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Review

Transgenesis in microalga *Chlamydomonas reinhardtii*: current approaches

Pavel A. Virolainen, Elena M. Chekunova

Saint Petersburg State University, Saint Petersburg, Russia

ABSTRACT

Microalgae are a rich source of biologically active substances of natural origin, which have potential for use in pharmaceutical, agricultural, food and industrial production. Genetic engineering of microalgae opens up great prospects for creating improved strains that produce various food additives, commercial enzymes, as well as proteins for therapeutic purposes — antibodies, hormones and vaccines. *Chlamydomonas reinhardtii* P.A. Dang. is a unicellular green alga, a reference organism for studying the genetics of photosynthesis and developing new genetic engineering approaches in microalgae. The advantages of *C. reinhardtii* include the ability to transform all three of its genomes (nuclear, mitochondrial and chloroplast), low cost and ease of cultivation, safety for humans and the presence of a system for post-translational modification of proteins, which makes this organism a potential platform for use in biotechnology. Over the past few years, significant advances have been made in transgenesis of *C. reinhardtii*, including the use of new techniques based on the CRISPR/Cas9 genome editing technology. In this review, we summarize the available information on current approaches to transgenesis of the unicellular green alga *C. reinhardtii*: 1) general principles of transgenic constructs design for transformation of the nuclear and chloroplast genome, 2) popular selection markers used, 3) methods of cell transformation, 4) methods of genome editing using the CRISPR/Cas9 system.

Keywords: microalgae; *C. reinhardtii*; chloroplast; transformation; genetic engineering; transgene design; GMO; CRISPR/Cas.

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Научный обзор

Трансгенез микроводоросли *Chlamydomonas reinhardtii*: актуальные подходы

П.А. Виролайнен, Е.М. Чекунова

Санкт-Петербургский государственный университет, Санкт-Петербург, Россия

АННОТАЦИЯ

Микроводоросли — богатый источник биологически активных веществ природного происхождения, которые находят применение в фармацевтическом, сельскохозяйственном, пищевом и промышленном производстве. Генетическая инженерия микроводорослей открывает большие возможности для создания штаммов-продуцентов различных пищевых добавок, коммерческих ферментов, а также белков терапевтического назначения — антител, гормонов и вакцин. Одноклеточная зеленая водоросль *Chlamydomonas reinhardtii* P.A. Dang. — модельный объект генетики фотосинтеза — оказалась удобной для разработки новых подходов в генетической инженерии микроводорослей. Преимущества *C. reinhardtii* состоят в возможности трансформации всех трех ее геномов (ядерного, митохондриального и хлоропластного), низкой стоимости и простоте культивирования, безопасности для человека и наличии системы посттрансляционной модификации белков, что делает этот организм потенциально интересной платформой для применения в биотехнологии. За последние несколько лет были достигнуты значительные успехи в трансгенезе *C. reinhardtii*, в том числе с применением новых методик редактирования генома. В этом обзоре мы представляем данные о современных достижениях в области модификации генома одноклеточной зеленой водоросли *C. reinhardtii*: принципы дизайна трансгенных конструкций, методики трансформации ядерного и хлоропластного геномов, используемые селективные маркеры и подходы к редактированию геномов с помощью системы CRISPR/Cas9.

Ключевые слова: микроводоросли; *C. reinhardtii*; хлоропласт; трансформация; генетическая инженерия; дизайн трансгенов; ГМО; CRISPR/Cas.

Как цитировать

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INTRODUCTION

Eukaryotic microalgae are an extremely diverse group of photosynthetic unicellular microorganisms adapted to a wide range of ecological niches [1]. They account for about 50% of the total carbon fixation worldwide [2]. Due to their high metabolic plasticity, microalgae contain a wide range of beneficial substances such as natural antioxidants, vitamins, lipids, proteins, pigments, carbohydrates and secondary metabolites for pharmaceutical, agricultural, food and industrial applications. Microalgae are a rich source of pharmacologically active metabolites with antitumor, antibacterial, antifungal and antiviral properties. In addition to the production of endogenous compounds using bioengineering methods, microalgae can potentially be used as an inexpensive platform for the biosynthesis of various industrial enzymes and therapeutic proteins such as antibodies, hormones and vaccines [3]. Genetic engineering offers great prospects for the creation of highly productive strains of microalgae; being unicellular organisms, many species of microalgae remain haploid for most of their cell cycle, which reduces the time for obtaining and selecting of transformants. The use of microalgae as "cell factories" is a promising approach to produce commercially valuable compounds [4]. Some species of microalgae (e.g., *Chlorella vulgaris* Beijerinck and *Chlamydomonas reinhardtii* P.A. Dang.) have been granted GRAS (Generally Recognized as Safe) status — they are safe to eat [5].

