce commercially relevant metabolites. This review provides updated background on current and future uses for microalgae molecular farming. We also discuss the development of genetic tools used in terrestrial plants to obtain genetically modified microalgae free of foreign DNA by means of (i) site-specific mutations, (ii) excision of selectable markers, (iii) zinc-finger nuclease and transcription activator-like effectors, and (iv) CRISPR/Cas9 systems. It is currently important to consider scientific debate not only from a technological standpoint but also in terms of conceptual, socioeconomic, ethical, and legal aspects.

Keywords Microalgae · Transgenesis · Cisgenesis · Intragenesis

✉ Vitalia Henríquez
vitalia.henriquez@pucv.cl

[1] Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

[2] Laboratorio de Biotecnología, Facultad de Ciencias del Mar, Universidad Estatal Península de Santa Elena, La Libertad, Ecuador

Introduction

Microalgae are a heterogenic group of eukaryotic and prokaryotic, unicellular, and colonial photosynthetic organisms that are highly important from an ecological standpoint. Microalgae primarily inhabit aquatic environments and can be found in waters extending from the tropics to the Polar Regions. Additionally, these organisms exhibit a wide tolerance range to different salinities, temperatures, pH levels, and nutrient availabilities (Borowitzka 1999; Cadoret et al. 2012; Singh et al. 2005; Spolaore et al. 2006).

Microalgae play a critical role in ecological terms, constituting the first link in the food chain. In particular, microalgae are the primary food source for zooplankton, which in turn, are a significant source of nutrition for higher aquatic organisms. Concomitant with this, close to half of worldwide photosynthesis and oxygen production is through microalgae. In fact, these organisms are strict photoautotrophs and necessarily require light energy for biological processes. Microalgae are extremely diverse, not only in terms of shape, size, and phylogeny, but also with regard to a wide range of highly valuable compounds. Currently, between 40,000 and 70,000 microalgae species have been identified from nine different phyla (Cadoret et al. 2012; Chisti 2007; Doan et al. 2011; Gimpel et al. 2015; Ghosh et al. 2016; Rindi et al. 2009). However, although many microalgae species have been identified, only a few have been successfully exploited for biotechnological ends (Olaizola 2003). The main cultured species are *Chlorella* and *Spirulina*, for human and animal consumption, and *Dunaliella salina* and *Haematococcus pluvialis*, for the extraction of β-carotene and astaxanthin, respectively. It is estimated that over 10,000 microalgae species remain undescribed and that these likely belong to biological groups completely different than those already defined (Sastre and Posten 2010).

The exploitation of microalgae for biotechnological purposes has increased in recent years, leading to diverse commercial applications for microalgae, including in pigments, nutritional supplements, antioxidants, fatty acids, cosmetics, and biofuels. These uses have resulted in the development of important pharmaceutical and nutraceutical products (Milledge 2011; Leu and Boussiba 2014; Pulz and Gross 2004; Qin et al. 2012). For example, microalgae are currently the primary sustainable source of carotenoids and omega-3 fatty acids (Borowitzka 2013; Leu and Boussiba 2014; Vílchez et al. 2011). Nutritional and biomedical applications are particularly adequate for microalgae as many of the biomolecules expressed by these organisms are generally recognized as safe for human consumption (Fletcher et al. 2007; Franklin et al. 2005; Walker et al. 2005).

The goals of this mini-review are to highlight the potential of microalgae as biotechnological "factories" for the production of commercially relevant metabolites and to describe alternative solutions for natural, sustainable, and environmentally friendly microalgae production. For this, two cutting-edge concepts in the field of genetically transforming microalgae will be discussed as new alternatives to transgenesis—cisgenesis and intragenesis (Abiri et al. 2015; Camacho et al. 2014; Espinoza et al. 2013; Holme et al. 2012; Singh et al. 2015).

Genetic engineering in microalgae

Microalgae have recently come to light as novel and attractive alternatives for producing recombinant proteins, including from therapeutic proteins to biofuels. Microalgae also have the capacity to over-express endogenous enzymes from particular metabolic pathways, thereby facilitating the accumulation of metabolites of interest, such as astaxanthin and polyunsaturated fatty acids (Adarme-Vega et al. 2012; Cadoret et al. 2012; Hamilton et al. 2014; Leu and Boussiba 2014; Steinbrenner and Sandmann 2006).

The high demand for, but productivity low, algae-based biotechnologies has promoted advanced research into the development of genetically engineered microalgae strains (Almaraz-Delgado et al. 2014). Transformation protocols exist for genetic manipulations of the nucleus (Kindle 1990), mitochondria (Remacle et al. 2006), and chloroplast (Boynton et al. 1988). Of these, chloroplasts have presented the best success in accumulating recombinant proteins for therapeutic uses, including oral vaccines, immunotoxins, and monoclonal antibodies, among others (Cadoret et al. 2008; Demurtas et al. 2013; Gimpel and Hyun 2015; Gregory et al. 2013; Jones et al. 2013; Jones and Mayfield 2013; Mussgnug 2015; Purton et al. 2013; Rasala and Mayfield 2015; Soria-Guerra et al. 2014; Specht and Mayfield 2014; Tran et al. 2013a, b).

To date, more than 30 different microalgae species have been successfully transformed (Radakovits et al. 2010), an encouraging figure for ongoing efforts to design algae that present selected characteristics. As compared with current systems used for producing recombinant proteins (e.g., terrestrial plants, mammalian cells, yeasts, and bacteria), microalgae present several comparative advantages. These advantages include low costs of production, ease of manipulation, and the fact that certain species of microalgae have been considered "generally regarded as safe" (GRAS) by the US Food and Drug Administration (Cadoret et al. 2008; Gutiérrez et al. 2012; Rasala and Mayfield 2015).

There is a growing interest in the use of microalgae as low-cost hosts for the synthesis of bioactive metabolites due to genomic, proteomic, and metabolomic advances in different species. Indeed, these advances have driven the development of novel technologies for microalgae genetic engineering (Doron et al. 2016; Hlavova et al. 2015). For instance, the manipulation of metabolic pathways can redirect cellular functions to the synthesis of determined products, giving rise to the direct control of a cellular organism through the introduction of extra copies of a particular enzyme (Gimpel et al. 2015; Henríquez et al. 2016; León et al. 2007). Innovation is further fueled by widespread interest in the commercial demand for the high-value compounds produced by microalgae, including for lutein from *Chlorella protothecoides*, β-carotene from *D. salina*, astaxanthin from *H. pluvialis* (Campenni et al. 2013; Kathiresan et al. 2015; Shi et al. 1997; Steinbrenner and Sandmann 2006; Sun et al. 2014; Ye and Jiang 2010), and biofuels (Burkart and Mayfield 2013; Ghosh et al. 2016; Gimpel et al. 2013; Glass 2015; Gouveia 2011; Grima et al. 2013; Hannon et al. 2010; Scranton et al. 2015).

