Genome editing using a DNA-free CRISPR-Cas9 system in green seaweed *Ulva prolifera*

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Short running title: Genome editing in *Ulva*

SUMMARY

Although the green seaweed *Ulva* is one of the most common seaweeds in the coastal regions with well-studied ecological characteristics, few reverse genetic technologies have been developed for it. The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system is a simple genome editing technology based on a ribonucleoprotein (RNP) complex composed of an endonuclease and programmable RNA to target particular DNA sequences. Genome editing makes it possible to generate mutations on a target gene in non-model organisms without established transgenic technologies. In this study, we applied the CRISPR-Cas9 RNP genome editing system to the green seaweed *Ulva prolifera*, using polyethylene glycol (PEG)-mediated transfection. Our experimental system disrupts a single gene (*UpAPT*) encoding adenine phosphoribosyl transferase (APT) and generates a resistant phenotype for gametophytes cultured in a medium with toxic compound 2-fluoroadenine. The PEG-mediated transfection used for gametes resulted in 2-fluoroadenine-resistant strains containing short indels or substitutions on *UpAPT*. Our results showed that the CRISPR-Cas9 system with PEG-mediated transfection was efficient for genome editing in *Ulva*.

Keywords: 2-fluoroadenine, adenine phosphoribosyl transferase, green alga, reverse genetics, ribonucleoprotein complex, transfection

ABBREVIATIONS

CRISPR clustered regularly interspaced short palindromic repeats

crRNA CRISPR RNA

GFP green fluorescent protein

gRNA guide RNA

HDR homology-directed repair

PAM protospacer adjacent motif

PEG polyethylene glycol

RNP ribonucleoprotein

ssODN single-stranded oligodeoxynucleotide

tracrRNA trans-activating crRNA

INTRODUCTION

The green seaweed *Ulva,* belonging to a green algal lineage of Ulvophyceae, is a common primary producer found in seashores, brackish estuaries, and freshwater rivers and ponds (Van Den Hoek *et al*. 1995, Shimada *et al*. 2008, Ichihara *et al*. 2009). *Ulva* spp. can cause environmental problems such as green tides (Largo *et al.* 2004, Zhao et al. 2013); however, their rapid proliferation ability may have applications in biofuels and bioremediation (Nielsen et al. 2012, Bikker et al. 2016). Recent studies have applied next-generation sequencing technology to discover genomic information (De Clerck *et al.* 2018) including mating-type loci in *Ulva* (Yamazaki *et al.* 2017). These findings have significantly advanced the evolutionary understanding of *Ulva* and improved the ecological applications of its genetic resources. Reverse genetics has also been applied in *Ulva* studies. The transient expression of an exogenous GFP gene driven by endogenous ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (*rbcS*) promoter was successfully applied in vegetative cells using the particle bombardment method (Kakinuma *et al.* 2009). Subsequently, the more efficient PEG-mediated transformation method has been developed for *Ulva* gametes (Oertel *et al*. 2015, Suzuki *et al.* 2016). Blomme *et al.* (2021) developed a useful molecular toolkit that enables the stable expression of fluorescent proteins, marker lines for different organelles, and tagged endogenous proteins in *Ulva mutabilis*; this toolkit is expected to play an important role in the study of gene functions in *Ulva*.

Genome editing is a powerful tool to understand gene function by causing a specific mutation on the target gene. CRISPR-Cas9 system is a simple genome editing system based on bacterial immunity. Cas9 serves as a DNA endonuclease, guided by programmable RNA that cleaves DNA upon crRNA and recognises the targets by