Nevertheless, microalgae still remain relatively difficult targets for genetic manipulations [6]. The main problems are related to the need for a species-specific approach: cell transformation may be complicated by the peculiarities of membrane and cell wall structure in representatives of a particular taxon, and the developed transgenic construct may not be expressed due to a powerful gene silencing system. These difficulties explain the low efficiency of microalgae transformation and the scarce list of successfully edited species [6, 7].

C. reinhardtii (*Chlamydomonas*) is one of the most studied eukaryotic green algae, a popular model object for studying the genetics of photosynthesis, chloroplast biology, structure and function of sensory photoreceptors and photobehavior. *Chlamydomonas* is also a model organism for developing new approaches for genetic engineering of microalgae. The combination of rapid growth and ease of cultivation with the ability to photosynthesize, a well-studied life cycle, fully sequenced three genomes (nuclear, chloroplast, and mitochondrial) [8, 9], the possibility of transformation of each genome [10], and safety for humans make *Chlamydomonas* a valuable object for biotechnology. A great diversity of *C. reinhardtii* strains is maintained in various bioresource collections around the world. The only genetic collection in Russia is maintained at the Department of Genetics and Biotechnology of the St. Petersburg State University in Peterhof.

Currently, the *Chlamydomonas* chloroplast is successfully used as a platform for the biosynthesis of recombinant proteins with therapeutic properties (Table 1); for example, it can synthesize the birch pollen allergen Bet v 1 for allergy therapy and the spike protein of SARS-CoV-2 for vaccine development. The transformation techniques for *C. reinhardtii* plastome are well developed and allow to obtain stable high transgene expression [11].

Until recently, the introduction of transgenes into the nuclear genome of *Chlamydomonas* and their expression were associated with certain difficulties due to an efficient gene silencing system. Over the past few years, significant progress has been made in this area, including the use of novel CRISPR/Cas9-based techniques [41, 42].

This article presents an overview of current transgenesis techniques used for the unicellular green alga *C. reinhardtii*. The general design principles of transgenic constructs for transformation of nuclear and chloroplast genomes, selectable markers used to select transformants, techniques of transgene introduction (transformation) and genome editing using the CRISPR/Cas9 system are considered.

TRANSGENIC CONSTRUCTS DESIGN PRINCIPLES

A transgenic construct is a DNA cassette containing all the necessary regulatory elements for proper expression in the target organism. All constructs typically contain a promoter sequence, a gene of interest (transgene), a selectable marker and a terminator sequence. Depending on the need, the transgenic construct may carry additional elements to allow directed insertion into the genome or its controlled deletion from the genome.

The foreign gene can be integrated in the nuclear and chloroplast genome of *C. reinhardtii*. The main characteristics of each are presented in Table 2. In both cases, the efficient production of transformants requires adequate design of the transgenic construct, including codon optimization, selection of techniques for transgene introduction and selection of transformants, and methods for removal of marker sequences.

Transgenic constructs for transforming the nuclear and chloroplast genomes of *C. reinhardtii* differ significantly from each other (Fig. 1).

Until recently, transformation of the *C. reinhardtii* nuclear genome was associated with certain difficulties due to the effective silencing system [41]. In addition, before the advent of CRISPR/Cas technique, the transgenic construct could be integrated into the nuclear genome only by random insertion, which made the chloroplast of *Chlamydomonas* a more attractive platform, since

Table 1. Therapeutic proteins synthesized in the chloroplast of *C. reinhardtii*

Таблица 1. Белки терапевтического назначения, синтезированные в хлоропласте *C. reinhardtii*

Purpose	Protein	Reference
Vaccine development	Foot-and-mouth disease virus VP1 protein	[12]
	E2 protein of classical swine fever virus (CSFV)	[13]
	p57 protein of the fish pathogen *Renibacterium salmoninarum*	[14]
	VP28 protein of shrimp white spot syndrome virus (WSSV)	[15]
	Fibronectin-binding domain D2 of *Staphylococcus aureus*	[16]
	acrV and vapA antigens of the fish pathogen *Aeromonas salmonicida*	[17]
	Pfs25, Pfs28, Pfs48/45, CelTOS antigens of *Plasmodium falciparum*	[18–21]
	Human papilloma virus 16 (HPV 16) protein E 7	[22, 23]
	MPT64 antigen of *Mycobacterium tuberculosis*	[24]
	Avian influenza virus (AIV) hemagglutinin H5	[25]
	p201 protein of ApoB100	[26]
	SARS-CoV-2 spike protein	[27]
Disease therapy	Vascular endothelial growth factor	[28]
	Interleukin 4	[29]
	TRAIL (TNFSF10)	[30]
	Human monoclonal antibodies	[28, 31–33]
	Human glutamate decarboxylase (hGAD65)	[34]
	Amphoterin (HMGB1)	[28]
	Immunotoxins	[35]
	Human somatotropin	[36]
	Cpl 1 and Pal endolysins of *Streptococcus pneumoniae* bacteriophage	[37]
	Interleukin 29	[38]
Allergy therapy	Peanut allergen Ara h 1	[39]
	Birch pollen allergen Bet v 1	[40]

