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Electroporation-mediated gene delivery in *Pyropia yezoensis* (Rhodophyta) without cell wall removal

Hikari Izumi*
Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori 036-8561, Japan
Section of Food Sciences, Institute of Regional Innovation, Hirosaki University, Aomori, Aomori 038-0012, Japan
ORCID: 0000-0001-5366-6250

Toshiki Uji
Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
ORCID: 0000-0002-2925-7913

Kojiro Matsumoto
Nepa Gene Co., Ltd., Ichikawa, Chiba 272-0114, Japan

Kaz Nagaosa
Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori 036-8561, Japan
Section of Food Sciences, Institute of Regional Innovation, Hirosaki University, Aomori, Aomori 038-0012, Japan

Satoru Fukuda
Regional Fisheries Co-Creation Center, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

*Correspondence author: e-mail: h-izumi@hirosaki-u.ac.jp; phone: +08-17-763-5047

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Author contributions

Hikari Izumi and Satoru Fukuda contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hikari Izumi, Toshiki Uji, Kojiro Matsumoto and Kaz Nagaosa. The first draft of the manuscript was written by Hikari Izumi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Abstract

This study aimed to assess the potential of a gene delivery technique in the red macroalga *Pyropia yezoensis* (Rhodophyta) using electroporation without removing the cell wall. An antibiotic resistance gene was introduced into *P. yezoensis* tissues containing cells with intact cell walls through electroporation, followed by selection with the corresponding antibiotic. No germlings survived in the non-electroporated control tissue fragments under antibiotic selection. In contrast, some germlings were observed to survive in the electroporated group. Furthermore, the presence of antibiotic resistance genes was confirmed in the genomic DNA of several antibiotic-resistant germlings. Although reporter genes such as β-glucuronidase (GUS) and green fluorescent protein (GFP) were also introduced as supplementary markers, their expression was not detectable under the tested conditions. These findings provide evidence supporting the successful introduction of antibiotic resistance genes into *P. yezoensis* cells via electroporation. This study offers a preliminary assessment of a gene delivery strategy in *P. yezoensis* that bypasses cell wall removal, presenting a straightforward method for introducing foreign genes into *Pyropia*. To the best of our knowledge, this is the first report to demonstrate successful gene transfer via electroporation in a macroalga without cell wall removal. These results provide valuable insights for the development of genetic transformation systems in red macroalgae.

Keywords: gene delivery, electroporation, red macroalgae, *Pyropia yezoensis*

Introduction

Red macroalgae (Rhodophyta) are the most extensively cultivated seaweeds globally, serving as a source of commercially valuable products such as food, food additives, and pharmaceuticals (García-Poza et al. 2020). To develop efficient breeding strategies and varieties enriched with beneficial compounds, a thorough understanding of their physiological processes is essential. Genetic transformation offers a powerful tool for uncovering gene functions and regulatory mechanisms underlying these processes.

In seaweeds, genetic transformation has largely been achieved through physical methods, particularly particle bombardment and electroporation (Mikami 2013; Khatri et al. 2023). Particle bombardment is a flexible method that can be applied across various types of receptor materials. In contrast, electroporation is advantageous for its speed, low cost, and consistent transformation efficiency (Kar et al. 2018; Ozyigit

2020). However, electroporation is constrained by its reliance on protoplasts, as it requires the removal of cell walls to enable material uptake. Protoplast preparation involves enzymatic digestion, which demands costly reagents and multiple, time-intensive steps. Moreover, regenerating whole plants from protoplasts remains a major challenge in many plant species (Reed and Bargmann 2021). Although genetic transformation studies in red macroalgae began over three decades ago (Kurtzman and Cheney 1991), successful gene transfer has been achieved in only a few species, and reports of consistently stable transformants remain scarce (Uji et al. 2014; Khatri et al. 2023). This underscores the need for new approaches in red macroalgal transformation research. In recent years, several studies have demonstrated the introduction of foreign materials into cells without cell wall removal in cultured plant cells (e.g., *Arabidopsis thaliana*, tobacco) and various microalgae (e.g., *Chlamydomonas*, diatoms, charophycean algae) using a multi-step electroporation system (Yamano et al. 2013; Miyahara et al. 2013; Furuhata et al. 2019; Kawai et al. 2022; Furuhata et al. 2023). Notably, a recent study showed that this method enables the direct introduction of materials into intact carnation plant tissues (Mori et al. 2024). This electroporation strategy holds promise for achieving more efficient and stable gene introduction in red macroalgae and offers a compelling alternative to traditional transformation techniques.

