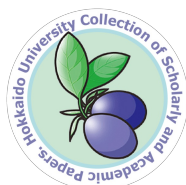




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Identification and Efficient Utilization of Antibiotics for the Development of a Stable Transformation System in *Porphyra yezoensis* (Bangiales, Rhodophyta)

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Abstract

Despite the development of transient gene expression in red algae *Porphyra* species, a stable transformation system has not been established in these algae yet. One reason for the difficulty in the transformation is the lack of a way to select transformed cells from the algae. Thus, to identify antibiotics suitable for the selection of stably transformed cells, we tested the gametophyte for sensitivity to 6 antibiotics, ampicillin (Am), kanamycin (Km), hygromycin (Hm), geneticin (G418), chloramphenicol (Cm) and paromomycin (Pm), which are frequently employed as selection agents in the transformation of land plants and microalgae. Sensitivity tests, which were carried out with different concentrations (0-10 mg mL⁻¹) of antibiotics, showed that Hm, G418, Cm and Pm exhibited a lethal effect at more than 1.0 mg mL⁻¹, suggesting the suitability of these four antibiotics for the selection of genetically transformed cells from *P. yezoensis* gametophytes, while *P. yezoensis* gametophytes were highly resistant to Am and Km. We also succeeded in reducing the antibiotic concentrations by lowering the pH value and salt concentration using an artificial synthetic medium. Increasing the sensitivity of gametophytic cells to Hm, G418, Cm and Pm in the modified synthetic medium will contribute to establishing a genetic transformation system in *P. yezoensis*.

Keywords: Antibiotics; Synthetic medium; Gametophyte; *Porphyra yezoensis*

Introduction

Microalgae and macroalgae are of great value both as organisms for basic biological research and as resources for bio-industry. That is why algae are now considered to be very promising organisms for economical and industrial applications and are the target of genetic transformation [1,2]. Recently, such transformation has been performed successfully in microalgae, and stably transformed microalgae have been employed to produce useful materials and to analyze the gene functions required for engineering these materials [3-5]. However, it is still hard to establish transgenic macroalgae, which is hindering our understanding of gene functions in various physiological regulations and also slowing our utilization of macroalgae in biotechnological applications.

The red macroalga *Porphyra yezoensis* is the most popular sea crop in Asia [6] and has recently received a great deal of attention as a model macrophyte for physiological and molecular biological studies in marine red algae [7,8]. To date, several studies have described the establishment of a transient gene expression system in *P. yezoensis* [9]; for example, we have succeeded in eliciting the efficient expression of the codon-modified β -glucuronidase (PyGUS) and humanized cyan and green fluorescent protein (AmCFP and ZsGFP) genes by transient transformation of *P. yezoensis* gametophytes using particle bombardment [10-13], which was then adapted to other Bangiophycean algae [14,15]. Despite the development of transient gene expression systems in *P. yezoensis*, a stable transformation system, which is needed for the fine analysis of gene function, has not been established, because of a lack of techniques to select and isolate stably transformed cells from gametophytes.

The availability of a useful selection protocol, which depends on the choice of a selection agent such as antibiotics and herbicides, is generally a prerequisite for the development of a stable transformation system [16]. Thus, the identification of selection agents such as antibiotics and herbicides was carried out in *P. yezoensis* [17], and

it resulted in the indication of the non-sensitivity of *P. yezoensis* gametophytes to many kinds of antibiotics at concentrations up to 5 mg mL⁻¹ in culture medium; however, the reproducibility of the test results was not examined. Therefore, we here identified a selection agent for a stable transformation system in *P. yezoensis* gametophytes by re-examining the sensitivity to 4 antibiotics used in the previous study, ampicillin, kanamycin, hygromycin B and geneticin [17], as well as that of chloramphenicol and paromomycin. In addition, since the high salt conditions often reduced the action of antibiotics in many marine algae [18] and high pH levels of the medium affected the toxicity of the antibiotic trimethoprim (TMP) for the willow tree *Salix viminalis* [19], we also investigated using an artificial synthetic medium to determine whether modification of the medium conditions in terms of pH value and salt concentration would affect the antibiotic sensitivity of *P. yezoensis* gametophytes.