Table 2. Main characteristics of the nuclear and chloroplast genomes of *C. reinhardtii*

Таблица 2. Основные характеристики ядерного и хлоропластного геномов *C. reinhardtii*

Characteristics	Genome	
	nuclear	chloroplast
Size, base pairs	~110×10^6	~205×10^3
GC composition, %	~64	~36
Number of genes	~17585	99
Number of chromosomes	17	1
Number of copies in one cell	1	One chloroplast per cell, ~80 copies of the genome per chloroplast
Method of transgene integration	Random insertion, homologous recombination (by genetic editing)	Homologous recombination

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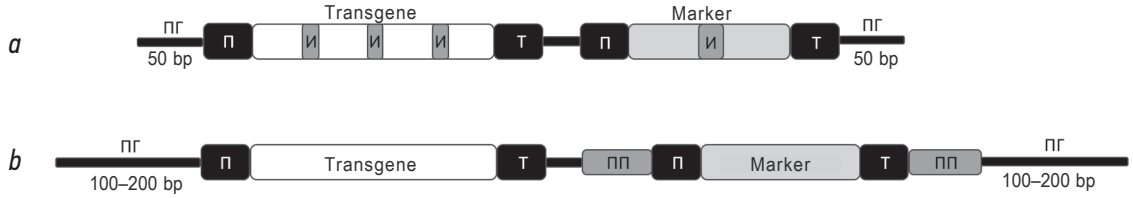

Fig. 1. Overall structure of transgenic constructs for transformation of the nuclear and chloroplast genome of *C. reinhardtii*: *a* — construct for transformation of the nuclear genome; *b* — construct for transformation of the chloroplast genome. ПГ — homology arms; П — promoter/5'-untranslated region; И — intron; Т — terminator/3'-untranslated region; ПП — direct repeats. Additional explanations are given in the text
Рис. 1. Общий план строения трансгенных конструкций для трансформации ядерного и хлоропластного генома *C. reinhardtii*: *a* — конструкция для трансформации ядерного генома; *b* — конструкция для трансформации хлоропластного генома. ПГ — плечи гомологии; П — промотор/5'-нетранслируемая область; И — интрон; Т — терминатор/3'-нетранслируемая область; ПП — прямые повторы. Дополнительные пояснения к рисунку приведены в тексте

transgene incorporation in it occurs only by homology. Several rules have been developed for the design of transgenes for their successful expression in the nuclear DNA of *C. reinhardtii*. First, it is necessary to use endogenous promoter (native or chimeric), terminator, 5'- and 3'-untranslated regions — the most frequently used are those from genes encoding the small subunit of ribulose bisphosphate carboxylase (*rbcS2*), heat shock protein *hsp70A*, and subunit II of the reaction center of photosystem I (*psaD*) [43]. Second, the codon composition of the transgene and the transforming genome should be taken into account [44]. Third, regular interruption of the transgene by introns [threefold repetition of intron 1 of *rbcS2* (145 bp) in the transgene sequence and a single use of intron 2 of *rbcS2* (329 bp) in the marker sequence] contributes to a high level of expression [45, 46]. This effect is achieved by a process called Intron-Mediated Enhancement (IME). The proposed mechanism is the interaction of RNA polymerase II with the spliceosome: if this interaction does not occur, RNA polymerase terminates transcription and the immature transcript is degraded. This phenomenon may underlie the ability to recognize the exogenous transcripts. The genes of *C. reinhardtii* are extremely rich in introns compared to other organisms: 92% of its genes contain introns with an average size of 373 bp. The average size of exons is 190 bp [41].

Directed insertion of a transgene into the nuclear genome of *Chlamydomonas* is possible only by homologous recombination along the homology arms (50 bp) through a reparation of double-stranded break induced by the CRISPR/Cas system [47]. The editing of the *C. reinhardtii* genome by CRISPR/Cas9 is discussed in detail below.

Transformation of the *C. reinhardtii* plastome is still more convenient and advantageous for the following reasons: 1) potential toxicity of the product to the cell is minimized, 2) targeted transgene integration by homologous recombination is possible without using CRISPR/Cas system, 3) high plastome ploidy and high level of chloroplast gene expression, 4) absence of a gene silencing system. One of the disadvantages of this approach is the lack of glycosylation of proteins synthesized in chloroplasts [11].

