Transient gene expression system established in *Porphyra yezoensis* is widely applicable in Bangiophycean algae

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Abstract

The establishment of transient gene expression systems in the marine red macroalga *Porphyra yezoensis* has been useful for the molecular analysis of cellular processes in this species. However, there has been no successful report about the expression of foreign genes in other red macroalgae, which has impeded a broader understanding of the molecular biology of these species. We therefore examined whether the *P. yezoensis* transient gene expression system was applicable to other red macroalgae. The results indicated that a codon-optimized GUS, designated PyGUS, and plant-adapted sGFP(S65T) were successfully expressed under the control of the *P. yezoensis PyAct1* promoter in gametophytic cells of six *Porphyra* species and also in *Bangia fuscopurpurea*, all of which are classified as Bangiophyceae. In contrast, there were no reporter-expressing cells in the Florideophycean algae examined. These results indicate the availability of PyGUS and sGFP as reporters and the 5' upstream region of the *PyAct1* gene as a heterologous promoter for transient gene expression in Bangiophycean algae, which could provide a clue to the efficient expression of foreign genes and transformation in marine red macroalgae.

Introduction

Marine red macroalgae are traditionally used as ingredients in foods, food additives and medicines (McHugh 2003). For instance, several species of *Porphyra* are cultivated on a large scale to produce the edible seafood products known as Nori in Japan (Niwa et al. 2008). In addition, other red macroalgae classified into Florideophyceae have also attracted attention as resources of polysaccharide such as kappa-carrageenan and agar (Villanueva et al. 2010). However, despite their great economic importance, our biological understanding of red

macroalgae is too primitive to apply these species to biotechnologies for molecular breeding. Therefore, there is currently a need for fundamental and applied research, including genetic manipulation, in marine red macroalgae.

Algal transformation has been developed as a method for investigating the function and regulation of genes and also for the production of commercially valuable molecules (Walker et al. 2005; Hallmann 2007). Stable transformation systems have already been established in microalgae (Hallmann 2007), such as volvocine green algae, unicellular *Chlamydomonas reinhardtii* (Debuchy et al. 1989; Kindle et al. 1989), *Dunaliella salina* (Geng et al. 2003, 2004) and multicellular *Volvox carteri* (Schiedlmeier et al. 1994), all of which have long been favorite experimental organisms for genetic and molecular biological studies. Recently, eukaryotic green alga *Ostreococcus tauri*, which is an attractive microalgal model because of its minimal cellular organization and compact genome, has also become transformable (Corellou et al. 2009; Moulager et al., 2010; Heidje et al., 2010). Furthermore, stable transformation is now available in diatom *Phaeodactylum tricornutum* (Apt et al. 1996; Zaslavskaia et al. 2000, 2001) and unicellular red alga *Cyanidioschyzon merolae* (Minoda et al. 2004). However, stable transformation has not yet been established in marine red and brown macroalgae due to the lack of efficient methods for transfecting foreign genes and the lack of selection markers to isolate transformants.

As an initial step in the development of a transformation system, establishment of transient gene expression systems has been attempted in red macroalgae. However, despite the accumulating reports on transient transformation in red macroalgae such as *Porphyra miniata*, *P. yezoensis* and *Gracilaria changii* (Kübler et al. 1994; Kuang et al. 1998; Gan et al. 2003), there remain problems with the efficiency of foreign gene expression and the reproducibility of these findings in other laboratories. In order to overcome these problems, we have established efficient transient gene expression systems in *P. yezoensis*. First, a

β-glucuronidase (GUS) reporter system was developed with a synthetic gene named *PyGUS*, whose codon usage was optimized to that in *P. yezoensis* genes, under the direction of endogenous strong promoters such as the *PyGAPDH* and *PyAct1* promoters (Fukuda et al. 2008; Takahashi et al. 2010). Subsequently, these two alterations were applied to fluorescent protein reporters such as humanized AmCFP and plant-adapted green fluorescent protein [sGFP(S65T); Niwa et al. 1999], which allowed successful visualization of the subcellular localization of phosphoinositides and transcription factors in *P. yezoensis* cells (Mikami et al. 2009; Uji et al. 2010). Thus, the availability of PyGUS and appropriate fluorescent proteins could contribute to the progress of molecular biological studies on various cellular regulations in *P. yezoensis*.

Red algae have diversified into a wide variety of 5,000-6,000 species (Thomas 2002), many of which are important for industrial and economic activities. Since transient transformation has not been made available for any marine red macroalgae except for *P. yezoensis* to date, the establishment of a genetic manipulation system for these algae also remains a goal for future studies. As mentioned above, we have previously demonstrated the importance of optimization of the codon usage for the host genome in combination with the use of an endogenous strong promoter for efficient and reproducible expression of foreign genes (Fukuda at al. 2008; Mikami et al. 2009; Uji et al. 2010; Takahashi et al. 2010). From this point of view, it is necessary to know the characteristics of the codon usage in Bangiophyceae. It has been reported that *P. haitanensis* has protein coding regions as rich in GC as those of *P. yezoensis* (Fan et al. 2007), and analysis of the GAPDH genes of *Chondrus crispus* has shown that the GC content of the coding region of *GapA* and *GapC* reaches 57% and 63%, respectively (Liaud et al. 1993), which are similar to the values in *P. yezoensis*. Thus, the red algae genome may be rich in both G and C residues. On the other hand, it has been shown that the 5' flanking sequence from *C. reinhardtii* could function in *V. carteri* and