tracrRNA. Target recognition by the Cas9 protein requires a conserved dinucleotide-containing PAM sequence (e.g., 5'-NGG–3' for *Streptococcus pyogenes* Cas9) following the crRNA targeting sequence (Jinek *et al*. 2012). Therefore, the CRISPR-Cas9 system can mutate almost any target gene using a PAM sequence by designing a target-specific crRNA, and the system has been successfully used in various organisms (Deltcheva *et al*. 2011, Hsu *et al*. 2014, Belhaj *et al*. 2015, Nødvig *et al*. 2015). In many organisms, genome editing is performed by delivery of DNA plasmids that encode Cas9 protein and gRNAs. A DNA-free method requiring delivery of pre-assembled Cas9/gRNA RNP complexes has also been developed in some plants and algae (Woo *et al*. 2015, Baek *et al*. 2016). The DNA-free method is used in cases when transgenic lines cannot be created, and Cas9 protein will be degraded after a certain period resulting in low off-target cleavage rates (Svitashev *et al*. 2016, Liang *et al*. 2017). In the model green alga *Chlamydomonas reinhardtii*, recent studies using pre-assembled Cas9/single-guide RNA RNP complexes have shown high mutation efficiency (Baek *et al*. 2016, Shin *et al*. 2016). In other microalgae, such as diatoms (Serif *et al*. 2018), *Nannochloropsis* (Naduthodi *et al*. 2019), and *Tetraselmis* (Chang *et al*. 2020), the DNA-free CRISPR-Cas9 system has been used to understand gene function and modify metabolic pathways. Recently, Cas9 RNPs method have been used in the filamentous brown alga *Ectocarpus*. Cas9 RNPs were delivered during two developmental stages using different methods (particle bombardment for gametes and laser-assisted thermal-expansion microinjection for unilocular sporangia), and both methods resulted in target-gene mutated strains (Badis *et al*. 2021).

In this study, we developed a genome-editing system using DNA-free CRISPR-Cas9 in the green seaweed *Ulva prolifera*. We used PEG-mediated transfection of Cas9

RNP complexes, using gamete cells obtained by synchronised induction and selection of mutants by disruption of an endogenous adenine phosphoribosyl transferase (*APT*) gene. APT is an enzyme involved in the purine salvage pathway that catalyses the conversion of free adenine into adenosine monophosphate (Ashihara *et al*. 2018). Some plants and algae exhibit a lethal phenotype under cultivation with adenine analogues, such as 2-fluoroadenine (2-FA), and loss of function for APT results in resistance to these analogues (Trouiller *et al*. 2007, Collonnier *et al*. 2017, Serif *et al*. 2018, Guzmán-Zapata *et al*. 2019, Badis *et al*. 2021). Therefore, we attempted to disrupt a *U. prolifera APT* gene (*UpAPT*) via genome editing and selection of mutants using 2-FA. PEG-mediated transfection was applied in *Ulva* gametes and generated 2-FA-resistant thalli. Sequencing analyses showed that these 2-FA-resistant mutants had various mutations including deletions, insertions, and substitutions on the *UpAPT* locus. Our genome editing technology is expected to advance biotechnology and gene function analysis by reverse genetics in seaweeds.

MATERIALS AND METHODS

Culture conditions

In this study, we used a previously described *Ulva prolifera* male gametophyte (strain E21; Shimada *et al.* 2008). Gamete germlings were cultured in petri dishes at 20°C with a 14:10-h light:dark cycle under fluorescent lights at 100 μmol m^{-2} s^{-1} in filtered seawater supplemented with 1/2 Provasoli's enriched seawater (PES) medium stock solution (Provasoli 1968). Upon reaching a size of 5 mm, some thalli were cultured in 1-L aerated flasks for 2–3 weeks to reach a size large enough to induce gametogenesis. The method for inducing gametogenesis has been described previously (Hiraoka & Enomoto 1998). To measure the growth inhibition effect of 2-FA, gamete suspensions were added to each

well of a 12-well culture plate. After 2 days, the medium was changed to 2-FA-supplemented 1/2 PES medium, prepared at 5, 10, 20, or 40 μM 2-FA by addition of 20 mM 2-FA stock solution dissolved in dimethyl sulfoxide (DMSO) to 1/2 PES medium; 1/2 PES medium supplemented with 0.2% (v/v) DMSO was used as the control.