The transgenic construct for plastid transformation consists of a transgene and a selectable marker flanked by 100–200 bp homology arms, which are homologous to a selected insertion site in the host chloroplast DNA. For transgene and marker expression in the chloroplast of *C. reinhardtii*, the regulatory sequences of the nuclear gene *rbcS2*, chloroplast genes *psaD*, *psaA*, and *psaB* (photosystem I apoproteins), *rbcL* (large subunit of ribulose bisphosphate carboxylase), and *psbA* (core protein D1 of photosystem II) are most often used [11]. The GC composition of the transgene and chloroplast DNA must also be considered when designing a transgenic construct for *Chlamydomonas* chloroplast transformation.

The marker gene can be removed from the transformed genome using the Cre/loxP recombinase system [48]. For the plastome, the marker gene should be flanked on both sides by direct repeats. Removal of the selection factor during further propagation of the transformant can lead to internal homologous recombination between repeats with removal of the selectable marker from the chloroplast genome [49].

SELECTABLE MARKERS

Selectable markers are used to create conditions for selection of transformed cells. The marker sequences most widely used in *C. reinhardtii* transformation are presented in Table 3.

The first selectable markers for nuclear genome transformation used in *C. reinhardtii* were its own genes that restore metabolic pathways in strains carrying mutations in the *ARG7* and *NIT1* genes. Auxotrophic mutants transformed with normal copies of these genes are selected as prototrophs. This “marker-free” approach based on the compensation of the mutation by a wild-type gene copy remains popular and is widely used in the transformation of nuclear and chloroplast DNA in the corresponding mutants [58, 59].

Table 3. The most widely used selection markers for the transformation of nuclear and chloroplast DNA of *C. reinhardtii*

Таблица 3. Наиболее широко используемые при трансформации ядерной и хлоропластной ДНК *C. reinhardtii* селекционные маркеры

Target	Gene	Product	Selection	Reference
Nuclear DNA	*ARG7*	Argininosuccinate lyase	Arginine-free medium	[50]
	NIT1	Nitrate reductase	Nitrate-free medium	[51]
	CRY1-1	Mutant ribosomal protein S14/rp59	Medium with emetine, cryptopleurin	[52]
	aph7"	Aminoglycoside phosphotransferase	Medium with hygromycin B	[43]
	aphVIII	Aminoglycoside phosphotransferase	Medium with paromomycin, neomycin, kanamycin	[53]
	ble	Bleomycin-binding protein	Fluomycin medium	[44, 54]
Nuclear and chloroplast DNA	*aadA*	Aminoglycoside adenylyltransferase	Medium with spectinomycin, streptomycin	[55, 56]
Chloroplast DNA	*aphA6*	Aminoglycoside phosphotransferase	Medium with kanamycin, amikacin	[57]

The *ARG7* gene encodes argininosuccinate lyase and restores the metabolic pathway of arginine biosynthesis during successful transformation of an arginine-dependent *C. reinhardtii* mutant strain, allowing the transformants to grow on minimal medium without arginine supplementation.

The *NIT1* gene encodes nitrate reductase, an enzyme that allows the successfully transformed *C. reinhardtii* mutant strain to utilize nitrate as the sole source of nitrogen in the medium.

A mutant strain of *C. reinhardtii* resistant to the translation inhibitors, emetin and cryptopleurin, was isolated and studied in 1994 [52]. This resistance attributed to a missense mutation (*CRY1-1*) in the *CRY1* gene. The sequence of the mutant *CRY1-1* gene under the control of the endogenous *rbcS2* promoter can be used for transformation of wild-type strains of *C. reinhardtii*.

The use of exogenous selectable markers has simplified the transformant selection process and eliminated the necessity of using only *C. reinhardtii* mutants as transgene acceptors. Currently, the most effective and widely used exogenous selectable markers are various antibiotic resistance genes.

The *aph7"* gene of *Streptomyces hygroscopicus* encodes a phosphotransferase that deactivates the antibiotic hygromycin B. A chimeric construct including the *aph7"* gene sequence of *S. hygroscopicus*, β2-tubulin gene promoter, and first intron with the 3'-untranslated region of *rbcS2* gene of *C. reinhardtii* is used for *Chlamydomonas* transformation [43].

The *aphVIII* gene of *Streptomyces rimosus* encodes a phosphotransferase that deactivates the antibiotics paromomycin, neomycin, and kanamycin. The highest transformation efficiency was achieved using a chimeric construct with the sequences of the 5'-untranslated region of the heat shock protein *hsp70A* gene of *C. reinhardtii*, first intron of the *rbcS2* gene of *C. reinhardtii*, the *aphVIII* gene of *S. rimosus*, and the 3'-untranslated region of the *rbcS2* gene of *C. reinhardtii* [53].

The *ble* gene *of Streptoalloteichus hindustanus* encodes an enzyme that binds the antibiotic phleomycin. A chimeric construct with this gene, including sequences of the first intron and the 5'- and 3'-untranslated regions of the *rbcS2* gene of *C. reinhardtii*, showed consistently high transformation and expression efficiency [44, 54].