vice versa (Hallmann and Wodniok 2006), and some strong or inducible promoters from *C. reinhardtii* or *V. carteri* have been proven functional in *Gonium pectoral*, which is one of the volvocine algae (Lerche and Hallmann 2009). Although endogenous promoters are not available for genetic transformation in marine macroalgae other than *P. yezoensis*, it is possible that the *PyAct1* promoter might be available as a heterologous promoter for red macroalgae other than *P. yezoensis*. Taken together, it is proposed that the PyGUS and GC-rich fluorescent proteins under the direction of the *PyAct1* promoter would be applicable for transient gene expression in Bangiophycean algae.

To confirm this possibility, we examined the applicability of PyGUS and sGFP as reporters for transient gene expression systems in several species of Bangiophycean algae under the direction of the *PyAct1* promoter. The results indicated that, with the *PyAct1* promoter, PyGUS and sGFP are widely applicable for transient gene expression in both *Porphyra* and *Bangia* species. These findings could stimulate methodological improvements in the functional analysis of genes in Bangiophyceae.

Materials and Methods

Algal strains and culture conditions

Gametophytes of culture strains of *P. yezoensis* TU-1 and *P. tenera* JTW were grown in ESL medium (Kitade et al. 2002) at 15°C under irradiation of 70 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps with a photo-period of 10 h light:14 h dark. The medium was bubbled continuously with filter-sterilized air and changed weekly. As shown in Fig. 1, both naturally growing Bangiophycean and Florideophycean algae were collected in a southern region of Hokkaido, Japan. The species, location and date for the collection of red algae used in the

present study are summarized in Table 1. These materials were washed twice and cultured under the conditions noted above except that a temperature of 10°C was used before the transformation experiments.

Transient transformation and expression analysis of reporter genes

We used two expression plasmids, pPyAct1-PyGUS and pPyAct1-sGFP (Takahashi et al. 2010; Uji et al. 2010), for transient transformation of the red macroalgae described above. Plasmids for which the *PyAct1* promoter was removed from two expression plasmids were used in control experiments. As materials for transient transformation, the filmy algae, like *Porphyra*, *Mazzaella japonica* and *Chondrus ocellatus*, were cut into approximately 1.0-cm squares and placed on filter paper, whereas rod or stalk-like algae such as *Bangia fuscopurpurea*, *Gloiopeltis furcata* and *Gracilaria vermiculophylla* were densely aligned on filter paper. Excess fluid was removed from the filters by an aspirator. For introduction of the foreign genes into Bangiophycean cells, we employed the particle bombardment devices IDERA GIE-III (Tanaka Inc., Sapporo, Japan) and PDS-1000/He (Bio-Rad, Foster City, CA). The protocol of transient transformation using an IDERA GIE-III system for Bangiophycean algae was the same as described previously (Mikami et al. 2009), for which the condition of bombardment was 700 mmHg of vacuum pressure, 0.4 Mpa of helium pressure, 6 cm of target distance, 0.6 cm in diameter of gold particles and 7 μg of plasmids/shot. For Florideophycean algae, the PDS-1000/He was used under the conditions of 700 mmHg of vacuum pressure, 1100 or 1550 psi of burst pressure for rupture disks, 3 cm target distance and 7 μg of plasmids/shot. After bombardment, the materials were replaced on an ESL medium plate and incubated at 10ºC for naturally growing algae or 15ºC for culture strains for 48 h with a 10 h light:14 h dark period. These transformation experiments were performed in

triplicate for all species. Thallus pieces transiently transformed in the laboratory were killed by autoclave and thus not returned to the sea.

Detection of plasmids introduced into algal cells

Plasmids introduced into algal cells were detected by amplification of a DNA fragment corresponding to a part of ORF of reporter genes using PyGUS- or sGFP-specific primer set (Table S1). Algal tissues were ground with chelex-100 (Bio-Rad, Foster City, CA) in 200 μl TE buffer and then incubated at 100ºC for 10 min. After centrifugation 10 min (12,000 *g*), the supernatant was collected and 2 μl of it was used as a template for PCR. The conditions of PCR were as follows: 98ºC for 2 min, subsequently 30cycles of 98ºC for 10 s, 68ºC for 1 min with MightyAmp DNA Polymerase (TaKaRa Bio Inc., Japan). The amplified PCR products were examined on a 1% agarose gel.

Fluorometric GUS assay and histochemical staining

The GUS enzymatic activity was quantified by the fluorometric GUS assay. Transiently transformed algal tissues were ground in extraction buffer consisting of 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton-X, 0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol. After centrifugation for 10 min (12,000 *g*) at 4ºC, the supernatant was collected and its protein concentrations were determined by the method of Bradford (1976). Fluorometric GUS assays were performed as described previously (Jefferson et al. 1987). Histochemical staining of GUS-expressing cells was performed almost the same as described previously (Fukuda et al. 2008). After bombardment, algal materials were incubated at 37 ºC for 6 h in staining solution containing 2 mM 5-bromo-4-chloro-3-indolylglucuronide (X-gluc), 5 mM potassium

ferricyanide ($K_3Fe(CN)_6$), 5 mM potassium ferrocyanide ($K_4Fe(CN)_6$), 0.5% Triton X-100, 1.5 M sorbitol and 50 mM sodium phosphate, pH 7.0.

