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

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13 Short running title: Genome editing in *Ulva*

14

15 **SUMMARY**

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

31

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

34

35 **ABBREVIATIONS**

36 CRISPR clustered regularly interspaced short palindromic repeats

37 crRNA CRISPR RNA

38 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

47 INTRODUCTION

48 The green seaweed *Ulva*, belonging to a green algal lineage of Ulvophyceae, is
49 a common primary producer found in seashores, brackish estuaries, and freshwater rivers
50 and ponds (Van Den Hoek *et al.* 1995, Shimada *et al.* 2008, Ichihara *et al.* 2009). *Ulva*
51 spp. can cause environmental problems such as green tides (Largo *et al.* 2004, Zhao *et al.*
52 2013); however, their rapid proliferation ability may have applications in biofuels and
53 bioremediation (Nielsen *et al.* 2012, Bikker *et al.* 2016). Recent studies have applied next-
54 generation sequencing technology to discover genomic information (De Clerck *et al.*
55 2018) including mating-type loci in *Ulva* (Yamazaki *et al.* 2017). These findings have
56 significantly advanced the evolutionary understanding of *Ulva* and improved the
57 ecological applications of its genetic resources. Reverse genetics has also been applied in
58 *Ulva* studies. The transient expression of an exogenous GFP gene driven by endogenous
59 ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (*rbcS*) promoter was
60 successfully applied in vegetative cells using the particle bombardment method
61 (Kakinuma *et al.* 2009). Subsequently, the more efficient PEG-mediated transformation
62 method has been developed for *Ulva* gametes (Oertel *et al.* 2015, Suzuki *et al.* 2016).
63 Blomme *et al.* (2021) developed a useful molecular toolkit that enables the stable
64 expression of fluorescent proteins, marker lines for different organelles, and tagged
65 endogenous proteins in *Ulva mutabilis*; this toolkit is expected to play an important role
66 in the study of gene functions in *Ulva*.

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

71 tracrRNA. Target recognition by the Cas9 protein requires a conserved dinucleotide-
72 containing PAM sequence (e.g., 5'-NGG-3' for *Streptococcus pyogenes* Cas9) following
73 the crRNA targeting sequence (Jinek *et al.* 2012). Therefore, the CRISPR-Cas9 system
74 can mutate almost any target gene using a PAM sequence by designing a target-specific
75 crRNA, and the system has been successfully used in various organisms (Deltcheva *et al.*
76 2011, Hsu *et al.* 2014, Belhaj *et al.* 2015, Nødvig *et al.* 2015). In many organisms, genome
77 editing is performed by delivery of DNA plasmids that encode Cas9 protein and gRNAs.
78 A DNA-free method requiring delivery of pre-assembled Cas9/gRNA RNP complexes
79 has also been developed in some plants and algae (Woo *et al.* 2015, Baek *et al.* 2016).
80 The DNA-free method is used in cases when transgenic lines cannot be created, and Cas9
81 protein will be degraded after a certain period resulting in low off-target cleavage rates
82 (Svitashev *et al.* 2016, Liang *et al.* 2017). In the model green alga *Chlamydomonas*
83 *reinhardtii*, recent studies using pre-assembled Cas9/single-guide RNA RNP complexes
84 have shown high mutation efficiency (Baek *et al.* 2016, Shin *et al.* 2016). In other
85 microalgae, such as diatoms (Serif *et al.* 2018), *Nannochloropsis* (Naduthodi *et al.* 2019),
86 and *Tetraselmis* (Chang *et al.* 2020), the DNA-free CRISPR-Cas9 system has been used
87 to understand gene function and modify metabolic pathways. Recently, Cas9 RNPs
88 method have been used in the filamentous brown alga *Ectocarpus*. Cas9 RNPs were
89 delivered during two developmental stages using different methods (particle
90 bombardment for gametes and laser-assisted thermal-expansion microinjection for
91 unilocular sporangia), and both methods resulted in target-gene mutated strains (Badis *et*
92 *al.* 2021).

93 In this study, we developed a genome-editing system using DNA-free CRISPR-
94 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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

119 well of a 12-well culture plate. After 2 days, the medium was changed to 2-FA-
120 supplemented 1/2 PES medium, prepared at 5, 10, 20, or 40 μ M 2-FA by addition of 20
121 mM 2-FA stock solution dissolved in dimethyl sulfoxide (DMSO) to 1/2 PES medium;
122 1/2 PES medium supplemented with 0.2% (v/v) DMSO was used as the control.

123

124 *UpAPT gene isolation and in vitro cleavage assay*

125 The *Chlamydomonas APT* gene (Gene ID: 5717232) was used as a query sequence to
126 search for the *U. prolifera APT* gene from a previously assembled transcriptomic dataset
127 (PRJDB7990) using the National Center for Biotechnology Information tblastx program.
128 DNA was extracted from *U. prolifera* thallus using the DNeasy Plant Mini Kit (Qiagen,
129 Valencia, CA, USA). Primers used in this study are summarised in Appendix S1. The
130 genomic *UpAPT* locus was amplified for examination of exon and intron structure by
131 KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan). The PCR program for each
132 gene consisted of an initial denaturation for 3 min at 95°C; followed by 30 cycles of
133 denaturation for 10 s at 98°C, annealing for 30 s at 62°C, and extension for 30 s at 68°C.
134 DNA sequences were assembled and analysed with the MEGA software (version 7.0.26;
135 Pennsylvania State University, State College, PA, USA) (Kumar *et al.* 2016). The amino
136 acid sequence of UpAPT was analysed using CDD/SPARCLE (Marchler-Bauer *et al.*
137 2017) to reveal the conserved domain and the active site. CRISPRdirect (Naito *et al.*
138 2015) was used to search for target sequences in *UpAPT*. crRNAs and tracrRNAs were
139 synthesised by Integrated DNA Technologies (IDT; Coraville, IA, USA). To assess the
140 cleavage activity of gRNAs, the partial *UpAPT* sequence was amplified using the protocol
141 described above. To prepare the synthetic gRNAs, crRNAs (100 pmol) and tracrRNA
142 (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 $5000 \times g$ 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 μ M) 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 μ M 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 *EcoRI*, *EcoRV*, and *BamHI*
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 (*MASI*) 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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278 **REFERENCES**