Cisgenic and intragenic technologies

Genetic transformation is a technology used to produce recombinant proteins and accumulate commercially valuable metabolites. This technology is primarily applied in terrestrial plants (Abiri et al. 2015; Gupta et al. 2013; Halford 2012; Kempinski et al. 2015; Molesini et al. 2012; Napier et al. 2015; Ortiz Rios 2015; Ulukan 2011) and, to a lesser extent, in microalgae (Cui et al. 2014; Gregory et al. 2013; Liu et al. 2013, 2014; Rasala et al. 2010; Sharon-Gojman et al. 2014; Zheng et al. 2014; Zhong et al. 2011). The optimization of desired characteristics has led to the generation of genetically modified crops that do not compromise human or animal health and that are environmentally friendly. Likewise, significant efforts have been made to apply genetic transformation in higher plants through cisgenic and intragenic technologies to attain products similar to those naturally produced (Cardi 2016; Cotter et al. 2015; Espinoza et al. 2013; Haverkort

2015; Jacobsen and Schouten 2007; Mlalazi et al. 2012; Napier et al. 2015; Schaart et al. 2015; Vanblaere et al. 2011). These technologies are analogous to self-cloning and natural occurrence processes in which fragments of non-recombinant and recombinant DNA, respectively, from a sexually compatible organism are introduced to a host (Kasai et al. 2015).

Genetic transformation through transgenesis, cisgenesis, and intragenesis has been widely reviewed by a number of authors (Holme et al. 2012, 2013a, b; León et al. 2004; Schouten et al. 2006, 2008; Singh et al. 2015). Transgenesis consists in the transference of foreign genes or transgenes to a particular organism, leading to the expression of new characteristics. Furthermore, transgenesis involves the recombination of genetic material that cannot naturally hybridize (Gupta et al. 2013; Molesini et al. 2012; Ortiz Rios 2015). In turn, the terms cisgenesis and intragenesis are used to describe species genetically modified using genes and genetic elements exclusively originating from a sexually compatible donor, usually from the same or a phylogenetically related species (Holme et al. 2012; Mlalazi et al. 2012; Molesini et al. 2012). These concepts were introduced over a decade ago, and to date, certain crops have been modified by these techniques and have been widely accepted by society (Holme et al. 2013a, b).

Cisgenesis only involves the use of genes and genetic elements from the same species or from a cross-breedable species (Camacho et al. 2014). Full coding sequences, including gene introns with the corresponding promoter and terminator regions, should be used in the transformation, thereby preventing recombination with genetic material from foreign species (Holme et al. 2013a; Schouten et al. 2006). In turn, the concept of intragenesis implies the use of genes and regulatory elements from phylogenetically related and/or cross-breedable species (Camacho et al. 2014). Thus, the genes are manipulated and/or reordered in vitro, including intron removal and sense or antisense orientation. These manipulations are driven by promoter and terminator regions of other endogenous genes that do not necessarily correspond to the same cisgene (Holme et al. 2012; Molesini et al. 2012; Rommens et al. 2007). These types of technologies accelerate gene transference among sexually compatible species, circumventing the linkage drag associated with conventional interbreeding while favoring the exchange of genetic material. Moreover, intragenesis favors expression levels by using more efficient promotors, thus decreasing the instance of gene silencing. Figure 1 shows the three types of gene modification: transgenesis, cisgenesis, and intragenesis.

These new technologies are highly promising tools for algae-based biotechnologies since the resulting products can be considered natural and conserved from one generation to the next one. These features are particularly relevant to consider for the accumulation of omega-3 polyunsaturated fatty acids and carotenoids with nutraceutical applications (Shew et al. 2015; Sticklen 2015). Furthermore, diverse studies in microalgae demonstrate the underlying potential of metabolic engineering using endogenous genes for the accumulation of these bioactive compounds. Nevertheless, metabolic engineering is classified more as a partial intragenic technology as it contains selection markers and vector backbones.

Genes coding for key enzymes involved in the biosynthesis of fatty acids have been identified in a number of species (Adarme-Vega et al. 2012; Schuhmann et al. 2012), including *Ostreococcus tauri* (Wagner et al. 2010), *Ostreococcus* RCC809 (Vaezi et al. 2013), *Ostreococcus lucimarinus* (Petrie et al. 2010), *Thalassiosira pseudonana* (Tonon et al. 2005; Xu et al. 2013), *Phaeodactylum tricornutum* (Domergue et al. 2003), and in the model organism *Chlamydomonas reinhardtii* (Chi et al. 2008).

The overexpression of DGAT2 in *P. tricornutum* showed a significant increase in the proportion of polyunsaturated fatty acids, with EPA in particular increasing by 76.2 % (Niu et al. 2013). Likewise, the overexpression of delta-5-desaturase in the same microalgae resulted in a higher accumulation of EPA, specifically a 58 % increase (Peng et al. 2014). The coding sequences of delta-6-desaturase OtD6 and delta-5-desaturase OtElo5 cloned from *O. tauri* in *P. tricornutum* resulted in an increased accumulation of DHA (Hamilton et al. 2014). These three studies used the specific pPha-T1 nuclear vector of the *P. tricornutum* diatom (Zaslavskaia et al. 2000). Similarly, *Nannochloropsis oceanica* cells engineered with DGAT2 show significantly increased (53.1 %) saturated fatty acid content as compared with wild-type cells. On the other hand, monounsaturated fatty acids decreased by 52.9 %. Similarly, polyunsaturated fatty acids showed an apparent decrease of 74.6 %, including for arachidonic acid (C20:4) and EPA (C20:5) (Li et al. 2016).

The microalgae *Chlorella zofingiensis* and *H. pluvialis* are promising candidates for the genetic engineering of carotenoids, particularly considering the commercial relevance of these pigments (Henríquez et al. 2016). Various enzymes have been identified from the metabolic pathways of carotenoids, including phytoene synthase (PSY), phytoene desaturase (PDS), plastid terminal oxidase, lycopene cyclase (LCY-b), and β-carotene ketolase (CRTR-B) in *H. pluvialis* (Henríquez et al. 2016; Huang and Chen 2006a; Kajiwara et al. 1995; Lotan and Hirschberg 1995; Tan et al. 2007; Steinbrenner and Linden 2001, 2003). The mutated *pds* gene has been used in *H. pluvialis* as a dominant selection marker and reporter gene for transformation (Steinbrenner and Sandmann 2006).

Similarly, the genes involved in the biosynthesis of astaxanthin in *C. zofingiensis* include PSY (Cordero et al. 2011), PDS (Huang et al. 2008), LCY-b (Cordero et al. 2010), LCY-e (Cordero et al. 2012), BKT (CrtO) (Huang et al. 2006b), and CrtR-b (Chy-b) (Li et al. 2008). The