The *aadA* gene of *Escherichia coli* encodes an enzyme that confers resistance to the antibiotics spectinomycin and streptomycin. For transformation of *Chlamydomonas* plastome, a construct with the sequences of the promoter of the chloroplast *atpA* gene and the 3'-untranslated region of *rbcL* gene of *C. reinhardtii* is used [55]. To transform nuclear DNA, the *aadA* gene of *E. coli* is combined with the 5'- and 3'-untranslated regions of the *rbcS2* gene of *C. reinhardtii* [56].

The *aphA6* gene of *Acinetobacter baumannii* encodes a phosphotransferase that deactivates the antibiotics kanamycin and amikacin. The highest expression of this gene in the chloroplast of *Chlamydomonas* was observed using a compact chimeric construct with the sequence of the 5'-untranslated region of the *psbA* gene of *C. reinhardtii*, the *aphA6* gene of *A. baumannii*, and the 3'-untranslated region of the *rbcL* gene of *C. reinhardtii* [57].

Fluorescent proteins, such as mCherry [60] and mVenus [45], can also be used as a selectable markers. Transformants are screened using a confocal microscope.

CHLAMYDOMONAS MODULAR CLONING KIT

Rapid and predictable modification of the *C. reinhardtii* genome can be achieved using standardized tools and techniques. For this purpose, a set of tools for modular cloning based on Golden Gate technology — *Chlamydomonas* Modular Cloning kit was developed [61]. The system uses *BsaI* and *BpiI* restrictases and consists of three sets of vectors for cloning (levels 0, 1, and 2). Level 0 vectors contain individual elements such as

promoters, 5'- and 3'-untranslated regions, coding sequences, terminators, selectable markers and introns. These modules are then assembling into one complete transcriptional unit within the level 1 vector during the restriction/ligation reaction. In the second step, several transcriptional units from level 1 vectors can be combined within a level 2 vector to create multigenic clusters encoding enzymes of new metabolic pathways [42].

A ready-to-use standardized toolkit allows rapid (in 4–5 weeks) creation of modified cells for basic research and biotechnology.

C. REINHARDTII TRANSFORMATION METHODS

Transformation is the process of integrating a functional DNA fragment — a transgenic construct — into target cells. Currently, three methods of transgene delivery into *Chlamydomonas* cells are widely used: glass beads, electroporation, and biolistic [10].

Glass beads are the most affordable, simple, and efficient method of nuclear [62] and plastome [63] genome transformation. The transgene, *C. reinhardtii* cells without cell wall (using autolysin or a cell wall-less mutant strain), and glass beads are mixed on a vortex. The glass beads rupture the membrane when shaken, allowing the transfer of exogenous DNA into the cell. They can also be used to deliver ribonucleoprotein (RNP) complexes formed by Cas nuclease and guide RNA (gRNA) into cells.

The electroporation method, first used on *Chlamydomonas* in 1991 [64], involves treating cells with high-voltage pulses on an electroporator, which forms pores with a diameter of 20–40 nm in the membrane, through which foreign DNA enters the cell. The method allows achieving a high yield of transformants, but requires adaptation of conditions depending on the electroporator and the specific strain of *C. reinhardtii* used [65]. Electroporation is performed by generation of exponential or square wave pulses. Square waves are more controllable and lead to the generation of a greater number of transformants [66]. In addition, square wave pulses may be used for transformation of cell-walled *C. reinhardtii* cells without its removal by autolysin [67], while exponential wave pulses are suitable only for cells lacking a cell wall [65]. Experiments on electroporation of cell-walled *C. reinhardtii* cells by exponential waves on the electroporator BTX ECM 600 without autolysin treatment resulted in transformation frequency of 3%, and with autolysin treatment — in 87%, confirming and supplementing the previously obtained data [47]. By optimizing parameters such as transgene concentration, wave type, and voltage, the transformation rate by electroporation can be higher than that of the glass bead method [68]. The overall efficiency of transformation is significantly increased by using the latest models of electroporators, such as the NEPA21. This device sequentially generates three types of square wave pulses: “poring pulse” with high voltage and short pulse length, creating pores in the cell membrane, series of “transfer pulses” with low voltage and long pulse length, and “transfer pulse” with reversed polarity for efficient delivery of DNA molecules into cells [67]. Electroporation is a standard method for *C. reinhardtii* transformation, especially for delivering the Cas/gRNA RNP complexes into cells.

The biolistic method involves bombarding recipient cells with heavy metal particles (mainly gold or silver) coated with transgenic DNA in a vacuum chamber. This method can be used to transform the DNA of both the cell nucleus and organoids, such as mitochondria and plastids. Currently, this technique is mainly used for transforming *Chlamydomonas* chloroplast.

Other methods of *Chlamydomonas* transformation, such as *Agrobacterium*-mediated transformation, have been developed and tested, but they have not been widely used [10]. This approach is much more complex and yet has no significant advantages over electroporation [69].