Pyropia yezoensis, a multicellular red alga in the class Bangiophyceae, is an economically valuable species in Japan and other parts of Asia. Among red macroalgae, it is the only species for which stable transformants have been consistently reported (Uji et al. 2014; Hirata et al. 2014; Cao et al. 2022; Izumi et al. 2025), making it a key model for genetic transformation studies. This suggests that essential functional data on transformation is already available. In this study, we introduced foreign genes into *P. yezoensis* tissues with intact cell walls using a multi-step electroporation system to assess its applicability for gene delivery without requiring cell wall removal.

Material and methods

Algal material and culture conditions

We used the *P. yezoensis* strain TU-1 (Kuwano et al. 1996). Gametophytic thalli were cultured in enriched sea life (ESL) medium (Kitade et al. 2002), aerated with filter-sterilized air at 15°C under a 10:14 h light:dark (L:D) photoperiod. Illumination was provided by cool-white fluorescent lamps at an intensity of 40–60 μmol photons/m^2/s. The medium was refreshed weekly. Thalli cultured for 5–7 weeks were used for

electroporation experiments.

Transformation

In this study, we evaluated the feasibility of gene delivery via electroporation in *P. yezoensis* without cell wall removal by introducing a hygromycin resistance gene and selecting for surviving germlings. Reporter genes such as β-glucuronidase (GUS) and green fluorescent protein (GFP) were used as supplementary tools to facilitate the optimization of electroporation conditions, as they allow rapid and direct observation of gene expression. We employed the plasmid pEA7-PyAct1::PyGUS, which carries both a hygromycin resistance gene and the GUS reporter and has been validated in previous studies (Hirata et al. 2014). Additionally, using established protocols (Uji et al. 2010; Hirata et al. 2014), we constructed the plasmid pEA7-PyAct1::sGFP by incorporating the functional GFP reporter construct pPyAct1-sGFP into pEA7. This plasmid was also used for transformation. The functionality of both plasmids was verified by introducing them into *P. yezoensis* via particle bombardment, following methods described previously (Fukuda et al. 2008; Uji et al. 2014), and assessing gene expression. Specifically, GUS expression was detected in tissues bombarded with pEA7-PyAct1::PyGUS, and GFP fluorescence was observed in tissues bombarded with pEA7-PyAct1::sGFP. In addition, antibiotic-resistant germlings were obtained following the introduction of both plasmids, confirming that the constructs were functional in *P. yezoensis* cells.

For electroporation-mediated transformation, algal tissue fragments were prepared (Fig. 1a, b). The tips of gametophytic thalli containing archeosporangia (>3 mm wide) were excised into ~3 mm squares. These fragments were washed in electroporation buffer (ESL medium with 0.6 M mannitol) and placed on filter paper. Electroporation was conducted using the NEPA21 Type II electroporator (Nepa Gene, Chiba, Japan). Electroporation buffer was added to the circular platinum electrode CUY700P3E (Nepa Gene). A Millipore filter (Merck, Darmstadt, Germany) was laid on the electrode, followed by placement of the tissue fragment. A plasmid solution (7 μg) in TE buffer was then applied directly onto the tissue. A covered circular platinum electrode CUY700P1L (Nepa Gene) was then placed in contact with the surface of the solution, and electric current was applied to the tissue fragment. Electroporation conditions were as follows: Poring pulses voltage: 75, 100, or 125V; pulse width: 0.1, 0.5, or 1.0ms; number of pulses: 3; pulse interval: 50 ms; polarity: (+). Transfer pulses—voltage: 7 V; pulse width: 50 ms; number of pulses: 3; pulse interval: 50 ms; polarity: both (+) and (–). All procedures were conducted at room temperature. Immediately after electroporation, the tissue fragment was transferred to a dish containing ESL medium for washing. It was