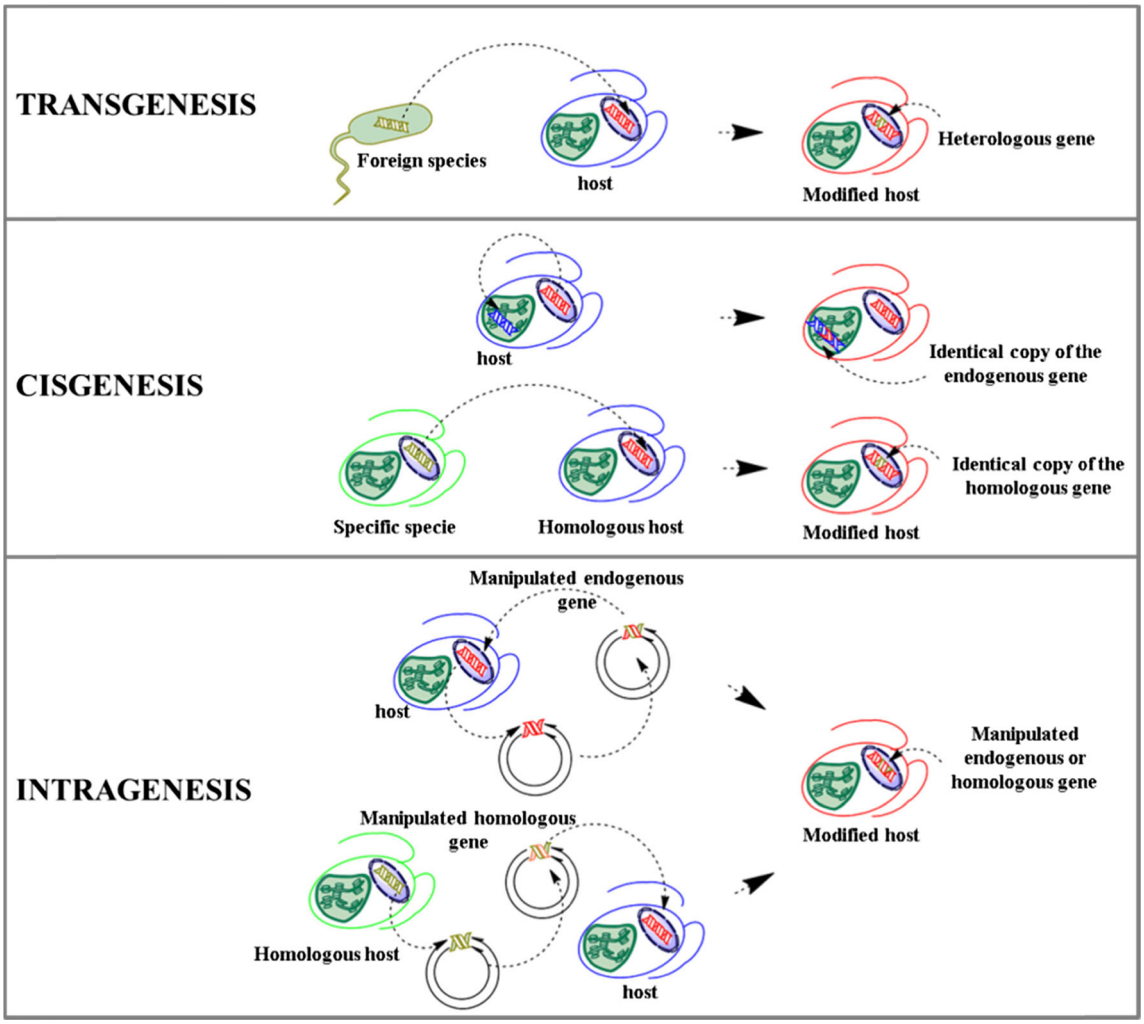

Fig. 1 General diagram for genetic transformation: transgenesis, cisgenesis, and intragenesis in microalgae

endogenous *pds* gene of *C. zofingiensis* was mutated and transformed, conferring resistance to norflurazon, and transformants accumulated 54.1 % more astaxanthin (Liu et al. 2014).

Cisgenesis and intragenesis have promising applications in microalgae. In particular, these technologies represent possible alternatives for increasing the accumulation of commercially relevant metabolites through the overexpression of native enzymes, in accordance with the needs of consumers and in line with environmental conservation (Adarme-Vega et al. 2012).

Generation of cisgenic and intragenic microalgae

The generation of new products through biotechnological means should not present human health risks; therefore, the presence of a selectable marker gene is inconvenient (Espinoza et al. 2013; Henley et al. 2013; Tuteja et al. 2012; Wannathong et al. 2016). The development of novel tools has facilitated an evident change in obtaining genetically modified organisms (GMOs) free of foreign DNA. These tools include (i) site-specific mutations, (ii) the excision of the selectable marker, (iii) zinc-finger nuclease and transcription activator-like effectors (TALEs), and (iv) Clustered Regularly Interspersed Short Palindromic Repeats System (CRISPR/Cas9) systems.

Site-specific mutations

Considering photoautotrophic growth, chloroplast transformations have used non-photosynthetic receptor strains with the mutated endogenous genes *atpB*, *psbA*, and *tscA* as reporters for recovering photosynthetic activities (Doron et al. 2016). Based on herbicide resistance, the *psbA* gene from *C. reinhardtii* encoding the D1 protein for the photosystem I has been mutated to modify the binding site for various herbicides (Johanningmeier et al. 1987). Indeed, resistance to the herbicide metribuzin was a key trait for the direct selection of *C. reinhardtii*

transformants (Przibilla et al. 1991). Similarly, and as previously mentioned, Steinbrenner and Sandmann (2006) mutated the *pds* gene by changing the leucine codon in position 504 for an arginine codon, which resulted in transformants exhibiting high resistance to the herbicide norflurazon. Furthermore, acquired resistance to the herbicide sulfometuron methyl has been demonstrated by mutating the AHAS gene, which codes for the enzyme acetohydroxyacid synthase (Lapidot 2002). On the other hand, and considering metabolic enzymes, reversing mutations of the *arg7* and *arg9* genes, which are both involved in arginine synthesis and respectively code for argininosuccinate lyase (Debuchy et al. 1989) and the *N*-acetyl ornithine aminotransferase protein (Remade et al. 2009), result in a restored ability to synthesize arginine. Likewise, other studies propose designing plasmids and using the endogenous *pds* gene as a dominant selectable marker for efficient nuclear transformations in *H. pluvialis* and *C. zofingiensis* (Sharon-Gojman et al. 2014). Genetic modifications using endogenous genes as selectable markers are an advancement towards successfully generating environmentally friendly cisgenic and transgenic microalgae.

Excision of selectable markers using direct repeats

Methods for the removal of selectable markers have already been described (Fischer et al. 1996). Selectable markers are inevitable in the selection of modified microalgae, resulting in the need to isolate stable transformants when cloning genes within the chloroplast. However, selectable markers can be eliminated when flanked by direct-repeat sequences that split the DNA sequence interspersed together with direct repeat copies. Excision of the selectable marker and restoration of the microalgal chloroplast genome is mediated by the native machinery of homologous recombination (Akbari et al. 2014; Day and Goldschmidt-Clermont 2011). Excision is a spontaneous process, where the frequency depends on the length of the direct repeat sequences, which should be between 400 and 650 base pairs (Fischer et al. 1996). Finally, a uniform population of chloroplast genomes free of the selectable marker will be produced.

According to Day and Goldschmidt-Clermont (2011), the split DNA is unstable and will be naturally lost. Also, the removed selection markers are not reintegrated nor does homologous recombination occur.

Roles of zinc-finger nucleases and transcription activator-like effector in editing the microalgal genome

Genes from and the genome of *C. reinhardtii* have been edited in situ by engineering nucleases via TALEs (Gao et al. 2014) and zinc-finger nucleases (Sizova et al. 2013). The principal difference between zinc-finger and TALE domains is the mechanism employed for recognizing DNA sequences. Zinc-finger modules always distinguish three base pairs, limiting the number of potential target sequences. In contrast, TALEs are composed of a variable number of four different DNA recognition domains, each of which specifically recognizes one of the four nucleotides. Therefore, 12–20 of these domains can fuse to create a TALE that binds to any desired target DNA sequence (Jinkerson and Jonikas 2015).

