Research in Health Science
ISSN 2470-6205 (Print) ISSN 2470-6213 (Online)
Vol. 10, No. 4, 2025
www.scholink.org/ojs/index.php/rhs

Original Paper

Research on Gene Editing Technology of Single Cell Microalgae

Nuobin Lin[1,2,*]

[1] College of Life Science, Fujian Normal University, Fuzhou, 350117, China

[2] College of Ocean Studies, Ningde Normal University, Ningde, 352106, China

[*] Corresponding Author

Received: November 12, 2025 Accepted: December 22, 2025 Online Published: January 5, 2026

doi:10.22158/rhs.v10n4p157 URL: http://dx.doi.org/10.22158/rhs.v10n4p157

Abstract

Microalgae, a kind of unicellular photoautotrophs that widely exist in marine and freshwater. Microalgae can accumulate a great deal of metabolites in the process of growth, then they have high-value in bioenergy, food production, health care and animal feed. Although microalgae has great potential of development, its practical application is still slow because of its various biological species, incomplete genetic information research, complex industrial production process and so on. In recent years, due to the rapid development of genomics research, especially the emergence of new gene editing techniques, people can quickly efficiently and fully figure out the genetic and molecular information of microalgae. Therefore, wide attention has been paid to the application of new gene editing technology in microalgae breeding and microalgae fermentation engineering. Based on this, this paper briefly introduces some new gene editing techniques, such as zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats, CRISPR-associated system, and summarizes the application progress of the above techniques in microalgae gene editing and engineering.

Keywords

gene editing, microalgae, ZFN, TALEN, CRISPR-Cas9

1. Introduction

Bacillariophyta, *Chlorophyta* and *Cyanobacteria* in microalgae have been widely studied for more than a century (Mann & Myers, 1968). Compared with higher plants, microalgae have the advantages of fast growth, high biomass yield, and no competition for arable land (Bowler, Allen, Badger, Grimwood, Jabbari, Kuo, Maheswari, Martens, Maumus, & Otillar, 2008). Microalgae can accumulate a large

amount of high value-added substances such as polysaccharides, proteins, and oils during growth (Zhai, Hong, Wang, Wang, Zhao, Liu, & Zhang, 2022). Although the microalgae industry has great prospects for development, their metabolic pathways and regulatory networks have not yet been elucidated due to their wide variety and diverse genetic information. The lack of these technologies has hindered the progress of microalgae bioengineering (Koussa, Chaiboonchoe, & Salehi-Ashtiani, 2014).

In order to apply microalgae to modern fermentation process and give full play to the production potential of microalgae, it is necessary to use modern molecular biotechnology to explore the genetic information of microalgae, so as to solve the problems of low carbon fixation efficiency (Bhola, Swalaha, Ranjith Kumar, Singh, & Bux, 2014), slow accumulation of high-value additional products and high cost of industrial production (Ho, Ye, Hasunuma, Chang, & Kondo, 2014). If the metabolic regulation mechanism of microalgae is fully revealed, microalgae can accumulate a large amount of biomass under suitable conditions and produce high-value products (Leu & Boussiba, 2014). In recent years, due to the rapid development of bioinformatics and new gene editing technology, the use of gene editing to artificially transform and breed algae has become the main way to solve the above problems. TALEN and CRISPR-Cas9 technologies are the most widely used gene editing technologies in recent years, and have achieved good research progress in other organisms. The genome of single-cell microalgae is small and easy to be modified and applied to industrial production (Wani, Akhtar, Singh, Prakash, Raza, Cavalu, Chopra, Madkour, Elolimy, & Hashem, 2023). The use of TALEN and CRISPR-Cas9 technology can efficiently study gene function and construct industrialized algae species to promote the rapid development of related industries.

2. Research Progress and Current Status

2.1 Gene Editing Technology

Gene editing is to change the target gene fragment and inherit it by inserting, modifying, knocking out or replacing the specific gene fragment of the organism, so as to realize the transformation of the target gene (Liu, Kong, Liu, Li, & Xiao, 2024). In recent years, nucleases known as molecular scissors have greatly promoted the development of gene editing. Currently. There are three main types of gene editing technology: zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR-associated systems) nuclease system.