then placed in a 24-well plate (AS ONE Corporation, Osaka, Japan) filled with fresh ESL medium and incubated overnight at 15℃ in the dark. This was followed by culture under a 10L:14D light:dark photoperiod for five days. A GUS histochemical assay was conducted following the protocol described by Fukuda et al. (2008). GFP fluorescence was observed using an inverted fluorescence microscope (CKX41, U-HGLGPS, Olympus, Japan). To assess hygromycin resistance, electroporated tissues were cultured in ESL medium supplemented with 2 mg/ml hygromycin B at 15℃ under a 10L:14D photoperiod. After two weeks of incubation in the selection medium, hygromycin-resistant germlings that regenerated from the electroporated tissues were identified and counted under a microscope at the bottom of the well. The concentration of hygromycin B used in this study was twice the level previously shown to eliminate all wild-type *P. yezoensis* strains (Takahashi et al. 2011; Uji et al. 2014). As demonstrated by Cao et al. (2022), transformants selected under these conditions consistently integrate the exogenous DNA into their genome.

Genomic polymerase chain reaction (PCR)

Genomic PCR was conducted to verify the presence of exogenous DNA in the genome of *P. yezoensis* transformants. Genomic DNA was extracted from gametophytes of hygromycin-resistant germlings cultured for six weeks in ESL medium containing 1 mg/ml hygromycin B, using Chelex 100 (Bio-Rad, Hercules, CA, USA) following the protocol described by HwangBo et al. (2010). PCR amplification was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). *PyElf1* served as the internal control gene, as reported by Uji et al. (2014). The primer pairs PyAph7-F/R (5′-TGGTGATGAGCCGGATGAC-3′/5′-AAGTCGTGCAGGAAGGTGAAG-3′) and PyElf1-F/R (5′-AAGGCCAAGGCACCCAAGCTG-3′/5′-ACCACACCAAGAGCGTCCAATC-3′) were used to amplify *PyAph7* (864 bp) and *PyElf1* (734 bp), respectively. The PCR products were separated on 1.5% agarose gels and post-stained with GR Red Post Stain 10000X (BioCraft Inc., Tokyo, Japan). DNA bands were visualized under UV illumination using a GelDoc imaging system (Bio-Rad).

Result and Discussion

In this study, we evaluated the feasibility of gene delivery into *P. yezoensis* via electroporation without cell wall removal, using hygromycin resistance as a selection marker. Germlings that survived selection were considered transformed with the hygromycin resistance gene. No surviving germlings were detected in wells containing non-electroporated control tissue fragments cultured in selection medium (Fig. 2a). In

153 contrast, some germlings survived in the electroporation-treated group (Fig. 2b, c). However, no germlings
154 persisted in electroporated samples lacking plasmid DNA, regardless of the electroporation parameters used.
155 Although no statistical analysis was performed, we observed a tendency for the number of hygromycin-
156 resistant germlings to increase with higher poring pulse voltages, despite variation under the same
157 conditions (Fig. 2d). To refine the energy level of poring pulses, we assessed the correlation between poring
158 pulse energy and transformation efficiency (Fig. 2e). While transformation efficiency showed substantial
159 variability, pulse energies between 0.04 and 0.1 J typically resulted in relatively improved outcomes. At
160 identical energy levels, higher voltages tended to yield more hygromycin-resistant germlings, indicating
161 that poring pulse voltage may be an important factor of electroporation success in *P. yezoensis*. These
162 findings offer the first empirical evidence that gene delivery via electroporation is achievable in *P. yezoensis*
163 without the need for cell wall removal.

164 Furthermore, electroporated samples were cultured in hygromycin-containing medium for six weeks,
165 following the protocol outlined by Uji et al. (2014). Under electroporation settings of 100 V and a 0.1 ms
166 pulse width for poring pulses, three surviving strains were successfully isolated. Genome PCR analysis
167 verified the presence of the hygromycin resistance gene in these strains (Fig. 2f). Although these findings
168 are derived from a limited sample set, they suggest that stable transformants may be generated in *P.*
169 *yezoensis* through electroporation.