UpAPT *gene isolation and* in vitro *cleavage assay*

The *Chlamydomonas APT* gene (Gene ID: 5717232) was used as a query sequence to search for the *U. prolifera APT* gene from a previously assembled transcriptomic dataset (PRJDB7990) using the National Center for Biotechnology Information tblastx program. DNA was extracted from *U. prolifera* thallus using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Primers used in this study are summarised in Appendix S1. The genomic *UpAPT* locus was amplified for examination of exon and intron structure by KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan). The PCR program for each gene consisted of an initial denaturation for 3 min at 95°C; followed by 30 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 62°C, and extension for 30 s at 68°C. DNA sequences were assembled and analysed with the MEGA software (version 7.0.26; Pennsylvania State University, State College, PA, USA) (Kumar *et al*. 2016). The amino acid sequence of UpAPT was analysed using CDD/SPARCLE (Marchler-Bauer *et al*. 2017) to reveal the conserved domain and the active site. CRISPRdirect (Naito *et al*. 2015) was used to search for target sequences in *UpAPT*. crRNAs and tracrRNAs were synthesised by Integrated DNA Technologies (IDT; Coraville, IA, USA). To assess the cleavage activity of gRNAs, the partial *UpAPT* sequence was amplified using the protocol described above. To prepare the synthetic gRNAs, crRNAs (100 pmol) and tracrRNA (100 pmol) were mixed and incubated for 5 min at 95°C. Then, 10 pmol Cas9 protein

(IDT) was added to synthetic gRNAs and incubated for 5 min at 37°C to make the RNP complex. The RNP complex was added to the *UpAPT* amplicon with 10× H buffer (Nippon Gene Co., Tokyo, Japan) and incubated for 30 min at 37°C. The reaction products were verified by 1% agarose gel electrophoresis.

PEG transfection of CRISPR-Cas9 RNPs to U. prolifera *gametes*

Gamete concentration was measured using Scepter 2.0 (Merck, Darmstadt, Germany). To prepare the RNP complex, 200 pmol crRNA, 200 pmol tracrRNA, and 40 pmol Cas9 protein were used. Two types of Cas9 proteins were used in this study: GFP-tagged Cas9 (Applied Biological Materials, Richmond, BC, Canada) and non-tagged Cas9 proteins (Alt-R S.p. Cas9 Nuclease 3NLS; IDT). RNP mixture, 100 μL of 1.0×10^6 gamete cell suspension, and 100 μL of 60% (weight/volume) PEG solution were gently mixed. After 20 min, the gametes were washed with filtered seawater and centrifuged at 5000 × *g* for 10 min to obtain the gametes pellet. The pellet was resuspended in 1/2 PES and added to a 60-mm dish. The dish was incubated at 20°C in dark for 36–48 h before 2-FA treatment. The 2-FA selection medium (final 2-FA concentration: 10 μM) was prepared by mixing equal volumes of PES medium supplemented with 2-FA (20 μM) with filtered, conditioned 1/2 PES medium, in which *U. prolifera* thalli had been cultured for 1–2 months. The 1/2 PES medium was removed, and the selection medium was added to the dish. After being cultured for 2–3 weeks, 2-FA-resistant thalli were transferred to a 12- or 24-well plate filled with the selection medium. The culture medium was changed every week. Two experiments were carried out independently.

Confirmation of APT *gene editing*

DNA was extracted from each 2-FA-resistant thallus using the Cica Geneus Extraction Reagent Series (Kanto Chemical Co., Tokyo, Japan). PCR was carried out under the cycling conditions described above. PCR products were sequenced using Eurofins Genomics (Ota-ku, Tokyo, Japan).