Observation of fluorescence

Observations of sGFP fluorescence and chloroplast autofluorescence were performed using a fluorescence microscope (DM5000B; Leica, Germany) equipped with fluorescence filter L5 (Excitation 480/40 nm, Emission 527/30 nm; Leica, Germany) and fluorescence filter N2.1 (Excitation 515-560 nm, Emission 590 nm; Leica, Germany).

Results

Characteristics of Bangiophycean algae used for analysis

We here used both laboratory-cultured and naturally growing Bangiophycean algae including *Porphyra* and *Bangia* species, which share many common developmental characteristics but which also have some morphological differences (Wang et al. 2008; Sommerfeld and Nichols 1970).

P. yezoensis TU-1 (Fig. S1a) and *P. tenera* JTW (Fig. S1f) have been available as laboratory-cultured strains in our laboratory. Gametophytes of both species consist of a single layer of vegetative cells containing one chloroplast and three chromosomes (Figs. S1b, S1c, S1e, S1g, S1h and S1j). For the sexual propagation, male and female reproductive cells are produced on gametophytes in a randomly localized fashion (Figs. S1d and S1i). Although there are no obvious morphological differences between *P. yezoensis* and *P. tenera*, CAPS analysis of the *ARP4* loci can distinguish TU-1 and JTW (Park et al. 2008).

Since laboratory culture strains have not been established for many of the marine macroalgae other than *P. yezoensis* and *P. tenera*, we collected naturally growing Bangiophycean algae such as five species of *Porphyra* (*P. yezoensis*, *P. okamurae*, *P. onoi*, *P. variegata*, and *P. pseudolinearis*) and *B. fuscopurpurea* in southern area of Hokkaido, Japan (Fig. 1; Table 1). Although the gametophyte of naturally growing *P. yezoensis* (Fig.S2a) appeared to be apparently different from that of TU-1, it was identified as *P. yezoensis* according to its characteristics showing not only a single layer of vegetative cells containing one chloroplast and three chromosomes (Figs. S2b, S2c and S2e) but also a monoecious nature with producing of male and female reproductive cells randomly distributed on gametophytes (Figs. S2d, S2f and S2g). Although both of *P. okamura*e (Fig. S2h) and *P. onoi* (Fig. S2m) have sexual and asexual life cycles as is in *P. yezoensis*, there are considerable differences in morphology; that is, *P. okamurae* consists of a single layer of vegetative cells having one chloroplast per cell and has small serrations along with the edge of the thallus (Figs. S2h-S2l), whereas vegetative cells of *P. onoi*, which grows by adhering to Florideophycean red algae such as *Mazzaella* and *Condrus* have two chloroplasts and form a single-layer sheet (Figs. S2m-S2q). In contrast, *P. variegata* has no asexual life cycle and grows on seagrass like *Phyllospadix* (Fig. S3a). The thallus of *P. variegata* is composed of a double-layer of vegetative cells, on which male and female reproductive cells are produced separately in each half of mature gametophytes (Figs. S3b-S3g).

All *Porphyra* species mentioned above were monoecious, we therefore collected one of the dioecious algae *P. pseudolinearis* (Figs. S3h-S3s). Its gametophyte consists of a single layer of vegetative cells containing one chloroplast and four chromosomes per cell (Figs. S3i, S3j, S3m, S3o, S3p and S3s). Although there are no apparent morphological differences in vegetative cells between male and female gametophyte of *P. pseudolinearis*, they could be distinguishable by their reproductive cells (Figs. S3k, S3l, S3q and S3r).

In addition to *Porphyra* species indicated above, we collected one of the *Bangia* species, *Bangia fuscopurpurea* (Fig. S4). Thallus of this alga represents uniseriate or multiseriate form of vegetative cells with a dioecious nature for sexual propagation, although asexual life cycle is also observed.

Transient expression of PyGUS and sGFP in laboratory-cultured *P. tenera*

Since transient gene expression systems had already been developed using a culture strain of *P. yezoensis* TU-1, we examined the applicability of this system to the culture strain of *P. tenera* JTW. Gene-transfer ability using four reporter plasmids (Fig. 2a, upper) by particle bombardment was first checked by the detection of plasmid DNA in algal cells using PCR. As shown in the lower part of Fig. 2a, DNA fragments in a correct size were amplified, indicating the successful introduction of all expression plasmids in *P. tenera* cells by particle bombardment. Transient nature of transferred plasmids was supported by nearly complete disappearance of these DNAs from cells within two weeks after bombardment (data not shown).

When pPyAct1-PyGUS was introduced into *P. tenera* cells, 2000 to 3000 of PyGUS-expressing cells were observed by staining with X-gluc solution (Figs. 2b and 2c), although there were no PyGUS-expressing cells when the promoter-less-PyGUS plasmid was introduced (Figs. 2b and 2c), which is not due to the degradation of plasmid (Fig. 2a, lower). The expression of the PyGUS gene on pPyAct1-PyGUS and promoter-less-PyGUS was confirmed by fluorometric GUS assay, in which the GUS enzymatic activity in gene-transferred cells was well correlated to the number of PyGUS-expressing cells in *P. yezoensis* TU-1 and *P. teneta* JTW (Figs. 2b and 2c).