TALEs were originally obtained from Gram-negative, pathogenic plant bacteria of the *Xanthomonas* genus. These bacterial TALEs inject proteins into plant cells through a type III secretion system mechanism (SST3). The injected proteins are translocated to the nucleus and specifically bind with the promoter regions of certain genes in the host DNA. Consequently, the genes are activated, and the transcriptome is manipulated, which contributes to bacterial survival and colonization for promoting further infection (Boch and Bonas 2010; Bogdanove et al. 2010; Moscou and Bogdanove 2009; Romer et al. 2009). TALEs have become the effectors of choice for increasing transcription, surpassing other options, such as zinc-fingers and meganucleases, due to the relatively easy and low-cost of laboratory synthesis, high specificities for the selected sequences, and low cellular toxicity, among other noted advantages (Bogdanove and Voytas 2011; Gaj et al. 2013; de Lange et al. 2014; Moscou and Bogdanove 2009).

Artificially designed TALEs have been used for gene-specific activation in *Chlamydomonas*. Specifically, Gao et al. (2014) selected two endogenous *C. reinhardtii* genes, ARS1 and ARS2, as activation targets for TALE domains. Through the TALE domains, both target genes exhibited noticeably increased transcript and protein expressions, which were confirmed via protein activity by ARS colorimetric assays. This genome-editing technique has also been used in the model diatom *P. tricornutum* to interrupt the urease enzyme encoding gene as a method for clarifying the role of urease in the urea cycle and improving the molecular toolkit for diatoms (Weyman et al. 2016).

Zinc-finger nucleases involve a fusion of three to four zinc-finger DNA-binding modules to a FokI DNA cleavage domain, forming an artificial restriction enzyme (Jinkerson and Jonikas 2015). A pair of these molecular scissors can be tailored to target specific DNA sequences, where dimerization of the FokI domain activates the nuclease, provoking site-specific, double-stranded DNA breaks that enable genome editing (Townsend et al. 2009). In *C. reinhardtii*, this technology has been used to interrupt genes of interest (Sizova et al. 2013). Furthermore, a *C. reinhardtii* genome database exists that contains over 330,000 identified target sites, accounting for 93 % of all model genes (Reyon et al. 2012). This technology could be useful for redirecting particular metabolic pathways.

Role of CRISPR/Cas in editing the microalgal genome

Currently, the traditional methods for genome editing in higher plants are being replaced by CRISPR technology (Ahuja 2015; Cong et al. 2013; Horvath and Barrangou 2010; Jiang et al. 2014; Jinek et al. 2012; Marraffini and Sontheimer 2010; Puchta and Fauser 2014). The development of this cutting-edge technology in activating and/or inactivating native genes is ongoing and seeks to circumvent the use of exogenous genes, thereby creating crops that are more socially acceptable (Araki et al. 2014; Araki and Ishii 2015; Hartung and Schiemann 2014; Kanchiswamy et al. 2015; Voytas and Gao 2014). CAS9 is an RNA-guided DNA nuclease that has been successfully used for targeted mutations in eukaryotic genomes (Belhaj et al. 2013; Cong et al. 2013; Dominguez et al. 2016; Gaj et al. 2013; Jinkerson and Jonikas 2015; Mali et al. 2013; Mussgnug 2015). Similarly, a CRISPR/Cas system was recently identified and characterized in the genome of the freshwater cyanobacterium *Microcystis aeruginosa* (Yang et al. 2015). In microalgae, preliminary studies in *C. reinhardtii* indicate that CRISPR/Cas technology can be feasibly used for editing the nuclear genome (Greiner 2014; Jiang et al. 2014). However, stable transformants of *C. reinhardtii* expressing the CAS9 protein were unobtainable (Guihéneuf et al. 2016; Mussgnung 2015). Recently, this technology has been employed to efficiently generate stable targeted gene mutations in the marine diatom *P. tricornutum* (Nymark et al. 2016). Genetic engineering advancements in higher plants using endogenous genes and applying CRISPR/Cas technology ensure the generation of non-GMOs (Araki and Ishii 2015; Cardi 2016). These studies could serve as strategies for standard genome editing to create cisgenic and intragenic microalgae.

Regulatory considerations for the development of cisgenic and intragenic microalgae

In general, the regulatory frameworks established by international entities for the testing and development of genetically modified plants have been rather drastic (Camacho et al. 2014; Devos et al. 2014; Sundaramurthy 2010; Wijffels 2015; Willems et al. 2016). Worries regarding the dissemination of GMOs are linked to ethical considerations and apprehensions concerning health risks and the propagation of new gene combinations in the environment (Shew et al. 2015; Sticklen 2015). However, most nationally established regulations have been fundamentally based only on transgenic organisms, without giving consideration to the concepts of transgenesis, cisgenesis, or intragenesis (Molesini et al. 2012; Schouten et al. 2006). In particular, evaluations of GMOs are primarily based on regulations given in the still standing 2000 Cartagena Protocol on Biosafety (Hartung and Schiemann 2014). Worth considering is that in Japan, the self-cloning microalga *Pseudochoricytis ellipsoidea* is considered natural, not GMO, and corresponding experiments are not governed by GMO limitations outlined in the Cartagena Protocol on Biosafety (Lusser et al. 2012; USDA Japan Report 2014).

Today, more than ever, researchers are making significant efforts to generate GMOs, particularly for plants, which are environmentally friendly and safe for human and animal health. Cis- and intragenesis involve the exchange of DNA between interbreeding groups, due to which, imposed regulations should be similar to those applied to conventional breeding. Regulations on cisgenic and intragenic organisms are currently under debate in various countries, including in the European Union and USA, where regulations are close to being defined (Kasai et al. 2015). While cisgenic and intragenic crops have already been developed and field-tested, only one intragenic crop has been approved for commercial use, the Innate™ potato developed by Simplot Plant Sciences. Likewise, the Wageningen University and Research Centre has applied cisgenesis to develop a potato resistant to the late blight fungus *Phytophthora infestans*. To date, regulations are pending for considering these organisms separate from transgenic GMOs.

In the near future, the innate properties of microalgae will become important resources for biotechnological products. Therefore, governmental authorities should take note of ongoing advancements and duly consider microalgae produced through cisgenic and/or intragenic technologies. Furthermore, self-cloning technology is important for outdoor open-pond algae breeding, which is currently considered the most viable option for the large-scale cultivation of microalgae (Brennan and Owende 2010).

Conclusions

The application of cisgenesis and intragenesis in microalgae represents promising biotechnological tools for rapidly and securely advancing the commercial exploitation of attractive and highly valued compounds, particularly of polyunsaturated fatty acids and carotenoids. Furthermore, these technologies could become socially accepted and can be considered "consumer friendly." Cis- and intragenesis have already been applied in crops, but microalgae remain to be exploited, despite the existence of the required genetic tools. Both technologies require that microalgae be transformed only with genomic sequences derived from the same or sexually compatible species, and it is worth noting that foreign sequences, such as selectable markers and vector backbones, would be absent from cis- and intragenic microalgae.

Cisgenesis and intragenesis have provided new opportunities for discussions between scientists, producers, and consumers regarding the safety of genetically modified crops.

These discussions should serve as a basis for more flexible governmental regulations, which would likely lead to new crops generated through modern technologies. Finally, less restrictive regulations should reasonably exist for cisgenic and intragenic organisms considering that the gene pools used in these technologies are identical to conventional interbreeding. Indeed, these technologies could facilitate the scaling of environmentally safe open-culture systems.