Materials and Methods

Gametophytes of *P. yezoensis* (strain TU-1;[20]), 2-3 mm long, were cultured for 4 weeks in 1 L of enriched sea life (ESL) medium [21] at 15°C with a cycle of 60 μ mol m⁻² s⁻¹ under irradiance for 10 h and darkness for 14 h. The ESL medium was continuously bubbled with filter-sterilized air and renewed weekly. Gametophytes of ca. 1.5

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cm length were used for sensitivity testing of antibiotics. Solutions of ampicillin (Am; Wako Pure Chemical Industries, Japan), kanamycin (Km; Wako Pure Chemical Industries, Japan), hygromycin (Hm; Wako Pure Chemical Industries, Japan), geneticin (Gm; Wako Pure Chemical Industries, Japan) and paromomycin (Pm; Sigma, USA) were prepared in ESL medium. Chloramphenicol (Cm; Wako Pure Chemical Industries, Japan) was prepared in ESL medium by dilution of stock solutions (500 mg mL⁻¹) resolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Japan) the concentration of which did not exceed 2% in the dilution. Media containing an antibiotic were finally adjusted to pH 8.0 with NaOH or HCl.

Sensitivity of *P. yezoensis* gametophytes to 6 antibiotics was first tested in standing culture as follows. Gametophytes were transferred into a well of 6-well plate (5 individuals/well)(Iwaki Sci Tech Div., Asahi Techno Glass, Japan) containing 5 mL ESL medium with one antibiotic (Am, Km, Hm, G418, Cm or Pm) and incubated for 2 weeks at 15°C, with the medium containing antibiotic renewed weekly. After cultivation, cell mortality of gametophyte was estimated by staining with 0.01% erythrosine (Wako Pure Chemical Industries, Japan)-containing ESL medium according to the previous report [22] with slight modifications. After staining for 5 min at room temperature, gametophytes were gently rinsed with ESL medium to remove excess erythrosine and mounted on a slide with ESL medium. Gametophytes were observed and photographed using a Leica DM 5000 B light microscope equipped with a Leica DFC 300 FX camera. In this study, we defined the sensitivity to antibiotics based on the death of gametophyte cells which were stained by the dye. The cell mortality was measured by counting the living and dead gametophyte cells using photographs.

To examine the effects of bubbling culture on the sensitivity of gametophytes to antibiotics, we transferred 5 gametophytes into a 100-mL glass flask (Iwaki Sci Tech Div., Asahi Techno Glass, Japan) in 50 mL of ESL medium containing various concentrations of antibiotics (Hm, G418, Cm and Pm) and incubated the samples for 2 weeks at 15°C under 60 µmol m⁻² s⁻¹. The culture medium was continuously bubbled with filter-sterilized air and renewed weekly. The sensitivity test was carried out as described above.

To identify factors influencing the susceptibility of *P. yezoensis* gametophytes to antibiotics, we examined the antibiotic sensitivity test using artificial synthetic medium ASPMT3 [23]. Low-salt medium was made by reducing NaCl from 28 g L⁻¹ to 14 g L⁻¹ in ASPMT3, and two pH-modified media were prepared by changing the buffering agent from HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma, USA, pH 8.0) to MES (2-(N-morpholino) ethanesulfonic acid; Dojin Laboratories, Japan, pH 6.0) or CHES (N-Cyclohexyl-2-aminoethanesulfonic acid; Dojin Laboratories, Japan, pH 10) in ASPMT3. Cultivation and sensitivity tests of *P. yezoensis* gametophytes to antibiotics were carried out as described above.

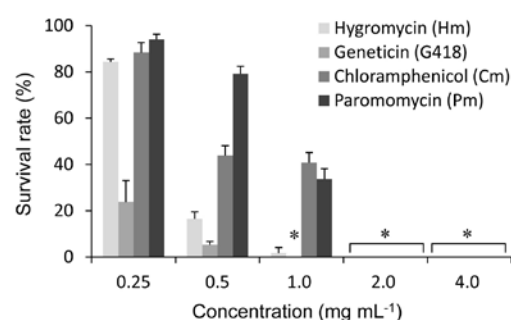
Results and Discussion

We first tested the sensitivity of *P. yezoensis* gametophytes to 6 antibiotics in standing culture. The gametophytes were insensitive to Am and Km at less than 10 mg mL⁻¹ (Figure 1A), although the somatic cells showed dark color or discoloration and also round shape (Supplementary Figure 1 A-C). In contrast, *P. yezoensis* gametophytes were highly sensitivity to Hm, G418, Cm and Pm (Figure 1A and Supplementary Figure 1D-G). Next, we examined the effects of bubbling culture on the sensitivity of gametophytes to Hm, G418, Cm and Pm. Survival rates of gametophytes decreased to 0% by using more than 2.0 mg mL⁻¹ of Hm, Cm and Pm and 1.0 mg mL⁻¹ of G418 (Figure 2),

