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

39 gRNA guide RNA

40 HDR homology-directed repair

41 PAM protospacer adjacent motif

42 PEG polyethylene glycol

43 RNP ribonucleoprotein

44 ssODN single-stranded oligodeoxynucleotide

45 tracrRNA trans-activating crRNA

46

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

95 RNP complexes, using gamete cells obtained by synchronised induction and selection of
96 mutants by disruption of an endogenous adenine phosphoribosyl transferase (*APT*) gene.
97 APT is an enzyme involved in the purine salvage pathway that catalyses the conversion
98 of free adenine into adenosine monophosphate (Ashihara *et al*. 2018). Some plants and
99 algae exhibit a lethal phenotype under cultivation with adenine analogues, such as 2-
100 fluoroadenine (2-FA), and loss of function for APT results in resistance to these analogues
101 (Trouiller *et al*. 2007, Collonnier *et al*. 2017, Serif *et al*. 2018, Guzmán-Zapata *et al*. 2019,
102 Badis *et al*. 2021). Therefore, we attempted to disrupt a *U. prolifera APT* gene (*UpAPT*)
103 via genome editing and selection of mutants using 2-FA. PEG-mediated transfection was
104 applied in *Ulva* gametes and generated 2-FA-resistant thalli. Sequencing analyses showed
105 that these 2-FA-resistant mutants had various mutations including deletions, insertions,
106 and substitutions on the *UpAPT* locus. Our genome editing technology is expected to
107 advance biotechnology and gene function analysis by reverse genetics in seaweeds.
108

109 **MATERIALS AND METHODS**

110 *Culture conditions*

111 In this study, we used a previously described *Ulva prolifera* male gametophyte (strain
112 E21; Shimada *et al.* 2008). Gamete germlings were cultured in petri dishes at 20°C with
113 a 14:10-h light:dark cycle under fluorescent lights at 100 μmol m^{-2} s^{-1} in filtered seawater
114 supplemented with 1/2 Provasoli's enriched seawater (PES) medium stock solution
115 (Provasoli 1968). Upon reaching a size of 5 mm, some thalli were cultured in 1-L aerated
116 flasks for 2–3 weeks to reach a size large enough to induce gametogenesis. The method
117 for inducing gametogenesis has been described previously (Hiraoka & Enomoto 1998).
118 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

143 (IDT) was added to synthetic gRNAs and incubated for 5 min at 37°C to make the RNP
144 complex. The RNP complex was added to the *UpAPT* amplicon with 10× H buffer
145 (Nippon Gene Co., Tokyo, Japan) and incubated for 30 min at 37°C. The reaction products
146 were verified by 1% agarose gel electrophoresis.
147
148 *PEG transfection of CRISPR-Cas9 RNPs to* U. prolifera *gametes*
149 Gamete concentration was measured using Scepter 2.0 (Merck, Darmstadt, Germany). To
150 prepare the RNP complex, 200 pmol crRNA, 200 pmol tracrRNA, and 40 pmol Cas9
151 protein were used. Two types of Cas9 proteins were used in this study: GFP-tagged Cas9
152 (Applied Biological Materials, Richmond, BC, Canada) and non-tagged Cas9 proteins
153 (Alt-R S.p. Cas9 Nuclease 3NLS; IDT). RNP mixture, 100 μL of 1.0×10^6 gamete cell
154 suspension, and 100 μL of 60% (weight/volume) PEG solution were gently mixed. After
155 20 min, the gametes were washed with filtered seawater and centrifuged at 5000 × *g* for
156 10 min to obtain the gametes pellet. The pellet was resuspended in 1/2 PES and added to
157 a 60-mm dish. The dish was incubated at 20°C in dark for 36–48 h before 2-FA treatment.
158 The 2-FA selection medium (final 2-FA concentration: 10 μM) was prepared by mixing
159 equal volumes of PES medium supplemented with 2-FA (20 μM) with filtered,
160 conditioned 1/2 PES medium, in which *U. prolifera* thalli had been cultured for 1–2
161 months. The 1/2 PES medium was removed, and the selection medium was added to the
162 dish. After being cultured for 2–3 weeks, 2-FA-resistant thalli were transferred to a 12- or
163 24-well plate filled with the selection medium. The culture medium was changed every
164 week. Two experiments were carried out independently.
165
166 *Confirmation of* APT *gene editing*