Table 1. Comparison of New Gene Editing Technologies

Edit Technology	ZFNs	TALENs	CRISPR-Cas9
Working mechanism	DNA-protein recognition	DNA-protein recognition	DNA-RNA recognition
DNA recognition domain	Zinc finger domain	Repeated variable amino acid sites (RVDs)	cr RNA / guide RNA (gRNA)
DNA cleavage domain	Fok I nuclease	Fok I nuclease	Cas9 protein
Advantages	High targeting, efficiency is better than homologous recombination	The specificity is stronger than ZFN, and the design is simple	Accurate targeting, low cytotoxicity and low cost
Defect	The design relies on upstream and downstream sequences, with high off-target rate and certain cytotoxicity	There are cytotoxicity problems and high cost	Targeting is limited by PAM sites
Reference	Smith, Bibikova, Whitby, Reddy, Chandrasegaran, & Carroll, 2000	Zu, Tong, Wang, Liu, Pan, Li, Hu, Luo, Huang, & Wu, 2013	Horvath & Barrangou, 2010

2.2 Application of Gene Editing Technology in Microalgae

Table 2. Application of Novel Gene Editing Techniques in Microalgae

Edit Technology	Algae species	Research gene	Research findings	Reference
ZFN	*Chlamydomonas reardtii*	*COP3*	For the first time, ZFN was used to edit Chlamydomonas reinhardtii genes and optimize ZFN technology for studying the function of microalgae genes	Sizova, Greiner, Awasthi, Kateriya, & Hegemann, 2013
TALEN	*Phaeodactylum tricornutum*	*UGPase*	For the first time, TALEN technology was applied to microalgae gene editing, and mutant strains with high expression of TAG were obtained	Daboussi, Leduc, Maréchal, Dubois, Guyot, Perez-Michaut, Amato, Falciatore, Juillerat, & Beurdeley, 2014

TALEN	*Phaeodactylum tricornutum*	*urease*	TALEN technology was used to knock out urease gene based on HDR pathway, and the role of urease in diatom metabolism was analyzed	Weyman, Beeri, Lefebvre, Rivera, McCarthy, Heuberger, Peers, Allen, & Dupont, 2015
TALEN	*Phaeodactylum tricornutum*	*PtAureo1α*	Knockout of *PtAureo1α* to obtain stable mutants, and establish a TALEN gene editing method suitable for diatoms	Serif, Lepetit, Weißert, Kroth, & Bartulos, 2017
CRISPR-Cas9	*Chlamydomonas reinhardtii*	*Mgfp, mGluc, Hygror*	CRISPR-Cas9 technology was used to edit green algae genes for the first time	Jiang, Brueggeman, Horken, Plucinak, & Weeks, 2014
CRISPR-Cas9	*Nannochloropsis spp*	*NR*	CRISPR-Cas9 technology was used to edit the genes of new oil-producing microalgae	Wang, Lu, Xin, Wei, Huang, & Xu, 2016
CRISPR-Cas9	*Nannochloropsis gaditana*	*ZnCys*	CRISPR-Cas9 technology was used to knock out 18 lipid synthesis regulatory factors of the target algae species to obtain high-yield oil algae species	Ajjawi, Verruto, Aqui, Soriaga, Coppersmith, Kwok, Peach, Orchard, Kalb, & Xu, 2017
CRISPR-Cas9	*Chlorella vulgaris*	*NR, APT*	To construct a new screening method for mutant algae after CRISPR-Cas9 gene editing	Kim, Chang, Lee, & Jin, 2021

2.2.1 Application of ZFN Technology in Microalgae

There are few studies on the application of ZFN technology in microalgae, only one case. Sizova first applied ZFNs technology to *Chlamydomonas reardtii* in 2013 (Sizova, Greiner, Awasthi, Kateriya, & Hegemann, 2013), targeting the *COP3* gene that encodes the photosensitive type I rhodopsin proton channel. The editing efficiency of ZFN was evaluated by knocking out the target cell resistance or fluorescent markers. According to the results of the reporter gene, the gene editing module was optimized, and the nuclease complex with the strongest specificity and affinity was selected to co-transform with exogenous DNA to achieve specific knockout of the target algae species.

2.2.2 Application of TALEN Technology in Microalgae

It can be seen from the existing reports that TALEN technology is less used in microalgae research, mainly studied in diatoms. In 2014, Daboussi first used TALEN technology to knock out seven genes related to lipid metabolism in the genome of *Phaeodactylum tricornutum* (Horvath & Barrangou, 2010). The knockout of UDP-glucose pyrophosphorylase (UGPase) gene resulted in a 45-fold increase in the

triacylglycerol (TAG) content of the mutant compared to the wild type, and the algae species with industrial potential were constructed.