A

Agents (Abbrev.)	Concentrations (mg mL ⁻¹)		
	2.0	6.0	10.0
Ampicillin (Am)	-	-	-
Kanamycin (Km)	-	-	-
Hygromycin (Hm)	+	+	+
Geneticin (G418)	+	+	+
Chloramphenicol (Cm)	+	+	+
Paromomycin (Pm)	+	+	+

B



* shows that the survival rate was 0%.

Figure 1: Effects of antibiotics on survival of *P. yezoensis* gametophytic cells. (A) Sensitivity to antibiotics of *P. yezoensis* gametophytic cells cultured in ESL medium with antibiotics after 2 weeks. The sensitivity of the gametophytes to each antibiotic is shown as + (observed) or - (not observed). (B) The survival rate of *P. yezoensis* gametophytic cells in various concentrations of antibiotics. The survival rate was measured by counting the living and dead cells of gametophytes cultured in ESL medium containing antibiotics at 15°C with a cycle of 60 µmol m⁻² s⁻¹ irradiance for 10 h and darkness for 14 h for 2 weeks. Values are the mean ± SD (n = 30).

although both living and divided cells were observed in gametophytes cultured with less than 1.0 mg mL⁻¹ of these antibiotics, except for G418 (Figure 2). Therefore, 1.0 mg mL⁻¹ of G418 and 2.0 mg mL⁻¹ of Hm, Cm and Pm are considered to be effective agents for the selection of transformed cells from gametophytes.

We also examined whether the salt concentration and pH value affected the sensitivity of gametophytic cells to develop an effective selection system by using artificial synthetic medium. The survival rate of gametophyte cells was significantly lower with 1.0 mg mL⁻¹ of Hm in low-salt ASPMT3 than that in standard ASPMT3 (Figure 2a) (Student's t-test p<0.05). In the case of changing pH values, survival rates of gametophyte cells were high in ASPMT3 at pH 10, except in the case of 4.0 mg mL⁻¹ of Hm (Figure 2b). In contrast, gametophytic cells cultured in ASPMT3 containing 1.0 mg mL⁻¹ of Hm at pH 6.0 showed a significantly lower survival rate compared with that in ASPMT3 at pH 10 and pH 8.0 (Figure 2b) (Student's t-test p<0.05). Moreover, the combination of low pH (6.0) and low salt decreased the survival rates of gametophytic cells to less than 20% in all concentrations of Hm (Figure 2c). The same results were also obtained by using other antibiotics (G418, Cm and Pm) in modified ASPMT3 (Table 1). Thus, we clarified that the antibiotic concentration for the selection of transformed cells can be reduced by using a low salt concentration and a low pH value in the culture medium.