Acknowledgements This work was funded by the Pontificia Universidad Católica de Valparaíso (grant no. PIUAS 037293).

Compliance with ethical standards

Conflict of interest JI Galarza declares that she has no conflict of interest. N Delgado declares that she has no conflict of interest. V Henríquez declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abiri R, Valdiani A, Mahmood SN, Sahebi M, Balia Z, Atabaki N, Talei D (2015) A critical review of the concept of transgenic plants: insights into pharmaceutical biotechnology and molecular farming. Mol Biol 18:21–42

Adarme-Vega TC, Lim DK, Timmins M, Vernen F, Li Y, Schenk PM (2012) Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microbiol Cell Fact 11:96. doi:10.1186/1475-2859-11-96

Ahuja MR (2015) Next generation plant biotechnology. In: Ramawat KG, Ahuja MR (eds) Biotechnology and biodiversity. Springer Int Publishing, Switzerland, pp 77–100

Akbari F, Morteza E, Ahmad YK (2014) The potential of transgenic green microalgae; a robust photobioreactor to produce recombinant therapeutic proteins. World J Microbiol Biotechnol 30:2783–2796

Almaraz-Delgado AL, Flores-Uribe J, Perez-España VH, Salgado-Manjarrez E, Badillo-Corona JA (2014) Production of therapeutic proteins in the chloroplast of *Chlamydomonas reinhardtii*. AMB Express 4(1):1–9

Araki M, Ishii T (2015) Towards social acceptance of plant breeding by genome editing. Trends Plant Sci 20:1–5

Araki M, Kumie N, Ishii T (2014) Caution required for handling genome editing technology. Trends Biotechnol 32:234–237

Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 9:39–49

Boch J, Bonas U (2010) *Xanthomonas* AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol 48:419–436

Bogdanove AJ, Voytas DF (2011) TAL effectors: customizable proteins for DNA targeting. Science 333(6051):1843–1846

Bogdanove AJ, Schornack S, Lahaye T (2010) TAL effectors: finding plant genes for disease and defense. Curr Opin Plant Biol 13:394–401

Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol 70:313–321

Borowitzka MA (2013) High-value products from microalgae-their development and commercialisation. J Appl Phycol 25:743–756

Boynton JE, Gillham NW, Harris EH (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534–1538

Brennan L, Owende P (2010) Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14:557–577

Burkart MD, Mayfield SP (2013) Biofuels for the twenty-first century editorial overview. Curr Opin Chem Biol 17:436–438

Cadoret J, Bardor M, Lerouge P, Cabigliera M, Henríquez V, Carlier A (2008) Les microalgues: usines cellulaires productrices de molecules commerciales recombinantes. Med Sci 24:375–382

Cadoret JP, Garnier M, Saint-Jean B (2012) Microalgae, functional genomics and biotechnology. Adv Bot Res 64:285–341

Camacho A, Allen V, Chi-Ham C, Bennett A (2014) Genetically engineered crops that fly under the US regulatory radar. Nat Biotechnol 32:1087–1091

Campenni L, Nobre BP, Santos CA, Oliveira AC, Aires-Barros MR, Palavra AMF, Gouveia L (2013) Carotenoid and lipid production by the autotrophic microalga *Chlorella protothecoides* under nutritional, salinity, and luminosity stress conditions. Appl Microbiol Biotechnol 97(3):1383–1393

Cardi T (2016) Cisgenesis and genome editing: combining concepts and efforts for a smarter use of genetic resources in crop breeding. Plant Breed. doi:10.1111/pbr.12345

Chi XY, Zhang XW, Guan XY, Ding L, Li YX, Wang MQ, Lin HZ, Qin S (2008) Fatty acid biosynthesis in eukaryotic photosynthetic microalgae: identification of a microsomal delta 12 desaturase in *Chlamydomonas reinhardtii*. J Microbiol 46:189–201

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Advances 25: 294–306

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

Cordero BF, Obraztsova I, Martín L, Couso I, León R, Vargas MA, Rodríguez H (2010) Isolation and characterization of a lycopene b-cyclase gene from the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). J Phycol 46:1229–1238

Cordero BF, Couso I, León R, Rodríguez H, Vargas MA (2011) Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*. Appl Microbiol Biotechnol 91:341–351

Cordero BF, Couso I, León R, Rodríguez H, Vargas MA (2012) Isolation and characterization of a lycopene e-cyclase gene of *Chlorella* (Chromochloris) *zofingiensis*. Regulation of the carotenogenic pathway by nitrogen and light. Mar Drugs 10:2069–2088

Cotter J, Zimmermann D, Bekkem H (2015) Application of the EU and Cartagena definitions of a GMO to the classification of plants developed by cisgenesis and gene-editing techniques. Greenpeace Res 1:2–18

Cui Y, Qin S, Jiang P (2014) Chloroplast transformation of *Platymonas* (*Tetraselmis*) *subcordiformis* with the bar gene as selectable marker. PLoS One. doi:10.1371/journal.pone.0098607

Day A, Goldschmidt-Clermont M (2011) The chloroplast transformation toolbox: selectable markers and marker removal. Plant Biotechnol J 9:540–553

de Lange O, Binder A, Lahaye T (2014) From dead leaf, to new life: TAL effectors as tools for synthetic biology. Plant J 78:753–771

Debuchy R, Purton S, Rochaix J-D (1989) The arginosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. EMBO J 8(10):2803–2809

Demurtas O, Massa S, Ferrante P, Venuti A, Franconi R, Giovanni G (2013) A *Chlamydomonas*-derived human *Papillomavirus* 16E7 vaccine induces specific tumor protection. PLoS One. doi:10.1371/journal.pone.0061473

Devos Y, Aguilera J, Liu Y, Paoletti C, Jardin P, Herman L, Perry J, Waigmann E (2014) EFSA's scientific activities and achievements on the risk assessment of genetically modified organisms (GMOs) during its first decade of existence: looking back and ahead. Transgenic Res 23:1–25

Doan T, Yen T, Sivaloganathan B, Obbard J (2011) Screening of marine microalgae for biodiesel feedstock. Biomass and Bioeng 35:2534–2544

Domergue F, Lerchl J, Zahringer U, Heinz E (2003) Cloning and functional characterization of *Phaeodactylum tricornutum* front–end desaturases involved in eicosapentaenoic acid biosynthesis. Eur J Biochem 269:4105–4113

Dominguez AA, Lim WA, Qi LS (2016) Beyond editing: repurposing CRSPR-Cas9 for precision genome regulation and interrogation. Nat Rev Mol Cell Biol 17:5–15

Doron L, Segal N, Shapira M (2016) Transgene expression in microalgae—from tools to applications. Front Plant Sci 7:505. doi:10.3389/fpls.2016.00505

Espinoza C, Schlechter R, Herrera D, Torres E, Serrano A, Medina C, Arce-Johnson P (2013) Cisgenesis and intragenesis: new tools for improving crops. Biol Res 46:323–331

Fischer N, Stampacchia O, Redding K, Rochaix J (1996) Selectable marker recycling in the chloroplast. Mol and General Genet 251: 373–380

Fletcher S, Muto M, Mayfield S (2007) Optimization of recombinant protein expression in the chloroplasts of green algae. Adv Exper Med Biol 616:90–98