When pPyAct1-sGFP was introduced in *P. tenera* thallus by particle bombardment,

efficient expression of the reporter gene was observed (Fig. 2d, left column), although no fluorescence was observed when promoter-less-sGFP(S65T) was transferred (Fig. 2d, right column). Since image of sGFP(S65T) fluorescence has no background derived from autofluorescence of chloroplasts (Fig. 2d, center column), expression and detection of the sGFP(S65T) gene was not interfered by the overlapping to chloroplast autofluorescence in our system.

Since results obtained by using *P. tenera* were the same as those in *P. yezoensis* (Figs. 2b, 2c and 2d), it was concluded that *PyAct1* promoter and PyGUS reporter gene were functional in *P. tenera* cells, indicating that the transient gene expression system developed in *P. yezoensis* TU-1 is applicable for *P. tenera* JTW.

Applicability of the *P. yezoensis* transient gene expression system in naturally growing Bangiophyceae

We next investigated the applicability of our transient gene expression system to other Bangiophycean algae collected in a southern region of Hokkaido (Fig. 1 and Table 1). According to the facts that the number of PyGUS-expressing cells reflects the PyGUS enzymatic activity and sGFP fluorescence was not overlapped with chloroplast autofluorescence in *P. tenera* cells (Figs. 2c and 2d, center column), two expression plasmids, pPyAct1-PyGUS and pPyAct1-sGFP(S65T), were used to judge whether the transient gene expression system developed in *P. yezoensis* TU-1 is applicable for other Bangiophyceae.

First, we compared the transient gene expression between cultured and naturally growing *P. yezoensis* (Fig. 3a). When pPyAct1-PyGUS was introduced into gametophytic cells of naturally growing *P. yezoensis*, expression of PyGUS was observed in 600 to 850 cells per bombardment (Figs. 3b and 5). In addition, the sGFP fluorescence was also detected

when pPyAct1-sGFP was introduced; however, the number of cells with a fluorescent signal was very small (Figs. 3c and 5). Thus, the transient expression system was useful for *P. yezoensis* grown in a natural ocean habitat, although the number of PyGUS- and sGFP-expressing cells was lower than those in the culture strain TU-1 (Fig. 5).

We next used *P. okamura*e (Fig. 3d) and *P. onoi* (Fig. 3g) for examination of transient gene expression. When pPyAct1-PyGUS was introduced into gametophytic cells of these two species, we could detect PyGUS-expressing cells in both species (Figs. 3e and 3h), although the number of PyGUS-expressing cells was 200 to 400 per bombardment (Fig. 5). The expression of sGFP was also detected in a small number of cells in both species (Figs. 3f, 3i and 5). Thus, both PyGUS and sGFP were successfully expressed under the control of the *PyAct1* promoter in *P. okamurae* and *P. onoi* cells. Moreover, when transient gene expression analysis using pPyAct1-PyGUS and pPyAct1-sGFP was conducted in *P. variegata* (Fig. 3j), ca. 300 PyGUS-expressing cells and ca. 10 sGFP-expressing cells were observed (Figs. 3k, 3l and 5). A dioecious species *P. pseudolinearis* (Figs. S3h-S3s), to which we are able to introduce pPyAct1-PyGUS and pPyAct1-sGFP into both male (Fig. 3m) and female (Fig. 3p) gametophytes separately, was then employed for transient gene expression. Although the number of PyGUS- and sGFP-expressing cells in male gametophytes was less than that in female gametophytes, we detected a significant expression of the two reporters in both algal materials (Figs. 3n, 3o, 3q, 3r and 5).

We next tested the transient gene expression in *Bangia fuscopurpurea* (Fig. 4a). When pPyAct1-PyGUS and pPyAct1-sGFP were introduced into *B. fuscopurpurea* cells, both PyGUS and sGFP expression was observed (Figs. 4b and 4c); however, the number of reporter-expressing cells in *B. fuscopurpurea* was even less than those in the natural species of *Porphyra* (Fig. 5).

Unsuitability of Bangiophycean transient gene expression systems in Florideophycean algae

Finally, we attempted to establish transient gene expression in four naturally growing Florideophycean algae: *Chondrus ocellatus*, *Gloiopeltis furcata*, *Gracilaria vermiculophylla* and *Mazzaella japonica*. However, we could not observe any PyGUS- or sGFP-expressing cells in these Florideophycean algae under various bombardment conditions with pPyAct1-PyGUS and pPyAct1-sGFP using a Tanaka IDERA GIE-III system (data not shown). Since this machine does not produce high helium pressure, we performed additional experiments using a Bio-Rad PDS-1000/He, which can produce high-burst pressure and vacuum pressure. However, no reporter-expressing cells were observed (data not shown). Thus, we concluded that the *PyAct1* promoter and/or codon-modified reporters are not functional in Florideophycean cells.