RESULTS

Growth inhibition assay at various 2-FA concentrations

Thalli were cultivated in 1/2 PES supplemented with four different concentrations of 2-FA (40, 20, 10, and 5 μM) and a control to determine the growth inhibitory concentration of 2-FA for *U. prolifera*. After 1 week of incubation, the growth of thalli was suppressed, and the colour of thalli changed from green to white for all experimental conditions (Fig. 1). Based on this experiment, we selected 10 μM 2-FA medium as the optimal medium in the following experiments.

Genomic structure of UpAPT *and cleavage testing of gRNAs*

UpAPT was found from previous transcriptomic data using the tblastx program. Amino acid alignment with CreAPT (XP_001691572.1) showed a well-conserved domain, including the active site regions (Appendix S2). Genomic PCR of the *UpAPT* gene (LC627065) revealed that it contained four exons and three introns (Fig. 2a). To disrupt the *UpAPT* gene, two crRNAs were designed to target the putative active site on the third exon (Fig. 2a). The *in vitro* cleavage assay showed that both gRNAs cleaved the *UpAPT* fragment (Fig. 2b).

PEG-mediated transfection experiment

GFP-tagged Cas9 was used to observe the delivery of Cas9 RNP complexes into the cells. However, one day after the PEG-mediated transfection experiment, no GFP signals were detected by observation of the attached gametes under a fluorescence microscope. Therefore, non-tagged Cas9 proteins were used in the second experiment. Two weeks after PEG-mediated transfection experiments using the two gRNA types, grown thalli were observed by naked eye under experimental conditions with Cas9 RNP complexes (Fig. 3; Table 1). Over 1000 thalli were consistently developed in all gRNA#2 experiments, but gRNA#1 experiments generated varying numbers of 2-FA-resistant thalli (Table 1). Some of these thalli were transferred to 12- or 24-well plates filled with selection medium as a second screening. In the first experiment, 5/16 strains obtained from the gRNA#1 experiment survived, while 18/18 strains obtained from the gRNA#2 experiment survived. In the second experiment, 22/24 strains from gRNA#1 and 24/24 strains from gRNA#2 experiments survived (Table 1). The DNA fragments size of the *UpAPT* gene amplified from 2-FA-resistant thalli were similar to the fragments amplified from the wild strain. Sequencing analysis of the *UpAPT* gene demonstrated that 2-FA-resistant strains had various mutations, including deletions, insertions, and substitutions (Fig. 4a; Appendix S3). These genome-edited strains grew well in both selection and 1/2 PES media (Fig. 4b; Appendix S4). The major mutations were the 2 bp deletion by gRNA#1 (n = 3) and the 4 bp deletion by gRNA#2 (n = 7).

DISCUSSION

This study showed that genome editing using the CRISPR-Cas9 system and PEG-mediated transfection can generate mutants in the green seaweed *U. prolifera*. The *APT*

gene is a key enzyme in the purine salvage pathway. It was found to be a suitable counter-selectable marker for genome editing methods in plants as well as macroalgae because only mutant strains can grow in the toxic 2-FA medium.

DNA-free Cas9 experiments in algae without established transgenic methods have an advantage because the loss of function mutants could be created. PEG-mediated direct delivery methods in *U. prolifera* showed high selection efficiencies (9.5×10^{-3}–1.6×10^{-1}) and target mutation efficiencies (3.0×10^{-3}–1.6×10^{-1}). The transient expression efficiencies of the exogenous GFP gene introduced using the PEG-mediated method were also high (9.0–15.1%) in *U. partita* gametes (Suzuki *et al*. 2016). These results suggest that the PEG-mediated method is efficient for delivering transgenic vectors or exogenous proteins to the cell wall-less *Ulva* gametes. We compared two gRNAs, and found gRNA#2 to be more efficient than gRNA#1 in obtaining mutant thalli. It has been reported that high-GC-content gRNAs were more efficient in causing mutations in model organism zebrafish (Gagnon *et al*. 2014). However, although the effects of the GC content of gRNAs on the efficiency of Cas9 protein activity remain unknown in *Ulva*, our experiment demonstrated that the higher-GC-content gRNA#1 (60%) was less efficient than gRNA#2 (45%). The efficiency of a gRNA depends on various factors, and further studies are needed to design more efficient gRNAs for *Ulva*.