167 DNA was extracted from each 2-FA-resistant thallus using the Cica Geneus Extraction
168 Reagent Series (Kanto Chemical Co., Tokyo, Japan). PCR was carried out under the
169 cycling conditions described above. PCR products were sequenced using Eurofins
170 Genomics (Ota-ku, Tokyo, Japan).
171
172 **RESULTS**
173 *Growth inhibition assay at various 2-FA concentrations*
174 Thalli were cultivated in 1/2 PES supplemented with four different concentrations of 2-
175 FA (40, 20, 10, and 5 μM) and a control to determine the growth inhibitory concentration
176 of 2-FA for *U. prolifera*. After 1 week of incubation, the growth of thalli was suppressed,
177 and the colour of thalli changed from green to white for all experimental conditions (Fig.
178 1). Based on this experiment, we selected 10 μM 2-FA medium as the optimal medium in
179 the following experiments.
180
181 *Genomic structure of* UpAPT *and cleavage testing of gRNAs*
182 *UpAPT* was found from previous transcriptomic data using the tblastx program. Amino
183 acid alignment with CreAPT (XP_001691572.1) showed a well-conserved domain,
184 including the active site regions (Appendix S2). Genomic PCR of the *UpAPT* gene
185 (LC627065) revealed that it contained four exons and three introns (Fig. 2a). To disrupt
186 the *UpAPT* gene, two crRNAs were designed to target the putative active site on the third
187 exon (Fig. 2a). The *in vitro* cleavage assay showed that both gRNAs cleaved the *UpAPT*
188 fragment (Fig. 2b).
189
190

191 *PEG-mediated transfection experiment*

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

211

212 **DISCUSSION**

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

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

218 DNA-free Cas9 experiments in algae without established transgenic methods
219 have an advantage because the loss of function mutants could be created. PEG-mediated
220 direct delivery methods in *U. prolifera* showed high selection efficiencies (9.5×10^{-3}–1.6
221 $\times 10^{-1}$) and target mutation efficiencies (3.0×10^{-3}–1.6×10^{-1}). The transient expression
222 efficiencies of the exogenous GFP gene introduced using the PEG-mediated method were
223 also high (9.0–15.1%) in *U. partita* gametes (Suzuki *et al*. 2016). These results suggest
224 that the PEG-mediated method is efficient for delivering transgenic vectors or exogenous
225 proteins to the cell wall-less *Ulva* gametes. We compared two gRNAs, and found
226 gRNA#2 to be more efficient than gRNA#1 in obtaining mutant thalli. It has been
227 reported that high-GC-content gRNAs were more efficient in causing mutations in model
228 organism zebrafish (Gagnon *et al*. 2014). However, although the effects of the GC content
229 of gRNAs on the efficiency of Cas9 protein activity remain unknown in *Ulva*, our
230 experiment demonstrated that the higher-GC-content gRNA#1 (60%) was less efficient
231 than gRNA#2 (45%). The efficiency of a gRNA depends on various factors, and further
232 studies are needed to design more efficient gRNAs for *Ulva*.

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

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

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

263 selectable marker gene and a gene of interest) has already been attempted in algae. In the
264 model diatom *P. tricornutum*, *PtAPT* was used as a counter-selectable marker gene, and
265 aureochrome (*PtAureo1a*) mutants were obtained (Serif *et al*. 2018). In the model brown
266 alga *Ectocarpus*, *APT* was used as a counter-selectable marker to obtain mutants of
267 FK501-binding-protein 12 (*FKBP12*), vanadium-dependent bromoperoxidase (*vBPO*),
268 and mastigoneme 1 (*MAS1*) genes (Badis *et al*. 2021). Because the targeted mutagenic
269 efficiency of the PEG-mediated Cas9 RNP delivery method was high in *Ulva*, use of
270 multiple RNPs may make it possible to analyse functions of genes using 2-FA selection.
271 Additionally, recently reported molecular toolkits for *Ulva* have allowed the creation of a
272 gain-of-function mutant line (Blomme *et al.* 2021). These molecular toolkits and our
273 genome editing methods will broaden the scope of functional genomic studies of *Ulva*.
274

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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

405 **FIGURES**

406 Fig. 1 Growth inhibition assay using various concentrations of 2-FA. Different amounts
407 of 2 mM 2-FA dissolved in dimethyl sulfoxide (DMSO) were added to 1/2 PES medium.
408

409 Fig. 2 Up*APT* gene and cleavage assay by gRNA. a. Schematic diagrams of the Up*APT*
410 gene. Gray boxes and dark gray lines indicate exons and introns, respectively, and the
411 black box indicates the targeted region for each crRNA. Underbar indicates PAM
412 sequence. Arrows indicate each primer position. b. The *in vitro* cleavage assay of Cas9
413 protein and individual gRNAs. The *APT* amplicon was digested into two fragments by
414 each Cas9 RNP, but not digested by Cas9 protein only.
415

416 Fig. 3 Thalli developed in 1/2 PES medium supplemented with 10 μM 2-FA after
417 delivering Cas9 RNP via PEG-mediation. Enlarged images are shown at the bottom right
418 of each plate.
419

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