

Brief Communication

High-throughput sequencing revealed low-efficacy genome editing using Cas9 RNPs electroporation and single-celled microinjection provided an alternative to deliver CRISPR reagents into *Euglena gracilis*

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The genus *Euglena* contains more than 1000 species of single-celled flagellated microorganisms with both plant and animal characteristics. As a model organism, *E. gracilis* has been studied well to address fundamental questions in chloroplast development and photosynthesis, with implications in physiology, biochemistry and cell biology (Schwartzbach and Shigeoka, 2017). However, research in bioengineering and biotechnology in *Euglena*, especially using genome editing, remains insufficient (Figure 1a). Nomura *et al.* (2019) reported successful CRISPR/Cas9-mediated genome editing in *Euglena* using electroporation of Cas9 ribonucleoproteins (RNPs) targeting *EgGSL2* and obtained mutant rates of approximately 70–90% based on morphology and amplicon-sequencing (Nomura *et al.* 2019). The protocol for implementing the *E. gracilis* CRISPR experiments was also described in further detail and published (Nomura *et al.*, 2020). To date, these are the only research articles related to CRISPR genome editing of *E. gracilis*. Since their publication, no other successful case or report has been published by other groups, including ours.

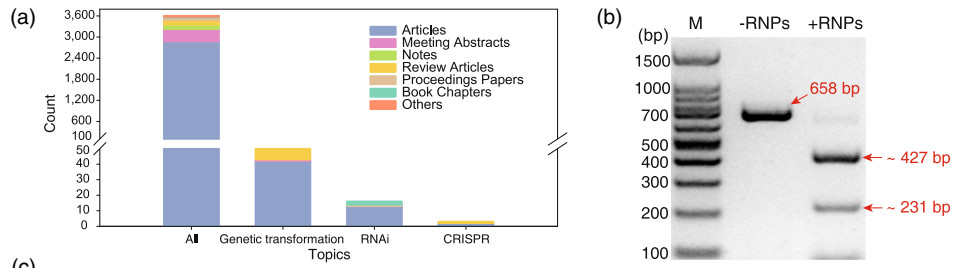
In this study, we repeated the CRISPR genome editing experiments on *E. gracilis* (Nomura *et al.*, 2019, 2020) and assessed their efficiency by high-throughput sequencing. Further, sgRNA targeting the *E. gracilis crtP1* gene for phytoene desaturase (PDS) was designed as a reference (Method S1). The EG300 experimental procedures and parameters strictly followed the protocol described by Nomura *et al.* (2020) (Figure 1c). Moreover, *E. gracilis* protoplast-like cells (Plas) treated with proteinase K were also used with extended voltage parameters from 150 to 600 V using two types of electroporation devices, NEPA21 and Bio-Rad Xcell (Figure 1c). Additional details of the process are described in Method S1.

The result showed that the CRISPR-RNPs system worked efficiently to induce enzymatic cleavage of the partial *EgGSL2* gene *in vitro* (Figure 1b). T7 Endonuclease I (T7E I) assays of three EG300 replications indicated their low editing efficiency from ‘target1’ on the partial *EgGSL2* by electroporation (Figure 1d).

Further, high-throughput sequencing of the ‘target1’ amplicons was performed using the Illumina NovaSeq 6000 platform. After data control, sequences were aligned and operational taxonomic units (OTUs) were clustered (FLASH v1.2.11 and USEARCH v10.0.240); three OTUs were found to be clustered in most high-quality sequences, which represented wild type, mutant1 and mutant2 (Figure 1e) (accessible data set in China National GeneBank (CNGB); accession no.: CNP0002995). The available sequences were obtained ranging from 29,541 to 353,307 in eight samples. However, only one sample, EG450, revealed mutant sequences with the mutant1 (0.81%) and mutant2 (0.17%), respectively (Figure 1f). The results showed that CRISPR genome editing of *E. gracilis* targeting the *EgGSL2* by electroporation was successful but had very low efficiency. The efficiency of EG300 and other samples except EG450 was 0% (Figure 1f). With the *crtP1* gene, eight mutant OTUs were obtained with an efficiency rate of approximately 3% in total (accessible data set in CNGB; accession no.: CNP0003142) (Figure S1). These results indicate that the delivery of RNPs into *E. gracilis* cells by electroporation is difficult.