Franklin SE, Mayfield SP (2005) Recent developments in the production of human therapeutic proteins in eukaryotic algae. Expert Opin Biol Ther 5:225–235

Gaj T, Gersbach C, Barbas C (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31:397–405

Gao H, Wrigh DA, Li T, Wang Y, Horken K, Weeks DP, Yang B, Spalding MH (2014) TALE activation of endogenous genes in *Chlamydomonas reinhardtii*. Algal Res 5:52–60

Ghosh A, Khanra S, Mondal M, Halder G, Tiwari O, Saini S, Bhowmick T, Gayen K (2016) Progress toward isolation of strains and genetically engineered strains of microalgae for production of biofuel and other value added chemicals: a review. Ener Convers Manage 113: 104–118

Gimpel JA, Hyun JS (2015) Production of recombinant proteins in microalgae at pilot greenhouse scale. Biotechnol and Bioengin 112:339–345

Gimpel JA, Specht EA, Georgianna DR, Mayfield SP (2013) Advances in microalgae engineering and synthetic biology applications for biofuel production. Curr Opi Chem Biol 17:489–495

Gimpel JA, Henríquez V, Mayfield SP (2015) In metabolic engineering of eukaryotic microalgae: potential and challenges come with great diversity. Front Microbiol. doi:10.3389/fmicb.2015.01376

Glass D (2015) Pathways to obtain regulatory approvals for the use of genetically modified algae in biofuel or biobased chemical production. Ind Biotechnol 11:71–83

Gouveia L (2011) Microalgae as a feedstock for biofuels. SpringerBriefs in Microbiology. doi:10.1007/978-3-642-17997-6_1

Gregory J, Topol A, Doerner D, Mayfield S (2013) Alga-produced cholera toxin-pfs25 fusion proteins as oral vaccines. App Enviroment Microbiol 79:3917–3925

Greiner A (2014) CRISPR/Cas9 und zinkfinger-nukleasen für die gezielte genstilllegung in *Chlamydomonas reinhardtii*. Dissertation, Humboldt University Berlin

Grima EM, González MJI, Giménez AG (2013) Solvent extraction for microalgae lipids. Algae for biofuels and energy. Edited by. In: Borowitzka MA, Moheimani NR (eds) New York. Springer, London, pp 187–205

Guihéneuf F, Khan A, Tran LP (2016) Genetic engineering: a promising toll to engender physiological, biochemical, and molecular stress resilience in green microalgae. Frontiers Plant Science 7. doi:10.3389/fpls.2016.00400

Gupta SM, Grover A, Nasim M (2013) Transgenic technologies in agriculture: from lab to field to market. CIBTech J Biotechnol 3(3):20–47

Gutiérrez C, Gimpel J, Escobar C, Marshall S, Henríquez V (2012) Chloroplast genetic tool for the green microalgae *Haematococcus pluvialis* (Chlorophyceae, Volvocales). J Phycol 48:976–983

Halford N (2012) Toward two decades of plant biotechnology: successes, failures, and prospects. Food Ener Security 1:9–28

Hamilton ML, Haslam RP, Napier JA, Sayanova O (2014) Metabolic engineering of *Phaeodactylum tricornutum* for the enhanced accumulation of omega-3 long chain polyunsaturated fatty acids. Metab Eng 22:3–9

Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S (2010) Biofuels from algae: challenges and potential. Biofuels 1(5):763–784

Hartung F, Schiemann J (2014) Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. Plant J 78:742–752

Haverkort AJ, Boonekamp PM, Hutten R, Jacobsen E, Lotz LAP, Kessel GJT, Vossen JH, Visser RGF (2016) Durable late blight resistance in potato through dynamic varieties obtained by cisgenesis: scientific and societal advances in the DuRPh project. Potato Res 59(1):35–66

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

Henríquez V, Escobar C, Galarza J, Gimpel J (2016) Carotenoids in microalgae. In: Stange C (ed) Carotenoids in nature, subcellular biochemistry, 79. Springer Int Publishing, Switzerland. doi: 10.1007/978-3-319-39126-7_8

Hlavova M, Turoczy Z, Bisova K (2015) Improving microalgae for biotechnology - from genetics to synthetic biology. Biotechnol Adv 33: 1194–1203

Holme I, Dionisio G, Brinch-Pedersen H, Wendt T, Madsen C, Vincze E, Holm P (2012) Cisgenic barley with improved phytase activity. Plant Biotechnol J 10:237–247

Holme I, Wendt T, Bach P (2013a) Current developments of intragenic and cisgenic crops. J Eastern Christian Studies 57:179–184

Holme I, Wendt T, Bach P (2013b) Intragenesis and cisgenesis as alternatives to transgenic crop development. Plant Biotechnol J 11:395–407

Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327:167–170

Huang JC, Chen F, Sandmann G (2006a) Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. J Biotechnol 122:176–185

Huang JC, Wang Y, Sandmann G, Chen F (2006b) Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 71:473–479

Huang J, Liu J, Li Y, Chen F (2008) Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). J Phycol 44:684–690

Jacobsen E, Schouten H (2007) Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends Biotechnol 25:219–223

Jiang W, Brueggeman AJ, Horken KM, Plucinak TM, Weeks DP (2014) Successful transient expression of cas9 and single guide RNA genes in *Chlamydomonas reinhardtii*. Eukaryot Cell 13:1465–1469

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

Jinkerson RE, Jonikas MC (2015) Molecular techniques to interrogate and edit the *Chlamydomonas* nuclear genome. Plant J 82:393–412

Johanningmeier U, Bodner U, Wildner GF (1987) A new mutation in the gene coding for the herbicide-binding protein in Chlamydomonas. Res Lett 211(2):221–224

Jones C, Mayfield S (2013) Steps toward a globally available malaria vaccine: harnessing the potential of algae for future low cost vaccines. Bioengineered 4:164–167

Jones C, Luong T, Hannon M (2013) Heterologous expression of the C-terminal antigenic domain of the malaria vaccine candidate Pfs48/45 in the green algae *Chlamydomonas reinhardtii*. App Microbiol Biotechnol 97:1987–1995

Kajiwara S, Kakizono T, Saito T, Kondo K, Ohtani T, Nishio N, Nagai S, Misawa N (1995) Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*. Plant Mol Biol 29(2): 343–352

Kanchiswamy CN, Malnoy M, Velasco R, Kim JS, Viola R (2015) Non-GMO genetically edited crop plants. Trends Biotechnol. doi:10.1016/j.tibtech.2015.04.002

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*. Biotechnol Biofuels 8:1–12

Kathiresan S, Chandrashekar A, Ravishankar G, Sarada R (2015) Regulation of astaxanthin and its intermediates through cloning and genetic transformation of β-carotene ketolase in *Haematococcus pluvialis*. J Biotechnol 197:33–41

Kempinski C, Jiang Z, Bell S, Chappell J (2015) Metabolic engineering of higher plants and algae for isoprenoid production. In advances in biochemical engineering/biotechnology. Springer Berlin, Heidelberg, p 161–99

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci U S A 87:1228–1232

Lapidot M (2002) Stable chloroplast transformation of the unicellular red alga *Porphyridium* species. Plant Physiol 129:7–12

León R, Couso I, Fernández E (2007) Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*. J Biotechnol 130:143–152