We detected only short indels (−7 bp to +6 bp) on the *UpAPT* locus. Large indels were not detected, indicating that only the NHEJ reaction occurred at the *UpAPT* locus. In *Ectocarpus*, the major type of mutation at the *APT* locus is short indels (1–4 bp) three nucleotides upstream from the PAM; few mutants had long indels (Badis *et al.* 2021). The majority of double-strand breaks generated by Cas9 in the *APT* locus of *Chlamydomonas* were repaired with large insertions (101 bp), similar to the MRC1 miniature

retrotransposon sequence (Kim *et al*. 2006). The insertion site was 24 nucleotides downstream from the PAM site (Guzmán-Zapata *et al*. 2019). Atypical gene sequence modifications found in *Chlamydomonas* were not detected on the *APT* locus in *Ulva*, indicating that different repair processes must be used for double-strand breaks in different organisms. HDR is a double-strand break repair mechanism that uses homologous donor sequences to introduce mutations or integrate exogenous genes into the genome. Knock-in and expression of the antibiotic resistance genes by homologous recombination using a long homology arm (1 kb) was successful in diatom *Phaeodactylum tricornutum* (Moosburner *et al*. 2020). Recently, other HDR methods using the ssODN for genome modification were developed in some microalgae. The efficient knock-in using Cas9 RNPs with ssODNs (142 bp) as DNA donors was used in *Euglena gracilis* (Nomura *et al*. 2019). In this case, a 50-bp homology arm played a role in HDR, and a knock-in DNA fragment (42 bp) including *Eco*RI, *Eco*RV, and *Bam*HI sites were inserted into the glucan synthase-like 2 gene. Another CRISPR endonuclease, Cpf1, made efficient gene editing possible using the HDR pathway in *C. reinhardtii* (Ferenczi *et al*. 2017). Co-delivery of CRISPR-Cpf1 RNP complex and ssODN repair templates induced precise, targeted DNA replacement, such as the insertion of His-tag or stop codon. A similar strategy might work in *Ulva*, making it possible to fuse fluorescent protein and epitope tag with the endogenous target gene by HDR, and to uncover the spatial and temporal expression of the target gene.

To develop transformants for a gene of interest, it was necessary to co-transform the gene and a selectable marker, and integrate the genes into the genome. However, the mutation on the *APT* gene makes screening using 2-FA possible without integration of an exogenous selectable marker. The generation of double-mutants (an endogenous counter-

selectable marker gene and a gene of interest) has already been attempted in algae. In the model diatom *P. tricornutum*, *PtAPT* was used as a counter-selectable marker gene, and aureochrome (*PtAureo1a*) mutants were obtained (Serif *et al*. 2018). In the model brown alga *Ectocarpus*, *APT* was used as a counter-selectable marker to obtain mutants of FK501-binding-protein 12 (*FKBP12*), vanadium-dependent bromoperoxidase (*vBPO*), and mastigoneme 1 (*MAS1*) genes (Badis *et al*. 2021). Because the targeted mutagenic efficiency of the PEG-mediated Cas9 RNP delivery method was high in *Ulva*, use of multiple RNPs may make it possible to analyse functions of genes using 2-FA selection. Additionally, recently reported molecular toolkits for *Ulva* have allowed the creation of a gain-of-function mutant line (Blomme *et al.* 2021). These molecular toolkits and our genome editing methods will broaden the scope of functional genomic studies of *Ulva*.

ACKNOWLEDGEMENTS

This work was supported by JSPS KAKENHI grant numbers JP18K14690 and JP21K06242 to K.I.