Discussion

Genetic engineering is a powerful tool both for elucidating the molecular mechanisms of physiological regulations and for producing transgenic organisms by alteration of physiological characteristics. However, in red macroalgae, there have only been reports on efficient transient transformation using *P. yezoensis* (Fukuda at al. 2008; Mikami et al. 2009; Uji et al. 2010; Takahashi et al. 2010). The objective of our study was to determine the applicability of transient gene expression systems established in *P. yezoensis* to other marine red macroalgae. We have shown that the *PyGUS* and *sGFP(S65T)* reporter genes under the control of the *PyAct1* promoter were successfully expressed in Bangiophycean algae, including one *Bangia* and six *Porphyra* species (Figs. 2-4), but not in Florideophycean algae (data not shown). Thus, we concluded that the transient gene expression system in *P.*

yezoensis is widely applicable for Bangiophycean algae.

It is noteworthy that the number of reporter-expressing cells was higher in the culture strains than in naturally growing algae (Fig. 5). For example, the number of PyGUS-expressing cells in laboratory-cultured *P. yezoensis* TU-1 was nearly three-fold higher than that in naturally growing *P. yezoensis*. There are two possible explanations for this phenomenon. One is that it is due to the transfection efficiency of DNA-coated gold particles by bombardment. Since the principle of transient gene expression using particle bombardment is that particles coated with DNA are introduced into cells, the strength and thickness of the cell wall would affect the efficiency of gene transfection. In addition, the cells of *Porphyra* species contain large chloroplasts and relatively small nuclei (Gibbs 1962), which also seems to prevent foreign genes from reaching the nucleus. The other possibility is that the observed phenomenon was related to transcription efficiency. The 5' upstream region of the *PyAct1* gene was able to drive both reporter genes in all Bangiophycean algae examined (Figs. 2-5), indicating its usefulness as a heterologous promoter in the transformation of Bangiophycean algae. However, the results shown in Fig. 5 suggested that the activity of the 5' upstream region of *PyAct1* may be significantly lower in naturally growing Bangiophycean algae than in *P. yezoensis*. Together, these findings indicate that the development of optimal conditions of particle bombardment and the use of endogenous strong promoters will be important for the efficient gene expression in individual red alga.

In contrast to Bangiophycean algae, there were no PyGUS- or sGFP-expressing cells in Florideophycean algae. The most likely reason for this involves the availability of the *P. yezoensis* promoter. It is important to isolate the 5' upstream region of the endogenous gene that is constitutively and strongly expressed in Florideophycean cells. In addition, since there is a possibility of a reduction in the efficiency of plasmid introduction, the bombardment parameters, such as the size of the gold particles, target distance, acceleration pressure and

amount of DNA per bombardment, should be adjusted.

In conclusion, PyGUS and sGFP were functional as reporters of transient gene expression and the *PyAct1* promoter was functional as a heterologous promoter in Bangiophycean algae. This applicability of *P. yezoensis* transient gene-expression systems in Bangiophycean algae can be expected to accelerate molecular biological studies in marine red algae. More importantly, we anticipate that these reporters and this promoter will be available for stable transformation in Bangiophycean algae. However, it should be resolved two major problems for establishing the stable transformation system in marine macroalgae in the future. Development of appropriate selective markers is one of the most important tasks. Although there are a few reports on the antibiotics or herbicides affecting on the growth and survival of *Porphyra* and drug resistance genes which can express in the *Porphyra* cells (Zuo et al. 2007; Wang et al. 2010), candidates of drugs and corresponding resistance genes available for the selection of transformants have not yet been declared. Furthermore, it is necessary to establish the method of foreign gene-integration into the genome, for which homologous recombination is suitable for targeted gene-disruption and insertion. Establishment of stable transformation system through resolution of these problems contributes further understanding of the molecular mechanisms regulating development and stress responses in Bangiophyceae.

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Figure legends

Fig. 1

Schematic representation of the sampling locations of red algae used in the present study. The southern region of Hokkaido is highlighted in the box at right, and the sampling locations are indicated. Algal samples collected at each location are summarized in Table 1.

Fig. 2

Transient gene expression in a laboratory-cultured strain *P. tnera* JTW. (**a**, upper) Schematic representation of reporter constructs used in the present study. Promoter-containing and promoter-less constructs were made for both PyGUS and sGFP(S65T) reporter gene. (**a**, lower) Confirmation of gene transfer into algal cells. After bombardment of four expression plasmids into *P. tenera* cells, DNA fragment corresponding to a part of PyGUS or sGFP(S65T) ORF was amplified by PCR using specific primers (Table S1). 857 bp and 706 bp fragments were amplified when PyGUS and sGFP(S65T) containing plasmids were introduced into cells, respectively (Fig. 2a, lower). Appearance of these bands were independent to the presence or absence of the promoter in the plasmids. (**b**) Successful expression of PyGUS directed by the *PyAct1* promoter in *P. tenera* cells. The results of histochemical staining using *P. tenera* JTW and *P. yezoensis* TU-1 are compared. For each species, the left panel shows a gametophytic blade, the center column shows cells expressing PyGUS by transient transformation with pPyAct1-PyGUS and stained with X-gluc solution after 48 h of bombardment, and the right column shows cells introduced promoter-less-PyGUS and stained with X-gluc solution after 48 h of bombardment. Upper and lower panels in the center and right columns show low- and high-magnification images, respectively. (**c**) Correlation of the number of PyGUS-expressing cells and the GUS

enzymatic activity. The number of PyGUS-expressing cells (left) and the GUS enzymatic activity (right) in *P. tenera* JTW (light gray) and *P. yezoensis* TU-1 (dark gray), into which pPyAct1-PyGUS or promoter-less-PyGUS was introduced. (**d**) Successful expression of pPyAct1-sGFP in *P. tenera* JTW. Upper and lower panels in the left show bright field and fluorescent images of sGFP-expressing cells, respectively. Center panels show bright field (upper), autofluorescence of chloroplast (middle), and fluorescent images (lower) of a sGFP-expressing cell, respectively. Right panels show bright field and fluorescent images of cells to which promoter-less-sGFP was introduced, respectively. Scale bar: 1 cm in left panels of **b**, 200 μm in upper of center and right panels of **b** and left and right panels of **d**, 10 μm in lower of center and right panels of **b** and center panels of **d**.