170 The GUS and GFP reporter assays were conducted as supplementary experiments to facilitate the
171 optimization of electroporation conditions. However, no GUS-positive cells were detected under any tested
172 conditions using histochemical assays. Likewise, GFP fluorescence was not clearly observed. These
173 findings indicate that transient expression of the reporter genes was not detectable under the electroporation
174 conditions employed in this study. Several factors may account for the absence of detectable expression.
175 One possibility is the low copy number of the introduced transgenes. Particle bombardment typically leads
176 to the integration of multiple transgene copies (Kohli et al. 2003), as seen in *P. yezoensis* transformants with
177 at least six copies (Izumi et al. 2025). Additionally, in *Closterium*, a positive correlation between transgene
178 copy number and expression level has been reported (Kawai et al. 2022). Therefore, it is possible that the
179 lower number of transgene copies introduced by electroporation contributed to weak expression and
180 difficulty in detecting reporter activity. Another possible factor is the difference in expression thresholds
181 between marker types. Antibiotic resistance genes generally can confer survival under selection pressure

even with low expression levels, whereas reporter genes like GUS and GFP require higher expression levels to be detected, which may have contributed to the lack of detection under the tested conditions. Transgene silencing may also have played a role. In *P. yezoensis*, transgene silencing has been reported, in which antibiotic resistance was retained while expression of a co-introduced reporter gene was suppressed (Shin et al. 2016). Consistently, in our laboratory, GUS activity was detected in only about half of the antibiotic-resistant transformants (Izumi et al. 2025). Our primary objective in this study was to evaluate whether foreign genes could be introduced into *P. yezoensis* cells via electroporation. To minimize vector-related effects, we used constructs previously validated in particle bombardment experiments (Uji et al. 2010; Hirata et al. 2014). Nevertheless, the possibility remains that vector design partially contributed to the undetectable reporter expression. Taken together, these factors may have contributed to the failure to detect reporter expression. Future studies should focus on quantifying transgene copy number, optimizing vector design specifically for electroporation, and developing new strategies to improve the sensitivity of transgene expression detection.

Traditionally, electroporation-based transformation in *P. yezoensis* has relied on generating protoplasts by enzymatically removing cell walls (Mei et al. 1998; Liu et al. 2003; Mizukami et al. 2004). This process is labor-intensive, requiring multiple enzymatic treatments and specialized expertise, as the approach remains underdeveloped for *P. yezoensis*. Additionally, regenerating whole macroalgae from protoplasts poses technical difficulties, a challenge that also persists in this species (Fujita and Migita 1985; Araki et al. 1987; Fukui et al. 2023; Khatri et al. 2023). As a result, electroporation has not yet been widely adopted for genetic transformation in *P. yezoensis*. The findings of this study provide the first indication that these longstanding barriers might be addressed. Although the current method shows variable efficiency and is still undergoing refinement, continued optimization of host tissue, donor DNA, and electroporation parameters is anticipated to establish electroporation as a reliable technique for genetic transformation in *P. yezoensis*. It should be noted that, due to the technical challenges associated with stable protoplast preparation and regeneration in *P. yezoensis*, we were unable to perform direct comparisons between cells with and without cell walls. However, such comparisons is essential to evaluate the practical applicability of this method and thus remains an important subject for future research.

Beyond *P. yezoensis*, electroporation-mediated transformation in macroalgae has been predominantly reported in the genus *Pyropia* (Kübler et al. 1994; Okauchi and Mizukami 1999; Zuo et al. 2007), using

enzymatically prepared protoplasts. Reports in other macroalgae remain very limited. In green macroalgae, transient expression following electroporation of protoplasts has been shown in *Ulva lactuca* (Huang et al. 1996), whereas no such reports have been confirmed in brown macroalgae. Our findings may contribute to broadening the applicability of electroporation-based approaches by suggesting that gene delivery is feasible even in macroalgal tissues with intact cell walls. While further validation is necessary, this work may serve as a step toward establishing more accessible transformation methods for macroalgae, including red algae.

Conclusions

In summary, we demonstrate the successful introduction of foreign genes into *P. yezoensis* cells using electroporation, without requiring cell wall removal. This study provides an initial assessment of an electroporation-based gene delivery method for *P. yezoensis*, offering a streamlined alternative to conventional approaches that rely on protoplast formation. To our knowledge, this is the first report to establish that gene transfer via electroporation is feasible in macroalgae without enzymatic removal of cell walls. This technique offers a promising foundation for advancing genetic transformation strategies in red macroalgae.