REFERENCES

Ashihara, H., Stasolla, C., Fujimura, T. and Crozier, A. 2018. Purine salvage in plants. *Phytochemistry* **147**: 89–124.

Badis, Y., Scornet, D., Harada, M. *et al.* 2021. Targeted CRISPR-Cas9-based gene knockouts in the model brown alga *Ectocarpus*. *New Phytol.* **231**: 2077-91.

Baek, K., Kim, D.H., Jeong, J. *et al*. 2016. DNA-free two-gene knockout in *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins. *Sci. Rep.* **6**: 30620.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N.J. and Nekrasov, V. 2015. Editing plant genomes with CRISPR/Cas9. *Curr. Opin. Biotechnol.* **32**: 76–84.

Bikker, P., Krimpen, M.M. Van, Wikselaar, P. *et al*. 2016. Biorefinery of the green seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels. *J. Appl. Phycol.* **28**: 3511–25.

Blomme, J., Liu, X., Jacobs, T.B. and De Clerck, O. 2021. A molecular toolkit for the green seaweed *Ulva mutabilis*. *Plant Physiol.* **186**:1442–54.

Chang, K.S., Kim, J., Park, H., Hong, S.J., Lee, C.G. and Jin, E.S. 2020. Enhanced lipid productivity in AGP knockout marine microalga *Tetraselmis* sp. using a DNA-free CRISPR-Cas9 RNP method. *Bioresour. Technol.* **303**: 122932.

Collonnier, C., Epert, A., Mara, K. *et al*. 2017. CRISPR-Cas9-mediated efficient directed mutagenesis and RAD51-dependent and RAD51-independent gene targeting in the moss *Physcomitrella patens*. *Plant Biotechnol. J.* **15**: 122–31.

De Clerck, O., Kao, S.-M., Bogaert, K.A. *et al*. 2018. Insights into the evolution of multicellularity from the sea lettuce genome. *Curr. Biol.* **28**: 2921–2933.

Deltcheva, E., Chylinski, K., Sharma, C.M. *et al*. 2011. CRISPR RNA maturation by

trans-encoded small RNA and host factor RNase III. *Nature* **471**: 602–7.

Ferenczi, A., Pyott, D.E., Xipnitou, A., Molnar, A. and Merchant, S.S. 2017. Efficient targeted DNA editing and replacement in *Chlamydomonas reinhardtii* using Cpf1 ribonucleoproteins and single-stranded DNA. *Proc. Natl. Acad. Sci. U. S. A.* **114**: 13567–72.

Gagnon, J.A., Valen, E., Thyme, S.B. *et al*. 2014. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLOS ONE*. **9**: e106396.

Guzmán-Zapata, D., Sandoval-Vargas, J.M., Macedo-Osorio, K.S. *et al*. 2019. Efficient editing of the nuclear APT reporter gene in *Chlamydomonas reinhardtii* via expression of a CRISPR-Cas9 module. *Int. J. Mol. Sci.* **20**: 1247.

Hiraoka, M. and Enomoto, S. 1998. The induction of reproductive cell formation of *Ulva pertusa* Kjellman (Ulvales, Ulvophyceae). *Phycol. Res.* **46**: 199–203.

Hsu, P.D., Lander, E.S. and Zhang, F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**: 1262–78.

Ichihara, K., Arai, S., Uchimura, M. *et al*. 2009. New species of freshwater *Ulva*, *Ulva limnetica* (Ulvales, Ulvophyceae) from the Ryukyu Islands, Japan. *Phycol. Res.* **57**: 94–103.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816–21

Kakinuma, M., Ikeda, M., Coury, D. A., Tominaga, H., Kobayashi, I. and Amano, H. 2009. Isolation and characterization of the *rbcS* genes from a sterile mutant of *Ulva pertusa* (Ulvales, Chlorophyta) and transient gene expression using the *rbcS* gene

promoter. *Fish. Sci.* **75**: 1015–28.