- 279 Ashihara, H., Stasolla, C., Fujimura, T. and Crozier, A. 2018. Purine salvage in plants.
280 *Phytochemistry* **147**: 89–124.
- 281 Badis, Y., Scornet, D., Harada, M. *et al.* 2021. Targeted CRISPR-Cas9-based gene
282 knockouts in the model brown alga *Ectocarpus*. *New Phytol.* **231**: 2077-91.
- 283 Baek, K., Kim, D.H., Jeong, J. *et al.* 2016. DNA-free two-gene knockout in
284 *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins. *Sci. Rep.* **6**:
285 30620.
- 286 Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N.J. and Nekrasov, V. 2015.
287 Editing plant genomes with CRISPR/Cas9. *Curr. Opin. Biotechnol.* **32**: 76–84.
- 288 Bikker, P., Krimpen, M.M. Van, Wikselaar, P. *et al.* 2016. Biorefinery of the green
289 seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels. *J. Appl.*
290 *Phycol.* **28**: 3511–25.
- 291 Blomme, J., Liu, X., Jacobs, T.B. and De Clerck, O. 2021. A molecular toolkit for the
292 green seaweed *Ulva mutabilis*. *Plant Physiol.* **186**:1442–54.
- 293 Chang, K.S., Kim, J., Park, H., Hong, S.J., Lee, C.G. and Jin, E.S. 2020. Enhanced lipid
294 productivity in AGP knockout marine microalga *Tetraselmis* sp. using a DNA-free
295 CRISPR-Cas9 RNP method. *Bioresour. Technol.* **303**: 122932.
- 296 Collonnier, C., Epert, A., Mara, K. *et al.* 2017. CRISPR-Cas9-mediated efficient
297 directed mutagenesis and RAD51-dependent and RAD51-independent gene
298 targeting in the moss *Physcomitrella patens*. *Plant Biotechnol. J.* **15**: 122–31.
- 299 De Clerck, O., Kao, S.-M., Bogaert, K.A. *et al.* 2018. Insights into the evolution of
300 multicellularity from the sea lettuce genome. *Curr. Biol.* **28**: 2921–2933.
- 301 Deltcheva, E., Chylinski, K., Sharma, C.M. *et al.* 2011. CRISPR RNA maturation by

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

303 Ferenczi, A., Pyott, D.E., Xipnitou, A., Molnar, A. and Merchant, S.S. 2017. Efficient
304 targeted DNA editing and replacement in *Chlamydomonas reinhardtii* using Cpf1
305 ribonucleoproteins and single-stranded DNA. *Proc. Natl. Acad. Sci. U. S. A.* **114**:
306 13567–72.

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

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

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

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

317 Ichihara, K., Arai, S., Uchimura, M. *et al.* 2009. New species of freshwater *Ulva*, *Ulva*
318 *limnetica* (Ulvales, Ulvophyceae) from the Ryukyu Islands, Japan. *Phycol. Res.* **57**:
319 94–103.

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

323 Kakinuma, M., Ikeda, M., Coury, D. A., Tominaga, H., Kobayashi, I. and Amano, H.
324 2009. Isolation and characterization of the *rbcS* genes from a sterile mutant of *Ulva*
325 *pertusa* (Ulvales, Chlorophyta) and transient gene expression using the *rbcS* gene

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

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

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

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

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

337 Marchler-Bauer, A., Bo, Y., Han, L. *et al.* 2017. CDD/SPARCLE: Functional
338 classification of proteins via subfamily domain architectures. *Nucleic Acids Res.*
339 **45**: D200–3.

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

343 Naduthodi, M.I.S., Mohanraju, P., Südfeld, C. *et al.* 2019. CRISPR-Cas
344 ribonucleoprotein mediated homology-directed repair for efficient targeted genome
345 editing in microalgae *Nannochloropsis oceanica* IMET1. *Biotechnol. Biofuels* **12**:
346 66.

347 Naito, Y., Hino, K., Bono, H. and Ui-Tei, K. 2015. CRISPRdirect: Software for
348 designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics*
349 **31**: 1120–3.

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

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

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

359 Oertel, W., Wichard, T. and Weissgerber, A. 2015. Transformation of *Ulva mutabilis*
360 (Chlorophyta) by vector plasmids integrating into the genome. *J. Phycol.* **51**: 963–
361 79.

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

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

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

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

373 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 Svitashv, 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 Nogu , 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.

392

393 **Supporting Information**

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

396 Appendix S1: Primers used in this study.

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

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

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

402

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 | 95 | 859 | 1020 | 1575 |
| (Survival rate in 1st screening cell ⁻¹) | (9.5×10^{-3}) | (8.6×10^{-2}) | (1.0×10^{-1}) | (1.6×10^{-1}) |
| Survival rate in 2nd screening | 31.3% | 91.6% | 100% | 100% |
| | (5/16) | (22/24) | (18/18) | (24/24) |
| Confirmed mutations on Up <i>APT</i> | 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

403

404

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 μ M 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 μ M 2-FA.