Fig. 3

Successful expression of PyGUS and sGFP reporters in naturally growing *Porphyra* species. (**a**-**c**) *P. yezoensis*. (**d**-**f**) *P. okamurae*. (**g**-**i**) *P. onoi*. (**j**-**l**) *P. variegate*. (**m**-**o**) Female gametophytes of *P. pseudolinearis*. (**p**-**r**) Male gametophytes of *P. pseudolinearis*. (**a**, **d**, **g**, **j**, m and **p**) A gametophytic blade. (**b**, **e**, **h**, **k**, **n** and **q**) Cells expressing PyGUS by transient transformation with pPyAct1-PyGUS. Upper and lower panels show low- and high-magnification images, respectively. (**c**, **f**, **i**, **l**, **o** and **r**) Cells expressing sGFP by transient transformation with pPyAct1-sGFP. Upper and lower panels show bright field and fluorescent images of an sGFP-expressing cell, respectively. Scale bar: 1 cm in **a**, **d**, **g**, **j**, **m** and **p**, 200 μm in **b** (upper), **e** (upper), **h** (upper), **k** (upper), **n** (upper) and **q** (upper) and 10 μm in **b** (lower), **e** (lower), **h** (lower), **k** (lower), **n** (lower), **q** (lower), **c**, **f**, **i**, **l**, **o** and **r**.

Fig. 4

Successful expression of the PyGUS and sGFP reporters in gametophytic cells of *B. fuscopurpurea*. (**a**) Gametophytes of *B. fuscopurpurea*. Upper and lower panels show low and high magnification images, respectively. (**b**) Cells expressing PyGUS by transient transformation with pPyAct1-PyGUS. Upper and lower panels show low and high magnification images, respectively. (**c**) Cells expressing sGFP by transient transformation with pPyAct1-sGFP. Upper and lower panels show bright field and fluorescent images of an sGFP-expressing cell, respectively. Scale bar: 1 cm in **a** (upper), 200 μm in **b** (upper), 20 μm in **a** (lower) and 10 μm in **b** (lower) and **c**.

Fig. 5

Comparison of the number of cells expressing PyGUS (upper) and sGFP (lower) reporters in Bangiophycean algae. Data are represented as the mean and SD of triplicate experiments. TU-1, *P. yezoensis* TU-1; JTW, *P. tenera* JTW; *Py*, *P. yezoensis*; *Pok*, *P. okamurae*; *Pon*, *P. onoi*; *Pv*, *P. variegate*; *Pp*♀, female gametophyte of *P. pseudolinearis*; *Pp*♂, male gametophyte of *P. pseudolinearis*.