Kim, K.S., Kustu, S. and Inwood, W. 2006. Natural history of transposition in the green alga *Chlamydomonas reinhardtii*: Use of the AMT4 locus as an experimental system. *Genetics* **173**: 2005–19.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–4.

Largo, D.B., Sembrano, J., Hiraoka, M. and Ohno, M. 2004. Taxonomic and ecological profile of 'green tide' species of *Ulva* (Ulvales , Chlorophyta) in central Philippines. *Hydrobiologia* **512**: 247–53.

Liang, Z., Chen, K., Li, T. *et al*. 2017. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* **8**: 14261.

Marchler-Bauer, A., Bo, Y., Han, L. *et al*. 2017. CDD/SPARCLE: Functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* **45**: D200–3.

Moosburner, M.A., Gholami, P., McCarthy, J.K. *et al*. 2020. Multiplexed knockouts in the model diatom *Phaeodactylum* by episomal delivery of a selectable Cas9. *Front Microbiol.* **11**: 5.

Naduthodi, M.I.S., Mohanraju, P., Südfeld, C. *et al*. 2019. CRISPR-Cas ribonucleoprotein mediated homology-directed repair for efficient targeted genome editing in microalgae *Nannochloropsis oceanica* IMET1. *Biotechnol. Biofuels* **12**: 66.

Naito, Y., Hino, K., Bono, H. and Ui-Tei, K. 2015. CRISPRdirect: Software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics* **31**: 1120–3.

Nielsen, M.M., Bruhn, A., Rasmussen, M.B., Olesen, B., Larsen, M.M. and Møller, H.B. 2012. Cultivation of *Ulva lactuca* with manure for simultaneous bioremediation and biomass production. *J. Appl. Phycol.* **24**: 449–58.

Nødvig, C.S., Nielsen, J.B., Kogle, M.E. and Mortensen, U.H. 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLOS ONE* **10**: e0133085.

Nomura, T., Inoue, K., Uehara-Yamaguchi, Y. *et al*. 2019. Highly efficient transgene-free targeted mutagenesis and single-stranded oligodeoxynucleotide-mediated precise knock-in in the industrial microalga *Euglena gracilis* using Cas9 ribonucleoproteins. *Plant Biotechnol J.* **17**: 2032–4.

Oertel, W., Wichard, T. and Weissgerber, A. 2015. Transformation of *Ulva mutabilis* (Chlorophyta) by vector plasmids integrating into the genome. *J. Phycol.* **51**: 963–79.

Provasoli, L. 1968. Media and prospects for the cultivation of marine algae. *In* Watanabe, A. and Hattori, A. (Eds) *Culture and Collections of Algae*. Japanese Society of Plant Physiologists, Tokyo, pp. 63–75.

Serif, M., Dubois, G., Finoux, A.L., Teste, M.A., Jallet, D. and Daboussi, F. 2018. One-step generation of multiple gene knock-outs in the diatom *Phaeodactylum tricornutum* by DNA-free genome editing. *Nat. Commun.* **9**: 3924.

Shimada, S., Yokoyama, N., Arai, S. and Hiraoka, M. 2008. Phylogeography of the genus *Ulva* (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. *J. Appl. Phycol.* **20**: 979–89.

Shin, S.E., Lim, J.M., Koh, H.G. *et al*. 2016. CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. *Sci. Rep.* **6**: 27810.