In order to improve the efficiency and stability of gene knockout, in the subsequent study, Weyman integrated the TALEN expression system and the antibiotic resistance gene containing the homologous sequence of the TALEN targeting site into the vector plasmid (Weyman, Beeri, Lefebvre, Rivera, McCarthy, Heuberger, Peers, Allen, & Dupont, 2015). TALEN binds and cuts the target gene, and the vector plasmid is integrated into the genome through homology-directed repair (HDR). Therefore, an exogenous marker gene was transferred into the target site through the HDR pathway, which not only improved the mutation efficiency, but also simplified the screening of mutant algae. Since the mutation rate of TALEN editing technology in diatoms is lower than that in animals, Serif knocked out the blue-light-dependent transcription factor of *Phaeodactylum tricornutum* by referring to the TALEN editing strategy successfully applied in animals (Serif, Lepetit, Weißert, Kroth, & Bartulos, 2017). In this study, two vectors were constructed to express two TALEN proteins and carry different promoters and resistance genes. The results showed that 50 % of the clones had gene mutations, and 21 % were double allelic mutations. The results were consistent with the phenotypic traits obtained by previous RNAi, indicating that this is an efficient and feasible gene editing technology in diatoms.

2.2.3 Application of CRISPR-Cas9 Technology in Microalgae

Jiang first applied CRISPR-Cas9 technology in microalgae research, and successfully expressed Cas9 protein and sgRNA in *Chlamydomonas reinhardtii* (Jiang, Brueggeman, Horken, Plucinak, & Weeks, 2014). The vector was introduced into cells by electrotransformation, and four target genes were edited by non-homologous end joining repair system and CRISPR-Cas9 technology, with a mutation frequency of 42.8%. However, the success rate is very low when the endogenous gene of *Chlamydomonas reinhardtii* is knocked out. In the following study, Wang successfully knocked out a nitrate reductase gene *NR* of oil-producing microalgae *Nannochloropsis spp* by plasmid vector-mediated CRISPR-Cas9 technology (Wang, Lu, Xin, Wei, Huang, & Xu, 2016), and the gene knockout efficiency was significantly improved. The CRISPR-Cas9 vector system was successfully used to knock out the endogenous gene of microalgae. At present. The application of CRISPR-Cas9 in microalgae has gradually matured. Ajjawi transferred two Cas9 system vectors into *Nannochloropsis gaditana* by electrotransformation through optimization experiments (Ajjawi, Verruto, Aqui, Soriaga, Coppersmith, Kwok, Peach, Orchard, Kalb, & Xu, 2017), successfully knocked out 18 possible negative regulators of lipid synthesis in *Nannochloropsis gaditana* (Smith, Bibikova, Whitby, Reddy, Chandrasegaran, & Carroll, 2000). It was found that the carbon-lipid distribution of the ZnCys mutant was up-regulated from 20% to 55%, and an industrial algae species with oil production twice that of the wild type was obtained. In recent studies, established a genome editing tool based on CRISPR-Cas9 technology in *Chlorella vulgaris*. The *NR* gene and adenine phosphoribosyltransferase (APT) gene of *Chlorella* were successfully knocked out to construct auxotrophic algae species, reduce the use of antibiotics, and avoid ecological pollution caused by algae species with antibiotic resistance as

selective marker, which provided a new method for mutant screening after gene editing of microalgae.

3. Outlook and Discussion

In the past decade, with the rapid development of new gene editing technologies, researchers have established a preliminary structural framework for the metabolic pathways of microalgae, but there are still many areas that are still blank, such as: under nitrogen stress conditions, how carbon in different biological macromolecules accumulated in microalgae is transformed, and what is its regulatory mechanism. Under different culture conditions, the metabolic transformation of microalgae varies greatly, and how it is regulated. Based on the new gene editing technology, site-directed mutagenesis of microalgae to study the function of specific genes is an important way to solve these problems. At present, new gene editing is only studied in several model microalgae, and more industrial production potential of microalgae is deeply studied through new omics methods such as transcriptome. It has great advantages and development prospects to apply microalgae to fermentation process in a more effective, energy-saving and economical way to produce high-value products.

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