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Data availability statements

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

References

Araki T, Aoki T, Kitamikado M (1987) Preparation and regeneration of protoplasts from wild-type of *Porphyra yezoensis* and green variant of *P. tenera*. Nippon Suisan Gakkaishi 53:1623-1627. https://doi.org/10.2331/suisan.53.1623 (in Japanese)

Cao X, Kong F, Sun B, Yin J, Ren H, Yue H, Yu C, Tang X, Du G, Wang D, Mao Y (2022) A toolbox for

240 constructing a stable genetic transformation platform allowing foreign fragment integration in the
241 genome of *Neopyropia yezoensis*. Front Mar Sci 9:856790. https://doi.org/10.3389/fmars.2022.856790
242 Fujita Y, Migita S (1985) Isolation and culture of protoplasts from some seaweeds. Bull Fac Fish Nagasaki
243 Univ 57:39-45. (in Japanese)
244 Fukuda S, Mikami K, Uji T, Park EJ, Ohba T, Asada K, Kitade Y, Endo H, Kato I, Saga N (2008) Factors
245 influencing efficiency of transient gene expression in the red macrophyte *Porphyra yezoensis*. Plant Sci
246 174:329-339. https://doi.org/10.1016/j.plantsci.2007.12.006
247 Fukui Y, Abe M, Kobayashi M (2023) Effects of hyphomonas strains on the growth of red algae *Pyropia*
248 species by attaching specifically to their rhizoids. Microb Ecol 86:2502-2514.
249 https://doi.org/10.1007/s00248-023-02257-z
250 Furuhata Y, Egi E, Murakami T, Kato Y (2023) A method for electroporation of Cre recombinase protein
251 into intact *Nicotiana tabacum* cells. Plants 12:1631. https://doi.org/10.3390/plants12081631
252 Furuhata Y, Sakai A, Murakami T, Morikawa M, Nakamura C, Yoshizumi T, Fujikura U, Nishida K, Kato
253 Y (2019) A method using electroporation for the protein delivery of Cre recombinase into cultured
254 *Arabidopsis* cells with an intact cell wall. Sci Rep 9:2163. https://doi.org/10.1038/s41598-018-38119-9
255 García-Poza S, Leandro A, Cotas C, Cotas J, Marques JC, Pereira L, Gonçalves AMM (2020) The evolution
256 road of seaweed aquaculture: cultivation technologies and the industry 4.0. Int J Environ Res Public
257 Health 17:6528. https://doi.org/10.3390/ijerph17186528
258 Hirata R, Uji T, Fukuda S. Mizuta H, Fujiyama A, Tabata S, Saga N (2014) Development of a nuclear
259 transformation system with a codon-optimized selection marker and reporter genes in *Pyropia yezoensis*
260 (Rhodophyta). J Appl Phycol 26:1863-1868. https://doi.org/10.1007/s10811-013-0234-x
261 Huang X, Weber JC, Hinson TK, Mathieson AC, Minocha SC (1996) Transient expression of the GUS
262 reporter gene in the protoplasts and partially digested cells of *Ulva lactuca* L. (Chlorophyta). Bot Mar
263 39:467-474.
264 HwangBo K, Son SH, Lee JS Min SR, Ko SM, Liu JR, Choi D, Jeong WJ (2010) Rapid and simple method
265 for DNA extraction from plant and algal species suitable for PCR amplification using a chelating resin
266 Chelex 100. Plant Biotechnol Rep 4:49-52. https://doi.org/10.1007/s11816-009-0117-4
267 Izumi H, Nakai Y, Uji T, Nagaosa K, Fukuda S, Mizuta H, Saga N (2025) Isolation and characterization of
268 a spore development mutant in *Pyropia yezoensis* (Rhodophyta) induced via insertional mutagenesis. J

Phycol. https://doi.org/10.1111/jpy.70026

Kar S, Loganathan M, Dey K, Shinde P, Chang HY, Nagai M, Santra TS (2018) Single-cell electroporation: current trends, applications and future prospects. J Micromech Microeng 28:123002. https://doi.org/10.1088/1361-6439/aae5ae