Suzuki, R., Ota, S., Yamazaki, T. *et al*. 2016. Morphological changes of giant

374 mitochondria in the unicellular to multicellular phase during parthenogenesis of
375 *Ulva partita* (Ulvophyceae) revealed by expression of mitochondrial targeting GFP
376 and PEG transformation. *Phycol. Res.* **64**: 176–84.
377 Svitashev, S., Schwartz, C., Lenderts, B., Young, J.K. and Cigan, A. M. 2016. Genome
378 editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat.*
379 *Commun.* **7**:13274.
380 Trouiller, B., Charlot, F., Choinard, S., Schaefer, D.G. and Nogué, F. 2007. Comparison
381 of gene targeting efficiencies in two mosses suggests that it is a conserved feature
382 of Bryophyte transformation. *Biotechnol. Lett.* **29**: 1591–8.
383 Van Den Hoek, C., Mann, D.G. and Jahns, H.M. 1995. *Algae: An introduction to*
384 *phycology.* Cambridge University Press, Cambridge.
385 Woo, J., Kim, J., Kwon, S. *et al*. 2015. DNA-free genome editing in plants with
386 preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* **33**: 1162–4.
387 Yamazaki, T., Ichihara, K., Suzuki, R. *et al*. 2017. Genomic structure and evolution of
388 the mating type locus in the green seaweed *Ulva partita*. *Sci. Rep.* **7**: 11679.
389 Zhao, J., Jiang, P., Liu, Z. *et al*. 2013. The Yellow Sea green tides were dominated by
390 one species, *Ulva* (*Enteromorpha*) *prolifera*, from 2007 to 2011. *Chin. Sci. Bull.*
391 **58**: 2298–302.
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Supporting Information

Additional supporting information can be found in the online version of this article at the publisher's web-site:

Appendix S1: Primers used in this study.

Appendix S2: Amino acid sequence alignment of the adenine phosphoribosyl transferase (*APT*) gene.

Appendix S3: Genome-edited strains obtained in this study.

Appendix S4: Male gametophyte (WT) and genome-edited strains in 1/2 PES and selection media.

Table 1 Up*APT* mutation efficiency using Cas9 RNP complexes.

	gRNA#1		gRNA#2	
	1st experiment	2nd experiment	1st experiment	2nd experiment
Number of thalli (Survival rate in 1st screening cell^{-1})	95 (9.5×10^{-3})	859 (8.6×10^{-2})	1020 (1.0×10^{-1})	1575 (1.6×10^{-1})
Survival rate in 2nd screening	31.3% (5/16)	91.6% (22/24)	100% (18/18)	100% (24/24)
Confirmed mutations on Up*APT*	5/5	7/7	18/18	7/7
Expected target mutation efficiency	3.0×10^{-3}	7.9×10^{-2}	1.0×10^{-1}	1.6×10^{-1}

Expected target mutation efficiency = (survival rate in 1st screening) × (survival rate in 2nd screening) × (confirmed mutations on Up*APT*)

Up*APT*: *Ulva prolifera APT* gene

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FIGURES

Fig. 1 Growth inhibition assay using various concentrations of 2-FA. Different amounts of 2 mM 2-FA dissolved in dimethyl sulfoxide (DMSO) were added to 1/2 PES medium.

Fig. 2 Up*APT* gene and cleavage assay by gRNA. a. Schematic diagrams of the Up*APT* gene. Gray boxes and dark gray lines indicate exons and introns, respectively, and the black box indicates the targeted region for each crRNA. Underbar indicates PAM sequence. Arrows indicate each primer position. b. The *in vitro* cleavage assay of Cas9 protein and individual gRNAs. The *APT* amplicon was digested into two fragments by each Cas9 RNP, but not digested by Cas9 protein only.

Fig. 3 Thalli developed in 1/2 PES medium supplemented with 10 μM 2-FA after delivering Cas9 RNP via PEG-mediation. Enlarged images are shown at the bottom right of each plate.

Fig. 4 Mutation pattern on the Up*APT* locus and the phenotype of some genome-edited strains. a. Substitutions (S) and indels (I) in the Up*APT* sequence detected in each genome-edited strain. Bold letters indicate gRNA sequences; PAM sequence is underlined. b. Genome-edited strains grew well in 1/2 PES medium supplemented with 10 μM 2-FA.