In the present study, we have succeeded in finding antibiotics,

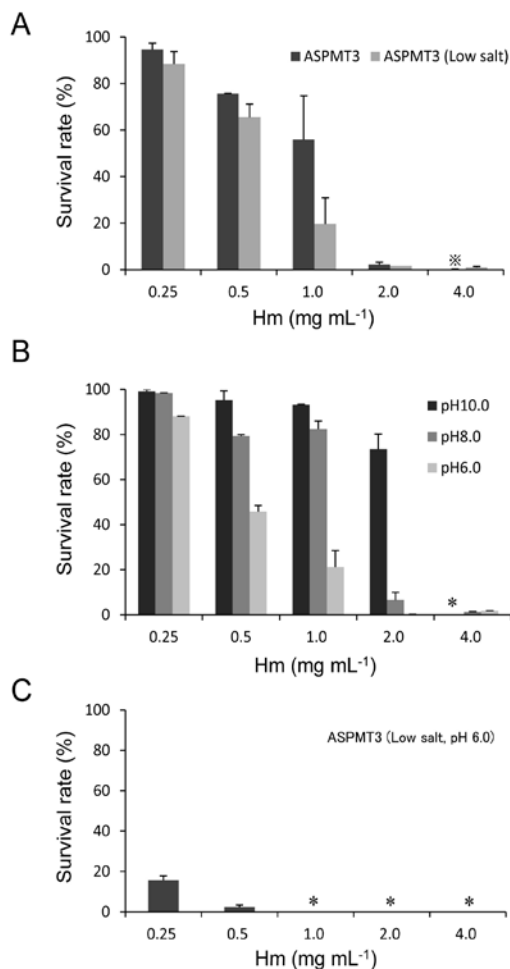


Figure 2: The survival rate of *P. yezoensis* gametophytic cells in various hygromycin B concentrations in modified ASPMT3. (A) Salt concentrations, (B) pH values and (C) combination of salt concentration and pH value. The survival rate was measured by counting the living and dead cells of gametophytes cultured in culture medium containing hygromycin B at 15°C with a cycle of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance for 10 h and darkness for 14 h for 2 weeks. Values are the mean $\pm$ SD (n = 30). * shows that the survival rate was 0%.

	Concentrations (mg mL ⁻¹)									
	ESL					Modified ASPMT3 (Low salt, pH 6.0)				
Agents	0.25	0.5	1.0	2.0	4.0	0.25	0.5	1.0	2.0	4.0
Geneticin (G418)	23.8	5.3	0	0	0	0	0	0	0	0
Chloramphenicol (Cm)	88.4	43.8	40.8	0	0	6.2	1.3	0	0	0
Paromomycin (Pm)	93.9	79.1	33.7	0	0	12.8	0	0	0	0

The number indicates survival rate of gametophyte cells (%).

Table 1: Comparison of the antibiotic effect to *P. yezoensis* gametophytes in different culture media between ESL and modified ASPMT3

Hm, G418, Cm and Pm, as candidates of selection agents useful for the stable transformation of *P. yezoensis* gametophytes (Figure 1B and Supplementary Figure 1). Furthermore, the aeration cultivation was considered to be effective for a selection system of transformed cells from gametophytes, since living gametophytic cells are often observed in standing culture compared with aeration culture at the

same antibiotic concentration (data not shown). In bubbling culture, *P. yezoensis* gametophytic cells were sensitive to more than 1.0-2.0 mg mL⁻¹ of antibiotic concentration (Figure 1B); however, this concentration is very high in comparison with the cases for other algae. For example, the red alga *Griffithsia japonica* and the green alga *Chlamydomonas reinhardtii* were highly sensitive to 50 mg mL⁻¹ and 1.0 mg mL⁻¹ of Hm [24,25], while *P. yezoensis* gametophytes cultured in ESL medium were sensitive to 2.0 mg mL⁻¹ of Hm (Figure 1B). It is possible that the differences in cell structure and cell wall composition between unicellular and multicellular algae might affect the sensitivity to antibiotics.

Changing the composition of the culture medium may be effective for reducing the antibiotic concentration for various algae [18]. Therefore, we tested whether salt and pH levels affect the sensitivity to antibiotics for the improvement of selection efficiency in transformed gametophytic cells and found that the antibiotics concentration for the selection of transformed cells can be reduced by using a low salt concentration and low pH value in the culture medium (Figure 2 and Table 1). In the unicellular microalga *Dunaliella bardawil*, a low salt concentration in the growth media can significantly affect the efficacy of Hm [26]. On the other hand, high-salt growth media of *Dunaliella* can significantly reduce the efficacy of various antibiotics and herbicides [18]. In addition, changing the salinity and pH values of culture medium also affects the growth rate and photosynthesis activity in *P. tenera* and *P. umbilicalis* [27,28]. Similar physiological changes are considered to occur in *P. yezoensis*, which results in enhancing the effective influence of antibiotics to gametophytic cells. Moreover, the lethal concentration was significantly lower in modified ASPMT3 than in ESL (Table 1). Using the antibiotic medium with low pH and low salt concentration is more economical, which is important, since large amounts of expensive antibiotics are used in this process.