Kawai J, Kanazawa M, Suzuki R, Kikuchi N, Hayakawa Y, Sekimoto H (2022) Highly efficient transformation of the model zygnematophycean alga *Closterium peracerosum-strigosum-littorale* complex by square-pulse electroporation. New Phytol 233:569-578. https://doi.org/10.1111/nph.17763

Khatri K, Patel J, Adams J, Jones H, Phillips D (2023) Functional genomic and transformation resources for commercially important red macroalgae (Rhodophyta). Algal Res 74:103227. https://doi.org/10.1016/j.algal.2023.103227

Kitade Y, Fukuda S, Nakajima M, Watanabe T, Saga N (2002) Isolation of a cDNA encoding a homologue of actin from *Porphyra yezoensis* (Rhodophyta). J Appl Phycol 14:135-141. https://doi.org/10.1023/A:1019584723331

Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247-258. https://doi.org/10.1023/a:1023941407376

Kübler JE, Minocha SC, Mathieson AC (1994) Transient expression of the GUS reporter gene in protoplasts of *Porphyra miniata* (Rhodophyta). J Mar Biotechnol 1:165-169.

Kurtzman AM, Cheney DP (1991) Direct gene transfer and transient expression in a marine red alga using the biolistic method. J Phycol 27:42.

Kuwano K, Aruga Y, Saga N (1996) Cryopreservation of clonal gametophytic thalli of *Porphyra* (Rhodophyta). Plant Sci 116:117-124. https://doi.org/10.1016/0168-9452(96)04380-4

Liu H, Yu W, Dai J, Gong Q, Yang K, Zhang Y (2003) Increasing the transient expression of *GUS* gene in *Porphyra yezoensis* by 18S rDNA targeted homologous recombination. J Appl Phycol 15:371-377. https://doi.org/10.1023/A:1026083920865

Mei K, Su-juan W, Yao L, Da-leng S, Cheng-kui Z (1998) Transient expression of exogenous gus gene in *Porphyra yezoensis* (Rhodophyta). Chin J Ocean Limnol 16: 56-61. https://doi.org/10.1007/BF02849081

Mikami K (2013) In: Rádis-Baptista G (ed) An integrated view of the molecular recognition and

298 toxinology— From analytical procedures to biomedical applications. IntechOpen, London
299 Miyahara M, Aoi M, Inoue-Kashino N, Kashino Y, Ifuku K (2013) Highly efficient transformation of the
300 diatom *Phaeodactylum tricornutum* by multi-pulse electroporation. Biosci Biotechnol Biochem 77:874-
301 876. https://doi.org/10.1271/bbb.120936
302 Mizukami Y, Hado M, Kito H, Kunimoto M, Murase N (2004) Reporter gene introduction and transient
303 expression in protoplasts of *Porphyra yezoensis*. J Appl Phycol 16:23-29.
304 https://doi.org/10.1023/b:japh.0000019050.54703.14
305 Mori K, Tanase K, Sasaki K (2024) Novel electroporation-based genome editing of carnation plant tissues
306 using RNPs targeting the *anthocyanidin synthase* gene. Planta 259:84. https://doi.org/10.1007/s00425-
307 024-04358-6
308 Okauchi M, Mizukami Y (1999) Transient β-Glucuronidase (GUS) gene expression under control of CaMV
309 35S promoter in *Porphyra tenera* (Rhodophyta). Bull Natl Res Inst Aquacult Suppl 1:13-18.
310 Ozyigit II (2020) Gene transfer to plants by electroporation: methods and applications. Mol Biol Rep
311 47:3195-3210. https://doi.org/10.1007/s11033-020-05343-4
312 Reed KM, Bargmann BOR (2021) Protoplast regeneration and its use in new plant breeding technologies.
313 Front Genome Ed 3:734951. https://doi.org/10.3389/fgeed.2021.734951
314 Shin YJ, Lim JM, Park JH, Choi DW, Hwang MS, Park EJ, Min SR, Kim SW, Jeong WJ (2016)
315 Characterization of *PyGUS* gene silencing in the red macroalga, *Pyropia yezoensis*. Plant Biotechnol
316 Rep 10:359-367. https://doi.org/10.1007/s11816-016-0408-5
317 Takahashi M, Mikami K, Mizuta H, Saga N (2011) Identification and efficient utilization of antibiotics for
318 the development of a stable transformation system in *Porphyra yezoensis* (Bangiales, Rhodophyta). J
319 Aquac Res Dev 2:1-4.
320 Uji T, Hirata R, Fukuda S, Mizuta H, Saga N (2014) A codon-optimized bacterial antibiotic gene used as
321 selection marker for stable nuclear transformation in the marine red alga *Pyropia yezoensis*. Mar
322 Biotechnol 16:251-255. https://doi.org/10.1007/s10126-013-9549-5
323 Uji T, Takahashi M, Saga N, Mikami K (2010) Visualization of nuclear localization of transcription factors
324 with cyan and green fluorescent proteins in the red alga *Porphyra yezoensis*. Mar Biotechnol 12:150-
325 159. https://doi.org/10.1007/s10126-009-9210-5
326 Yamano T, Iguchi H, Fukuzawa H (2013) Rapid transformation of *Chlamydomonas reinhardtii* without