León-Bañares R, González-Ballester D, Galván A, Fernández E (2004) Transgenic microalgae as green cell-factories. Trends Biotechnol 22:45–52

Leu S, Boussiba S (2014) Advances in the production of high value products by microalgae. Ind Biotechnol 10:169–183

Li Y, Huang JL, Sandmann G, Chen F (2008) Glucose sensing and the mitochondria alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown *Chlorella zofingiensis* (Chlorophyceae). Planta 228:735–743

Li DW, Cen SY, Liu YK, Balamurugan S, Zheng XY, Alimujiang A, Yang WD, Liu JS, Li HY (2016) A type 2 diacylglycerol acyltransferase accelerates the triacylglycerol biosynthesis in heterokont oleaginous microalga *Nannochloropsis oceanica*. J Biotechnol 229: 65–71

Liu J, Gerken H, Huang J, Chen F (2013) Engineering of an endogenous phytoene desaturase gene as a dominant selectable marker for *Chlamydomonas reinhardtii* transformation and enhanced biosynthesis of carotenoids. Proc Biochem 48:788–795

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. App Microbiol Biotechnol 98:5069–5079

Lotan T, Hirschberg J (1995) Cloning and expression in *Escherichia coli* of the gene encoding β-C- 4-oxygenase, that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. FEBS Lett 364:125–128

Lusser M, Parisi C, Plan D, Rodriguez-Cerezo E (2012) Deployment new biotechnologies in plant breeding. Nat Biotechnol 30:231–239

Mali P, Esvelt K, Church G (2013) Cas9 as a versatile tool for engineering biology. Nat Methods 10:957–963

Marraffini L, Sontheimer E (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet 11:181–190

Milledge JJ (2011) Commercial application of microalgae other than as biofuels: a brief review. Rev Environ Sci Biotechnol 10(1):31–41

Mlalazi B, Welsch R, Namanya P, Khanna H, Geijskes R, Harrison M, Harding R, Dale J, Bateson M (2012) Isolation and functional characterisation of banana phytoene synthase genes as potential cisgenes. Plant 236:1585–1598

Molesini B, Pii Y, Pandolfini T (2012) Fruit improvement using intragenesis and artificial microRNA. Trends Biotechnol 30:80–88

Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. Science 326:1501

Mussgnug J (2015) Genetic tools and techniques for *Chlamydomonas reinhardtii*. App Microbiol Biotechnol 99:5407–5418

Napier J, Usher S, Haslam R, Ruiz-Lopez N, Sayanova O (2015) Transgenic plants as a sustainable, terrestrial source of fish oils. J Sci Technol 117:1317–1324

Niu YF, Zhang MH, Li DW, Yang WD, Liu JS, Bai WB, Li HY (2013) Improvement of neutral lipid and polyunsaturated fatty acid biosynthesis by overexpressing a type 2 diacylglycerol acyltransferase in marine diatom *Phaeodactylum tricornutum*. Mar Drugs 11(11): 4558–4569. doi:10.3390/md11114558

Nymark M, Sharma AK, Sparstad T, Bones AM, Winge P (2016) A CRISPR/Cas9 system adapted for gene editing in marine algae. Scientific Rep 6. doi:10.1038/srep24951

Olaizola M (2003) Commercial development of microalgal biotechnology: from the test tube to the marketplace. Biomol Eng 20:459–466

Ortiz Rios R (2015) Plant breeding in the Omics Era. In: Ortiz Rios R (ed) Genetic engineering and transgenic breeding. Springer Int Publishing, Switzerland. doi: 10.1007/978-3-319-20532-8_7

Peng KT, Zheng CN, Xue J, Chen XY, Yang WD, Liu JS, Bai W, Li HY (2014) Delta 5 fatty acid desaturase upregulates the synthesis of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum*. J Agric Food Chem 62(35):8773–8776

Petrie JR, Liu Q, Mackenzie AM, Shrestha P, Mansour MP, Robert SS, Frampton DF, Blackburn SI, Nichols PD, Singh SP (2010) Isolation and characterisation of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. Mar Biotechnol 12:430–438

Przibilla E, Heiss S, Johanningmeier U, Trebst A (1991) Site-specific mutagenesis of the D1 subunit of photosystem II in wild-type *Chlamydomonas*. Plant Cell 3:169–174

Puchta H, Fauser F (2014) Synthetic nucleases for genome engineering in plants: prospects for a bright future. Plant J 78:727–741

Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 65:635–648

Purton S, Szaub J, Wannathong T (2013) Genetic engineering of algal chloroplasts: progress and prospects. J Plant 60:491–499

Qin S, Lin H, Jiang P (2012) Advances in genetic engineering of marine. Biotechnol Advances 30(6):1602–1613

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

Rasala BA, Mayfield SP (2015) Photosynthetic biomanufacturing in green algae; production of recombinant proteins for industrial, nutritional, and medical uses. Photosynthesis Res. doi:10.1007/s11120-014-9994-7

Rasala BA, Muto M, Lee PA, Jager M, Cardoso RMF, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. Plant Biotechnol J 8:719–733

Remacle C, Cardol P, Coosemans N, Gaisne M, Bonnefoy N (2006) High-efficiency biolistic transformation of *Chlamydomonas* mitochondria can be used to insert mutations in complex I genes. Proc Natl Acad Sci U S A 103:4771–4776

Remade C, Cline S, Boutaffala L, Gabilly S, Larosa V, Barbieri R, Coosemans N, Hamel P (2009) The ARG9 gene encodes the

plastid-resident N-acetyl ornithine aminotransferase in the green alga *Chlamydomonas reinhardtii*. Eukaryotic Cell 8:1460–1463

Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK (2012) FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol 30:460–465

Rindi F, Lam D, López-Bautista J (2009) Phylogenetic relationships and species circumscription in Trentepohlia and Printzina (Trentepohliales, Chlorophyta). Mol Phylogen and Evol 52:329–339

Romer P, Recht S, Lahaye T (2009) A single plant resistance gene promoter engineered to recognize multiple TAL effectors from disparate pathogens. Proc Natl Acad Sci U S A 106:20526–20531

Rommens C, Haring M, Swords K, Davies H, Belknap W (2007) The intragenic approach as a new extension to traditional plant breeding. Trends Plant Sci 12:397–403

Sastre RR, Posten C (2010) The variety of microalgae applications as renewable source. Chemie Ingenieur Technik 82:1925–1939

Schaart J, van de Wiel C, Lotz L, Smulders M (2015) Opportunities for products of new plant breeding techniques. Trends Plant Sci. doi:10.1016/j.tplants.2015.11.006

Schouten HJ, Jacobsen E (2008) Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends Plant Sci 13:260–261

Schouten H, Krens J, Jacobsen E (2006) Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. EMBO Rep 7:750–753

Schuhmann H, Lim DKY, Schenk PM (2012) Perspectives on metabolic engineering for increased lipid contents in microalgae. Biofuels 3(1):71–86

Scranton MA, OStrand JT, Fields FJ, Mayfield SP (2015) Chlamydomonas as a model for biofuels and bio-products production. Plant J 82:523–531

Sharon-Gojman R, Maimon E, Leu S, Zarka A, Boussiba S (2014) Advanced methods for genetic engineering of *H. pluvialis*. App Microbiol Biotechnol 10:8–15