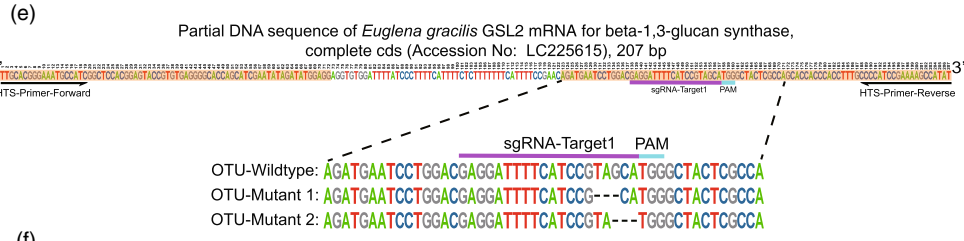
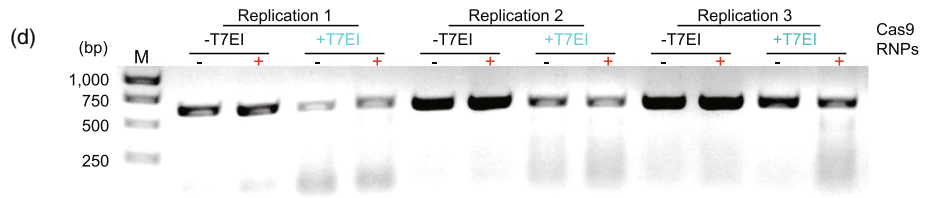
Microinjection is a physical method to deliver a small volume of substances into cells at the appropriate location, such as the cytoplasm or nucleus (Zhang and Yu, 2008). It is a visible and real-time traceable method that has been widely used in zebrafish embryos and mouse zygotes because of its high efficiency and low lethality (Gordon *et al.*, 1980; Yuan and Sun, 2009). However, the application of microinjection to microalgae, although reported, is very rare (Nichols and Rikmenspoel, 1978), especially if the cell size is less than 100 µm. Here, we present a microinjection method to deliver exogenous materials into *E. gracilis* cells. For example, the dihydrochloride (DAPI) stain was successfully injected into *E. gracilis* cells using TransferMan 4r and FemtoJet 4i (Eppendorf, Germany), and blue fluorescence was observed in the nuclear region at the appropriate excitation/emission wavelengths (364 nm/454 nm) under a DMi3000B epifluorescence microscope (Leica, Germany) (Figure 1g). For genome editing *E. gracilis*, the sgRNA targeting the *crtP1* gene for PDS and sgRNA of target1 on the *EgGSL2* gene were used.

In the *crtP1* gene group, six cells among 100 injected cells survived, and each clone was sequenced to verify specific mutation. According to the DNA sequencing results, one clone showed precise genome editing on the *crtP1* gene. The cytosine base was deleted at 295 in the partial DNA sequence of the *crtP1* gene. The colour differences between the WT and *crtP1*-mutant were evident under the same cell density at 2.4×10^6 cells/ml



(c)

Pulse No.	Sample Name	Poring Pulse/Transfer Pulse						Device	Reference
		Voltage (V)	Pulse length (ms)	Pulse interval (ms)	Number of pulses	Decay rate (%)	Polarity switching		
1	EG150	150/20						NEPA21 Super Electroporator	This study
2	EG300	300/20	5/50	50/50	2/5	40/40	+/-		Nomura et al., 2019
3	EG450	Square wave protocol: Voltage 450 V, Pulse length 50 ms, Number of pulses 5, Pulse interval 50 ms						Bio-rad Gene Pulser Xcell	This study
4	EG600	Square wave protocol: Voltage 600 V, Pulse length 50 ms, Number of pulses 5, Pulse interval 50 ms							
5	Plas150	150/20							
6	Plas300	300/20	5/50	50/50	2/5	40/40	+/-		
7	Plas450	Square wave protocol: Voltage 450 V, Pulse length 50 ms, Number of pulses 5, Pulse interval 50 ms						NEPA21 Super Electroporator	
8	Plas600	Square wave protocol: Voltage 600 V, Pulse length 50 ms, Number of pulses 5, Pulse interval 50 ms						Bio-rad Gene Pulser Xcell	



(f)

Items	Samples							
	EG150	EG300	EG450	EG600	Plas150	Plas300	Plas450	Plas600
OTU-Wildtype	353,307/100%	84,438/100%	183,187/99.02%	29,541/100%	273,804/100%	61,015/100%	140,930/100%	199,173/100%
OTU-Mutant1	0/0.00%	0/0.00%	1,508/0.81%	0/0.00%	0/0.00%	0/0.00%	0/0.00%	0/0.00%
OTU-Mutant2	0/0.00%	0/0.00%	314/0.17%	0/0.00%	0/0.00%	0/0.00%	0/0.00%	0/0.00%
Total seqs	353,307	84,438	185,009	29,541	273,804	61,015	140,930	199,173
Total mutant rate	0%	0%	0.98%	0%	0%	0%	0%	0%