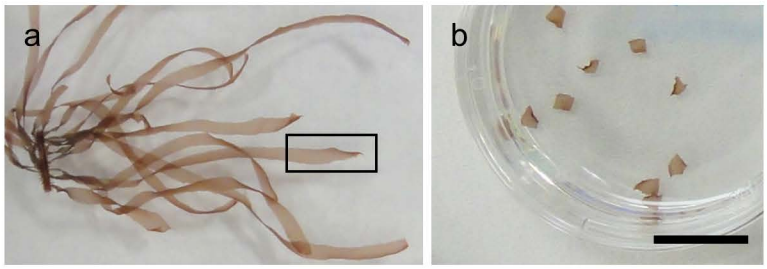
cell-wall removal. J Biosci Bioeng 115:691-694. https://doi.org/10.1016/j.jbiosc.2012.12.020

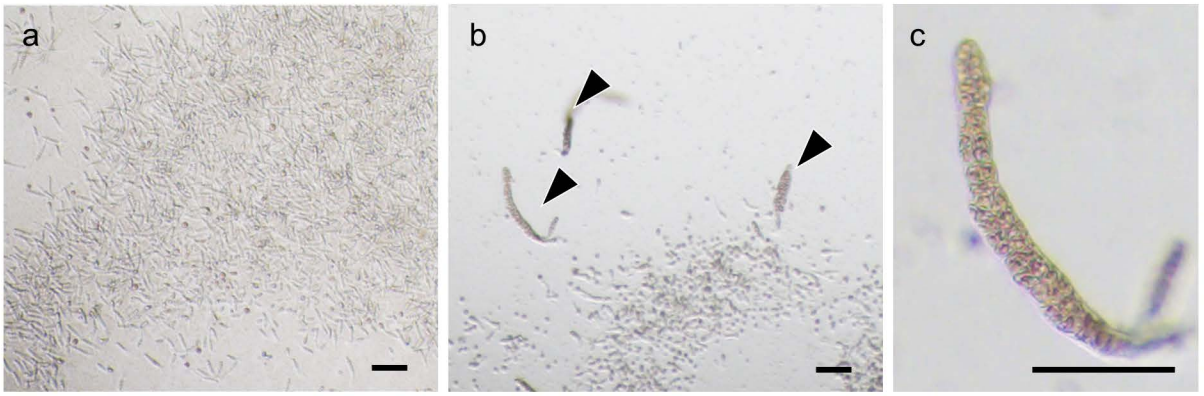
Zuo Z, Li B, Wang C, Cai J, Chen Y (2007) Increasing transient expression of CAT gene in *Porphyra haitanensis* by matrix attachment regions and 18S rDNA targeted homologous recombination. Aquac Res 38:681-688. https://doi.org/ 10.1111/j.1365-2109.2006.01645.x.