Recently, a novel system for the delivery of Cas9/gRNA RNP complexes into *C. reinhardtii* cells was developed using the cell-penetrating peptide pVEC (amino acid sequence: LLIILRRRRRIRKQAHAHSK). It can be used as an alternative to electroporation in terms of availability and efficiency [70].

GENOME EDITING USING CRISPR/CAS9 IN *C. REINHARDTII*

CRISPR/Cas9 is a system of Cas9 protein with nuclease activity and a non-coding gRNA complementary to DNA in the target site. The CRISPR/Cas9 genome editing system generally functions as follows: the assembled Cas9/gRNA complex scans the DNA sequence for a match of the gRNA spacer sequence to the target site (protospacer) on the DNA and the presence of the protospacer adjacent motif (PAM) sequence nearby. After formation of the gRNA–DNA complex, the Cas9 endonuclease makes a double-stranded break 3–4 nucleotides upstream of the PAM. A transgenic construct carrying homology arms to the restriction site can be targeted into the resulting *in vivo* gap (homologous recombination) [71].

Novel methods of *Chlamydomonas* transformation are based on the CRISPR/Cas9 technology. The components of the system can be delivered into the cell by the following ways: 1) plasmid vectors carrying sequences encoding Cas9 protein and gRNA; 2) *in vitro* assembled Cas9/gRNA RNP complex (Fig. 2). The use of the RNP complex has several advantages including avoidance of random insertional mutagenesis and reduction of off-target editing probability, consequently demonstrating higher efficiency.

The integrative strategy uses one or two plasmids encoding the *Cas9* and *gRNA* genes. Once introduced

into the cell, the plasmid is linearized by cellular restriction enzymes and integrated into a random location in the *C. reinhardtii* genome. Upon its expression, the Cas9/gRNA complex is assembled, which produces a double-stranded break in the target site [72]. The first attempt to produce Cas9 endonuclease and gRNA from the same plasmid in *C. reinhardtii* cells yielded a single colony of transformants for the *FKB12* gene from >10^9 cells taken [73]. Co-transfection of cells with two independent plasmids resulted in *APT* gene knockout lines with an efficiency of 3–30% [74].

A method was developed to prevent integration of *Cas9* and *gRNA* gene sequences into the genome. It was shown that plasmids containing the yeast centromeric sequence, replication initiation site and yeast selectable marker (CEN6-ARSH4-HIS3) replicate autonomously in cells and are not incorporated into the genome of the diatoms *Thalassiosira pseudonana* Cleve and *Phaeodactylum tricornutum* Bohlin [75, 76]. Experiments with this technique were continued in 2019 and stable transformants of the green microalgae *Acutodesmus obliquus* (Turpin) Hegewald & Hanagata and *Neochloris oleoabundans* S. were obtained. However, later the integration of the episomal plasmid into the nuclear genome occurred [77]. In another study [78], the nitrate reductase gene in the microalgae *Nannochloropsis oceanica* Suda & Miyashita was successfully knocked out using an episomal vector. The episomal plasmid was eliminated from the transformant cells after approximately 30 cell division cycles, yielding a nontransgenic line. The use of an episomal plasmid has not yet been tested for transformation of *C. reinhardtii*. Nevertheless, the development of such plasmid is easy, and the possibility of constructing nontransgenic lines without markers makes this method comparable in its applicability to the RNP system [72].

As the expression of foreign genes in *C. reinhardtii* is often inefficient, especially if they encode large proteins, such as Cas9 [60, 73], the most commonly used delivery method involves *in vitro* preassembly of the Cas9/gRNA RNP complex, which is directly used for transformation. The RNP complex rapidly degrades in the cell, which limits endonuclease activity and minimizes the risks of off-target effects, compared to constitutive expression of Cas9 [79].

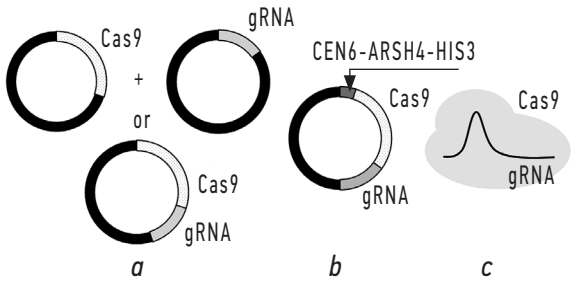

Fig. 2. Strategies for delivering components of the CRISPR/Cas9 system into *C. reinhardtii* cells: *a* — integrative system; *b* — episomal system; *c* — ribonucleoprotein system. Additional explanations are given in the text

Рис. 2. Стратегии доставки компонентов системы CRISPR/Cas9 в клетки *C. reinhardtii*: *a* — интегративная система; *b* — эписомная система; *c* — рибонуклеопротеиновая система. Дополнительные пояснения к рисунку приведены в тексте

The CRISPR/Cas9 technique can be used for targeted gene knockout and knockin in *Chlamydomonas*. The components of the system are delivered into the cell together with donor DNA cassette (transgene construct), which can be incorporated into the double-stranded break site. Addition of homology arms flanking the restriction site of Cas9 nuclease to the donor DNA sequence are required for homologous recombination, providing controllability and predetermination of the result. This method increases the yield of transformants up to 85% [47, 60].