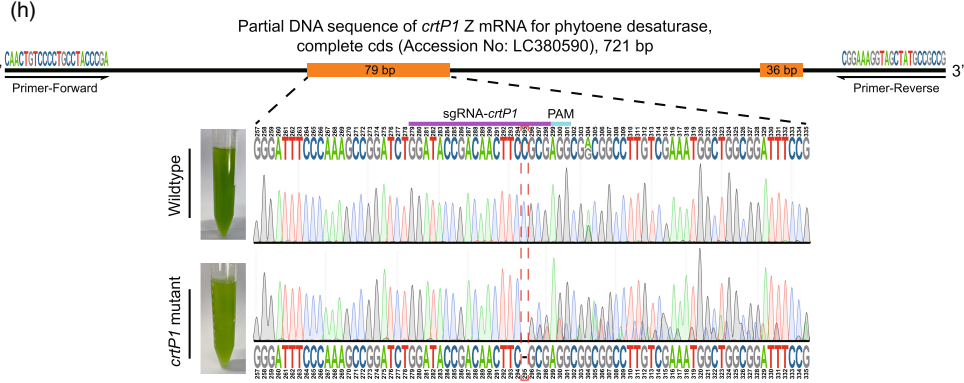
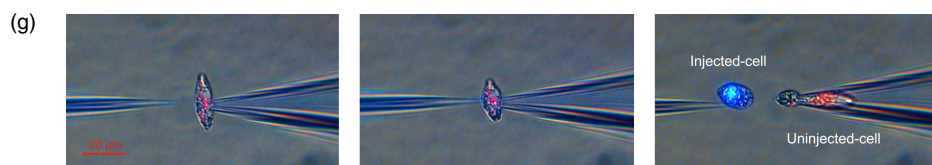


Figure 1 CRISPR genome editing to target the *EgGSL2* gene by electroporation and the *crtP1* gene using microinjection to deliver Cas9-RNPs into *E. gracilis*. (a) Overview of research status on *E. gracilis*. The data on the chart are derived from *Web of Science* and are not strictly proportional to the values of each entry. (b) The cleavage experiment on the partial *EgGSL2* gene using RNPs with the sgRNA-target1 designed by Nomura *et al.* (c) Overview of electroporation parameters to deliver RNPs into *E. gracilis*. (d) Detection of mutations in three EG300 replicates at *EgGSL2* target sites after electroporation using the T7E I assay at 72 h. (e) Detection of mutant sequences in the *EgGSL2* gene after electroporation by high-throughput sequencing. (f) Detection of the CRISPR genome edited mutant sequences and mutant rate of the *EgGSL2* gene after electroporation, by high-throughput sequencing. (g) Representative images of delivering DAPI stain into an *E. gracilis* cell by microinjection. (h) Schematic of CRISPR genome editing targeting the *crtP1* gene.

(Figure 1h). The efficiency of genome editing was as high as 16.7% based on the surviving cells, whereas 1.0% was calculated based on the number of processed cells. The *crtP1* mutant obtained using CRISPR technology coupled with microinjection was maintained even after repeated cultivation for a year. However, no mutant could be obtained in the *EgGSL2* gene group, even though twelve clones survived. Microinjection is not a newly emerging technique but rather an advanced and sophisticated technique, primarily when performed on cells smaller than 20 μm (Chen *et al.*, 2022). *E. gracilis* presents sphere-shaped cells smaller than 20 μm with a flexible pellicle, which hinders manipulation by microinjection. The size of the open tip of the injection pipette should be no more than 500 nm, and tips ranging from 50 to 100 nm are suitable to reduce cell mortality. The injection pressure varies between 100 and 2500 hPa depending on the specific circumstances. Several industrial microalgae cells are tiny with thick cell walls and movability. This may explain why the application of microinjection to microalgae compared with mammalian cells or zygotes is rare. This study represents the first successful report of a microinjection method for delivering CRISPR/Cas9 RNPs into microalgal cells.

In summary, we repeated the experiment parameters from Nomura *et al.* (2020), and obtained suboptimal results, with a maximum editing efficiency of 0.98% by electroporation. Moreover, we conducted CRISPR/Cas9-mediated genome editing of *E. gracilis* and successfully knocked out the *crtP1* gene by microinjection with relatively high efficiency (16.7%). The generated stable *crtP1* mutant can be a good candidate for studying carotenoid metabolism in *E. gracilis*. To the best of our knowledge, this is the first application of microinjection to genome editing in microalgae. Overall, we demonstrate that microinjection-based single-celled manipulation has potential exogenous material delivery to facilitate the bioengineering and biotechnology of microalgae.

Acknowledgements

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

ZC and JW conceived and designed the experiments. JZ, ZC, MD, RY and WF helped to perform experiments. AL and JW helped revise the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Mutant types of the sequence targeted on the *crtP1* gene after electroporation, determined by high-throughput sequencing

Method S1. Methods for Cas9 RNPs electroporation and single-celled microinjection on *E. gracilis*

Movie S1 Short movie of single-celled microinjection on *E. gracilis*