Shew A, Nalley L, Danforth D, Dixon B, Nayga R, Delwaide A, Valent B (2015) Are all GMOs the same? Consumer acceptance of cisgenic rice in India. Plant Biotechnol J 14:4–7

Shi XM, Chen F, Yuan JP, Chen H (1997) Heterotrophic production of lutein by selected chlorella strains. J Appl Phycol 9:445–450

Singh S, Kate BN, Banerjee UC (2005) Bioactive compounds from cyanobacteria and microalgae: an overview. Crit Rev Biotechnol 4:73–95

Singh A, Meenakshi J, Lamalakshmi D (2015) Alternative to transgenesis: cisgenesis and intragenesis. In: Al-Khayri JM (ed) Advances in plant breeding strategies: breeding, biotechnology and molecular tools. Springer Int Publishing, Switzerland, pp 345–367

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

Soria-Guerra R, Ramírez-Alonso J, Ibáñez-Salazar A, Govea-Alonso D, Paz-Maldonado L, Bañuelos-Hernández B, Korban S, Rosales-Mendoza S (2014) Expression of an HBcAg-based antigen carrying angiotensin II in *Chlamydomonas reinhardtii* as a candidate hypertension vaccine. Plant Cell 116:133–139

Specht E, Mayfield S (2014) Algae-based oral recombinant vaccines. Frontiers Microbiol 5:1–7

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 4:87–96

Steinbrenner J, Linden H (2001) Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. Plant Physiol 125(2):810–817

Steinbrenner J, Linden H (2003) Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: regulation by photosynthetic redox control. Plant Mol Biol 52(2):343–356

Steinbrenner J, Sandmann G (2006) Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. App Environm Microbiol 72:7477–7484

Sticklen M (2015) Transgenic, cisgenic, intragenic and subgenic crops. Adv Crop Science Technol 3:2–3

Sun Z, Liu J, Y B, Zhou Z (2014) Microalgae as the production platform for carotenoids. In: Liu J, Sun Z, Gerken H (eds) Recent advances in microalgal biotechnology. OMICS Group eBooks, Foster City

Sundaramurthy VT (2010) The impacts of the transgenes on the modified crops, non-target soil and terrestrial organisms. J Biotechnol 9: 9163–9176

Tan C, Zhao F, Su Z, Liang C, Qin S (2007) Expression of β-carotene hydroxylase gene (crtR-B) from the green alga *Haematococcus pluvialis* in chloroplasts of *Chlamydomonas reinhardtii*. J App Phycol 19:347–355

Tonon T, Sayanova O, Michaelson LV, Qing R, Harvey D, Larson TR, Li Y, Napier JA, Graham IA (2005) Fatty acid desaturases from the microalga *Thalassiosira pseudonana*. FEBS J 272:3401–3412

Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, Voytas DF (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459:442–445

Tran M, Ryan H, Siefker D, Van C, Newkirk G, Kim J, Bui J, Mayfield S (2013a) Production of anti-cancer immunotoxins in algae: ribosome inactivating proteins as fusion partners. Biotechnol Bioengin 110: 2826–2835

Tran M, Van C, Barrera D, Pär P, Peinado C, Bui J, Mayfield S (2013b) Production of unique immunotoxin cancer therapeutics in algal chloroplasts. Proc Natl Acad Sci U S A 110:15–22

Tuteja N, Verma S, Sahoo R, Raveendar S, Reddy B (2012) Recent advances in development of marker-free transgenic plants: regulation and biosafety concern. J Biosci 37:167–197

Ulukan H (2011) Plant genetic resources and breeding: current scenario and future prospects. J Agric Biol:447–454

USDA Japan Reports (2014) United States Department of Agriculture. Japan takes step forward to improve its GE product review process. Available at: usdajapan.org/en/reports/reports2014.html

Vaezi R, Napier JA, Sayanova O (2013) Identification and functional characterization of genes encoding omega-3 polyunsaturated fatty acid biosynthetic activities from unicellular microalgae. Mar Drugs 11(12):5116–5129

Vanblaere T, Szankowski I, Schaart J, Schouten H, Flachowsky H, Broggini G, Gessler C (2011) The development of a cisgenic apple plant. J Biotechnol 154:304–311

Vílchez C, Forján E, Cuaresma M, Bédmar F, Garbayo I, Vega J (2011) Marine carotenoids: biological functions and commercial applications. Mar Drugs 9:319–333

Voytas D, Gao C (2014) Precision genome engineering and agriculture: opportunities and regulatory challenges. PLoS Biol 12:–e1001877

Wagner M, Hoppe K, Czabany T, Heilmann M, Daum G, Feussner I, Fulda M (2010) Identification and characterization of an acyl-CoA: diacylglycerol acyltransferase 2 (DGAT2) gene from the microalga *O. tauri*. Plant Physiol Biochem 48:407–416. doi:10.1016/j.plaphy.2010.03.008

Walker T, Purton S, Becker D, Collet C (2005) Microalgae as bioreactors. Plant Cell Rep 24:629–641

Wannathong T, Waterhouse J, Young R, Economou C, Purton S (2016) New tools for chloroplast genetic engineering allow the synthesis of human growth hormone in the green alga *Chlamydomonas reinhardtii*. App Microbiol Biotechnol. doi:10.1007/s00253-016-7354-6

Weyman PD, Beeri K, Lefebre SC, Rivera J, Mccarthy JK, Heuberger AL, Peers G, Allen AE, Dupont CL (2016) Inactivation of *Phaeodactylum tricornutum* urease gene using transcription activator-like effector nuclease-based targeted mutagenesis. Plant Biotechnol J 13(4):460–470

Wijffels R, (2015) The need and risks of using transgenic microalgae for the production of food, feed, chemicals and fuels. In: Netherlands OECD (ed) Biosafety and the environmental uses of micro-organisms. Netherlands, p 60–71

Willems S, Fraiture M, Deforce D, De Keersmaecker S, De Loose M, Ruttink T, Herman P, Van F, Roosens N (2016) Statistical framework for detection of genetically modified organisms based on next generation sequencing. Food Chem 192:788–798

Xu J, Kazachkov M, Jia Y, Zheng Z, Zou J (2013) Expression of a type 2 diacylglycerol acyltransferase from *Thalassiosira pseudonana* in yeast leads to incorporation of docosahexaenoic acid b-oxidation intermediates into triacylglycerol. FEBS J 280:6162–6172

Yang C, Lin F, Li Q, Li T, Zhao J (2015) Comparative genomics reveals diversified CRISPR-Cas systems of globally distributed *Microcystis aeruginosa*, a freshwater bloom-forming cyanobacterium. Frontiers Microbiol 6:394. doi:10.3389/fmicb.2015.00394

Ye ZW, Jiang JG (2010) Analysis of an essential carotenogenic enzyme: ζ-carotene desaturase from unicellular alga *Dunaliella salina*. J Agri Food Chem 58:11477–11482

Zaslavskaia LA, Lippmeier JC, Kroth PG, Grossman AR, Apt KE (2000) Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. J Phycol 36:379–986

Zheng KJ, Wang CG, Xiao M, Chen J (2014) Expression of *bkt* and *bch* genes from *Haematococcus pluvialis* in transgenic *Chlamydomonas*. Sci China Life 57:1028–1033

Zhong Y, Huang J, Liu J, Li Y, Jiang Y, Xu Z, Sandmann G, Chen F (2011) Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic *Arabidopsis*. J Exper Bot 62:3659–3669