The general protocol for *C. reinhardtii* transformation was significantly refined in 2017. According to observations, heat shock treatment of cells prior to transformation is a critical parameter to maximize the number of transformants obtained. It is assumed that some physiological changes favorable for the activity of the Cas9/gRNA complex and/or for the processes associated with DNA repair occur in cells. The optimal parameters for post-transformation cell recovery have also been determined [60].

In 2020, a highly efficient method of targeted insertional mutagenesis (TIM) was developed. The authors conducted a series of paired experiments and identified parameters crucial for high efficiency of *Chlamydomonas* transgenesis based on the CRISPR/Cas RNP strategy. Optimization of the nuclear genome transformation protocol allowed them to achieve mutagenesis efficiency from 40 to 95% in separate experiments, even with multiplex editing [47].

In 2023, a protocol was developed for the routine generation of mutant *Chlamydomonas* lines without residual selectable markers [80]. In the same year, using the CRISPR/Cas RNP strategy, transformants expressing an optimized bacterial phytase transgene embedded in the second exon of the nuclear gene *NIT1* and stable for 100 days of cultivation were obtained for the first time. Notably, cells transformed by conventional methods (without using the RNP complex or CRISPR/Cas method) lost the transgene after two generations (about 20 days of cultivation) [81].

Many attempts were made in order to optimize conditions for *Chlamydomonas* transformation and increase the efficiency of gene editing. These attempts involved evaluation of various factors such as growth conditions and cell preparation methods, required density of the cell culture, concentration of the Cas9/gRNA RNP complex, technique of cell wall removal, selection and optimization of transformation methods for various *Chlamydomonas* strains and equipment, and techniques for recovery and

selection of transformants. The protocol for nuclear genome transformation of the microalgae *C. reinhardtii* via CRISPR/Cas RNP complex is summarized below.

The development of advanced gene editing techniques has played an important role in the establishment of *C. reinhardtii* as a model organism for the study of microalgae. Achievements of recent years, including the development of transgene design rules, creation of a *Chlamydomonas* Modular Cloning kit, and adaptation of the CRISPR/Cas9 system for targeted genome editing, have significantly increased the efficiency of transgene expression in *C. reinhardtii* cells and the overall potential of microalgae genetic engineering. This opens prospects for the creation of new producer strains, valuable for both fundamental research and practical use, and will make it possible to replenish the resources of the Peterhof genetic collection of *C. reinhardtii* strains, which is the only one in Russia.

CONCLUSION

The unicellular green alga *C. reinhardtii* is a well-established model organism in molecular biology and plant genetics. The degree of study of genetic systems of *Chlamydomonas* is a standard for other microalgae. The availability, ease of cultivation, and the possibility of manipulation of all three genomes (nuclear, chloroplast, and mitochondrial) have allowed to make significant contributions to the development of new approaches for genetic engineering in microalgae.

The CRISPR/Cas9 genome editing system has opened up a new chapter in *C. reinhardtii* genetic engineering by enabling directed transgene insertion into the nuclear genome. In recent years, significant advances have been made in the development of highly efficient *Chlamydomonas* transformation protocols and the production of valuable enzymes and proteins. The *Chlamydomonas* Modular