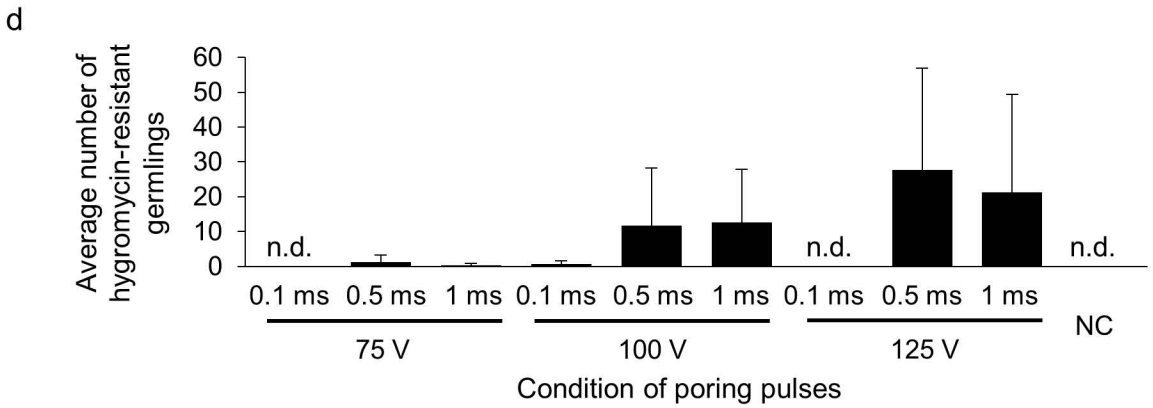
Figure Captions

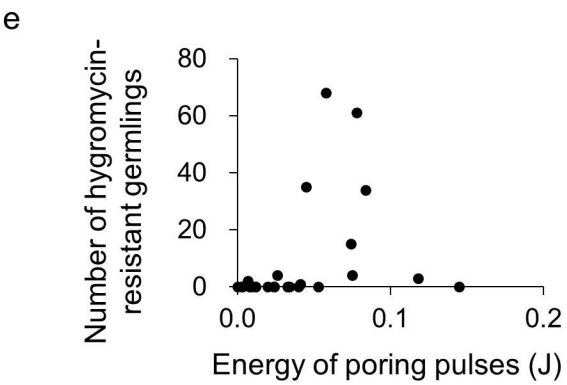
Fig. 1 Sample preparation for electroporation in *Pyropia yezoensis*. (a) TU-1 strain. (b) Algal tissue sections prepared for electroporation. Bar = 10 mm

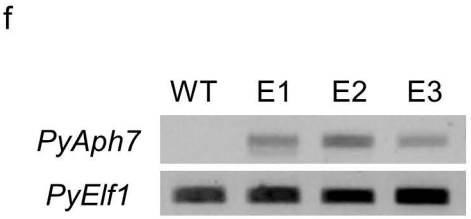
Fig. 2 Transformation of *Pyropia yezoensis* using the hygromycin-resistant gene (*PyAph7*) via electroporation. (a-c) Germlings regenerated from algal tissue cultured for 2 weeks in selection medium containing hygromycin B (2 mg/ml). (a) Non-electroporated tissue (negative control). (b) Electroporated tissue. Arrowheads indicate hygromycin-resistant germlings regenerated via archespores. (c) Hygromycin-resistant germling. Scale bar =100 μm. (d, e) Impact of poring pulse parameters on transformation efficiency. (d) Relationship between voltage, pulse width, and transformation efficiency. The average number of hygromycin-resistant germlings was determined by counting surviving germlings of *P. yezoensis* across three trials. NC represents the negative control. The error bar indicates standard deviation. "n.d." denotes no detection (no surviving germlings were observed). (e) Relationship between pouring pulse energy and transformation efficiency. Each black circle represents data from one experiment. (f) Polymerase chain reaction (PCR) amplification of the *PyAph7* and *PyElf1* genes in genomic DNA from *P. yezoensis* transformants. Genomic DNA from the wild type (WT) and three hygromycin-resistant germlings (E1–E3) was analyzed using PCR


a
b


a
b
c


d
Average number of hygromycin-resistant germlings
60
50
40
30
20
10
0
n.d.
n.d.
n.d.
0.1 ms
0.5 ms
1 ms
0.1 ms
0.5 ms
1 ms
0.1 ms
0.5 ms
1 ms
NC
75 V
100 V
125 V
Condition of poring pulses


e
Number of hygromycin-resistant germlings
80
60
40
20
0
0.0
0.1
0.2
Energy of poring pulses (J)


f
WT
E1
E2
E3
PyAph7
PyElf1