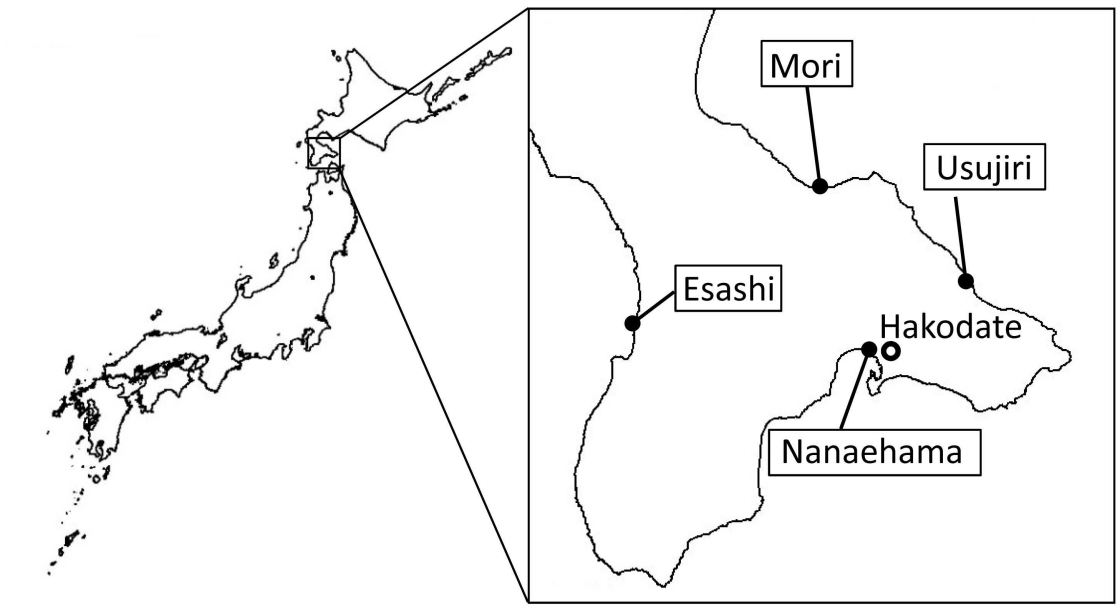
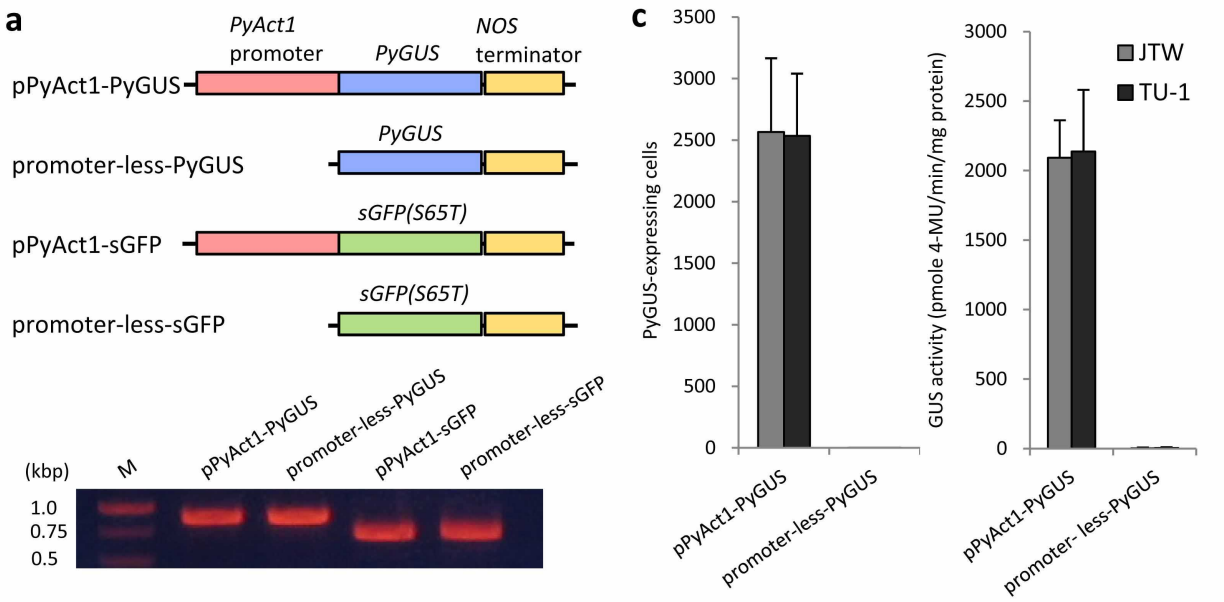


Fig. 1


a
PyAct1 promoter
PyGUS
NOS terminator
pPyAct1-PyGUS
promoter-less-PyGUS
sGFP(S65T)
pPyAct1-sGFP
promoter-less-sGFP
(kbp)
M
1.0
0.75
0.5
c
PyGUS-expressing cells
GUS activity (pmole 4-MU/min/mg protein)
JTW
TU-1
pPyAct1-PyGUS
promoter-less-PyGUS
promoter- less-PyGUS