1. Cell cultivation and preparation	
Grow cells on solid TAP medium (1.5% agar, arginine, yeast autolysate) at 20°C–25°C in a 14 h light (200–300 μE/m^2 × s):10 h dark cycle	
Harvest cells on the third day (exponential growth phase), resuspend in sterile water and centrifuge at 5,000 rpm for 5 min	
Resuspend the cells of cell-walled strains in autolysin to a cell density of 1 × 10^8 cells/mL and incubate at room temperature for 30 min	Resuspend the cells of cell wall-less strains in liquid TAP + 40 mM sucrose medium to a cell density of 1 × 10^8 cells/mL
2. Heat shock	
Centrifuge cell suspension at 5,000 rpm for 5 min	
Resuspend cell pellet in liquid TAP + 40 mM sucrose and centrifuge at 5,000 rpm for 5 min. Resuspend cell pellet in liquid TAP + 40 mM sucrose to a cell density of 1 × 10^{8}cells/mL	
3. Transformation	
Glass beads	Electroporation (BioRad GenePulser Xcell electroporator)
Mix 300 μL of resuspended cells (3 × 10^7 cells/mL) with 5 μL of Cas9/gRNA RNP complex (3 μM Cas9:gRNA) and 1 μg of transgenic construct in a tube with 0.45 nm sterile glass beads (500–600 μg)	For one 2 mm cuvette mix 40 μL of resuspended cells (4 × 10^6 cells/mL) with 4 μL of Cas9/gRNA RNP complex (3 μM Cas9:gRNA) and 1 μg of the transgenic construct
Vortex at top speed for 15 sec, rest for 10 sec, vortex again for 10 sec	Electroporate in 2 mm cuvette at square wave, 300 V, 6 pulses, pulse length 4 ms, pulse interval 100 ms. The cuvette with the mixture should be shortly cooled in ice before and after electroporation
Resuspend each reaction mixture in 600 μL of fresh liquid TAP + arginine medium in a separate tube	
4. Cell recovery and selection	
Cultivate cell suspension at 20°C–25°C under dim light for 24 h	
Vortex the culture mixture and pipette onto plates with solid TAP selective medium (1.5% agar, 10 μg/mL antibiotic)	
Grow cells at 20°C–25°C in a 14 h light (200–300 μE/m^2 × s):10 h dark cycle until the transformant colonies formation (1–2 weeks)	

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Cloning kit, introduced in 2018, aims to unify the design process for transgenic constructs. Standardized genetic elements can be readily substituted and assembled into complete expression units, which further enable the creation of new multigene constructs. This opens up the possibility of introducing new metabolic pathways into algae cells [42, 61].

Microalgae are an interesting object for introduction and adaptation of new approaches to genome engineering. They contain a wide range of useful substances: antioxidants, lipids, proteins, carbohydrates and secondary metabolites. For example, the green alga *Chlorella vulgaris* can be used as a producer of 18 amino acids, 20 vitamins, and many minerals [82]. Microalgae are an exceptionally rich source of pharmacologically active metabolites with antitumor, antibacterial, antifungal, and antiviral properties, and are also suitable for wastewater treatment and biofuel production.

Currently, heterotrophic platforms are usually used for biosynthesis of valuable compounds: bacteria, yeasts, cell cultures. Nevertheless, the development of bioeconomy and the need for sustainable alternatives to petrochemical products stimulate interest in the search and use of new producers, such as photosynthetic microalgae. Advances in the biosynthesis of recombinant proteins in *C. reinhardtii* chloroplasts along with the ever-improving transformation techniques have indicated the high commercial potential of this alga [36]. There are a number of specific properties that make microalgae ideal candidates for the production of various food additives, commercial enzymes, and therapeutic proteins: 1) affordable cultivation, fast biomass build-up, and easily controlled and scalable; 2) quick production of transgenic microalgae in 4–5 weeks; 3) the ability to transform the plastome and nuclear genome allows the production of multiple transgenic proteins (or an entire protein complex) in a single organism. Although bacterial and yeast systems are more economical, they have several disadvantages. It is not always possible to obtain functional proteins due to specific features in the post-translational modifications and protein folding. The use of another photosynthetic alternative platform — terrestrial plants — for large scale cultivation is economically not viable as it poses economic burden along with the long time required to obtain transgenic lines (4–6 years), the possibility of gene flow through pollen into the environment, and the ban on open cultivation of transgenic plants [4, 31, 83].

There is every reason to believe that microalgae in the future may become an alternative platform for the development of economically valuable compounds in industrial and medical biotechnology. As the most studied microalga, *C. reinhardtii* has the genetic potential to become a major platform in the microalgae biotechnology [84].

ADDITIONAL INFORMATION

Authors' contribution. All authors have made a significant contribution to the development of the concept and preparation of the article, as well as read and approved the final version before its publication. Personal contribution of the authors: P.A. Virolainen — search and analysis of literature, writing the main part of the text; E.M. Chekunova — development of the concept, making final edits.

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AUTHORS' INFO

Pavel A. Virolainen; ORCID: 0000-0001-5918-9395; SPIN: 6564-9350; e-mail: st085618@student.spbu.ru

***Elena M. Chekunova,** Dr. Sci. (Biology); address: 7–9 Universitetskaya emb., Saint Petersburg, 199034, Russia; ORCID: 0000-0001-8942-4771; eLibrary SPIN: 2788-6386; e-mail: e.chekunova@spbu.ru

ОБ АВТОРАХ

Павел Алексеевич Виролайнен; ORCID: 0000-0001-5918-9395; eLibrary SPIN: 6564-9350; e-mail: st085618@student.spbu.ru

***Елена Михайловна Чекунова,** д-р. биол. наук; адрес: 199034, Россия, Санкт-Петербург, Университетская наб., д. 7–9; ORCID: 0000-0001-8942-4771; eLibrary SPIN: 2788-6386; e-mail: e.chekunova@spbu.ru

* Corresponding author / Автор, ответственный за переписку