The antibiotics such as Hm, G418, Cm and Pm are frequently used selection agents for obtaining transgenic prokaryotic and eukaryotic cells, for which selectable marker genes have been established. For example, in the green alga *Chlamydomonas reinhardtii*, the aminoglycoside phosphotransferase *aph^r* gene from *Streptomyces hygroscopicus* and the aminoglycoside phosphotransferase *aph^{VIII}* (*aphH*) gene from *Streptomyces rimosus* had been reported as selectable marker genes for hygromycin B and paromomycin, respectively [25,29]. Similarly, in the multicellular green alga *Volvox carteri*, the aminoglycoside phosphotransferase *aph^{VIII}* (*aphH*) gene from *S. rimosus* had been used as a paromomycin-resistance gene [30,31]. In the diatom *Phaeodactylum tricornutum*, the expressed chloramphenicol acetyltransferase gene (*CAT*) detoxifies chloramphenicol [32], and the neomycin phosphotransferase II (*nptII*) gene confers resistance to the aminoglycoside antibiotic G418 [33]. Likewise, the *nptII* gene gives resistance to the antibiotic G418 in the diatoms *Navicula saprophila* and *Cyclotella cryptica* [34]. However, it is unknown whether these selection marker genes are available for *P. yezoensis*. Thus, it is necessary to confirm whether *P. yezoensis* gametophytes will obtain the antibiotic tolerance by introducing plasmid constructs containing the antibiotic-resistance genes mentioned above. Such efforts are expected to contribute to the establishment of a stable transformation system in *P. yezoensis*.

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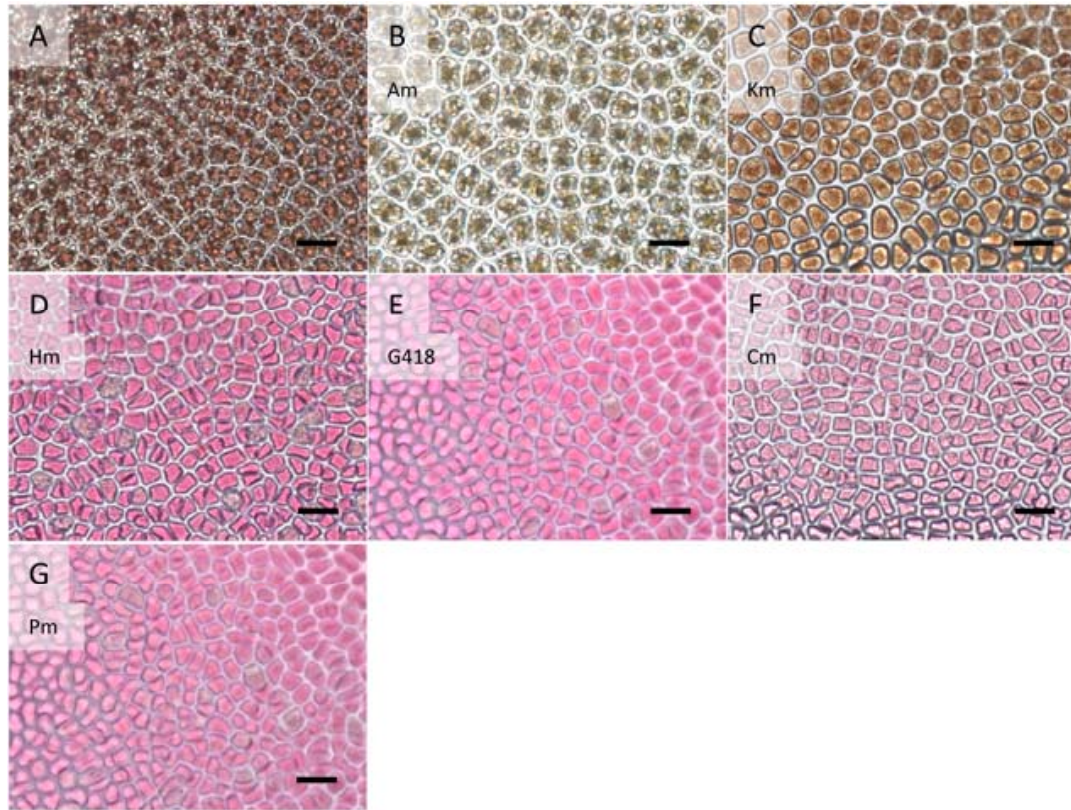
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Supplementary Figure 1. Effects of antibiotics on survival of *P. yezoensis* gametophytic cells. Gametophytes were cultured in ESL medium with 10 mg mL⁻¹ concentration of antibiotics at 15°C with a cycle of 60 μmol m⁻² s⁻¹ irradiance for 10 h and darkness for 14 h for 2 weeks and then stained with 0.01% erythrosine solution. (A) control, (B) ampicillin, (C) kanamycin, (D) hygromycin B, (E) geneticin, (F) chloramphenicol and (G) paromomycin. The cells stained pink are dead. Scale bars: 20 μm.