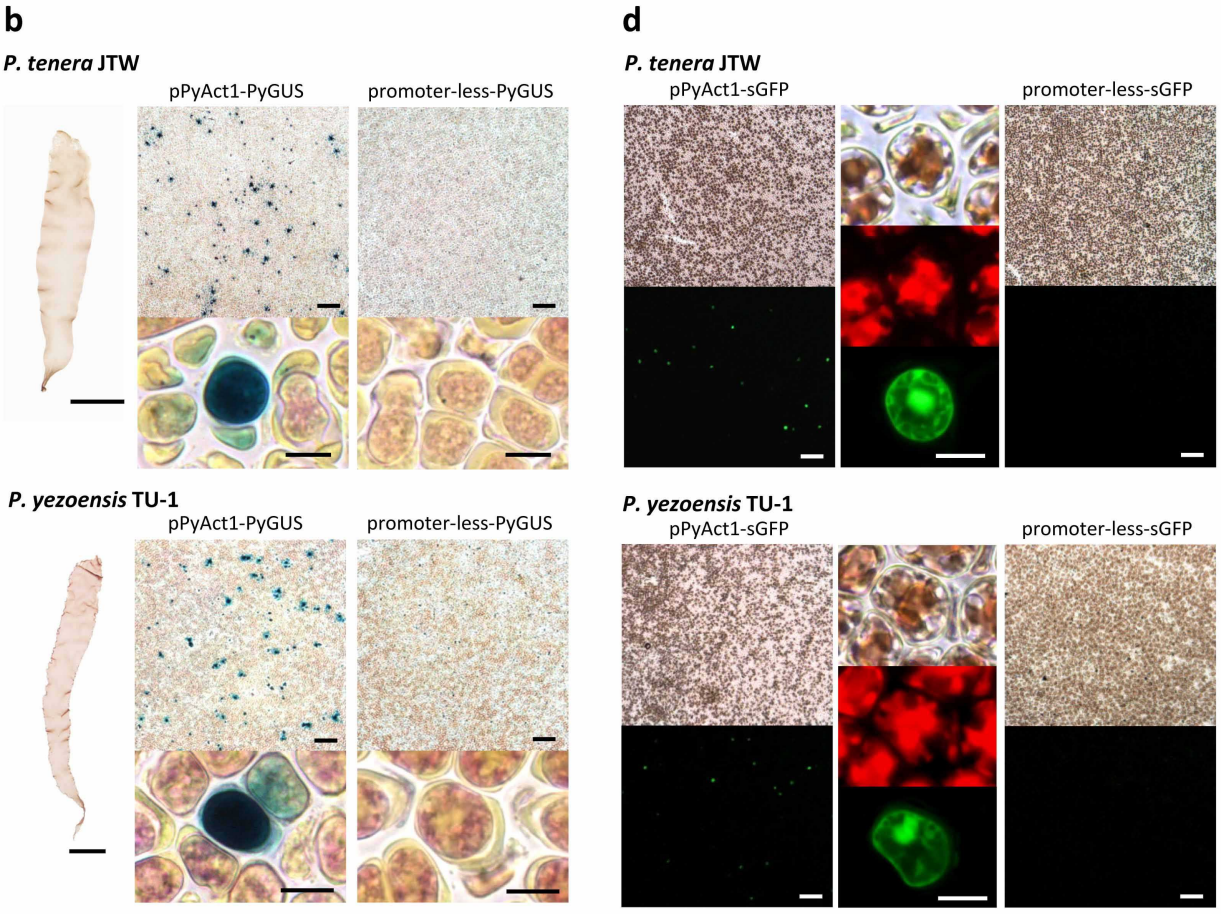

b
P. tenera JTW
pPyAct1-PyGUS
promoter-less-PyGUS
P. yezoensis TU-1
d
P. tenera JTW
pPyAct1-sGFP
promoter-less-sGFP
P. yezoensis TU-1

Fig. 2

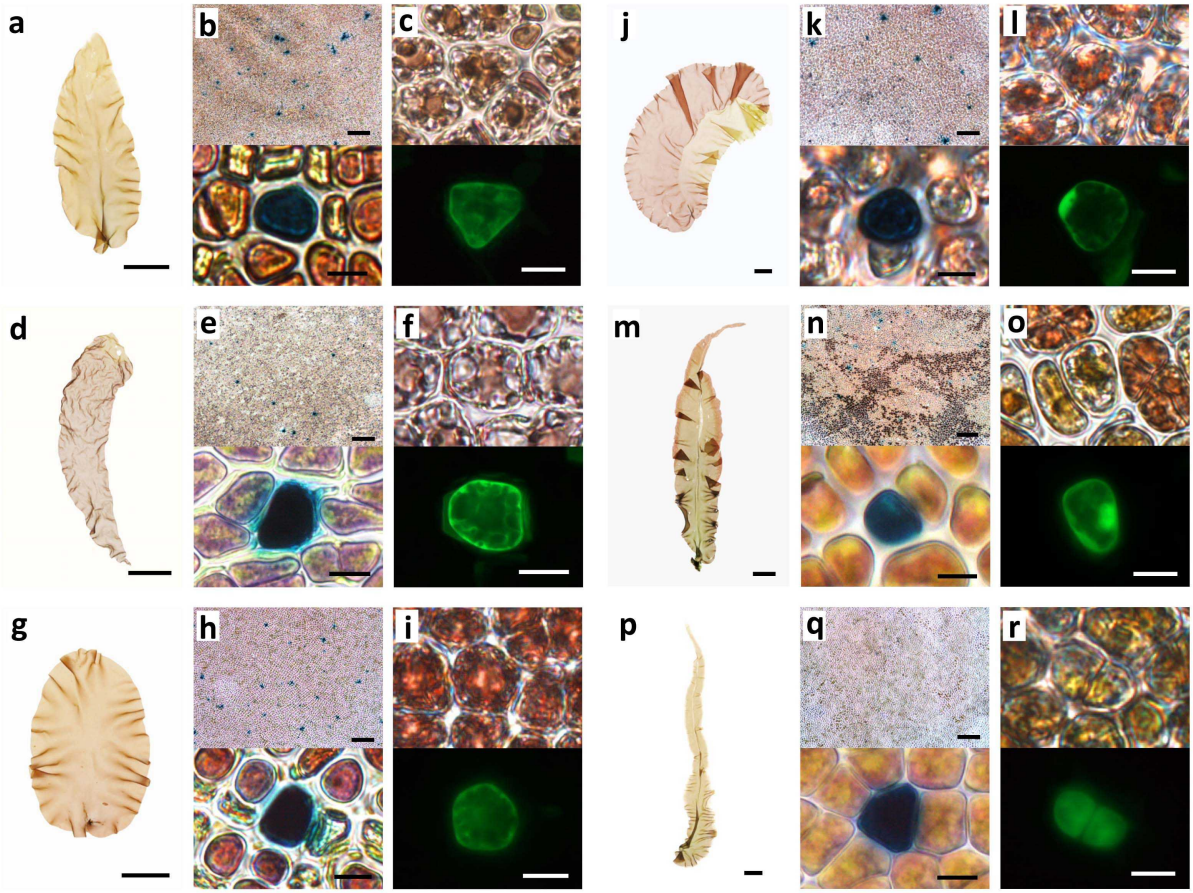


Fig. 3

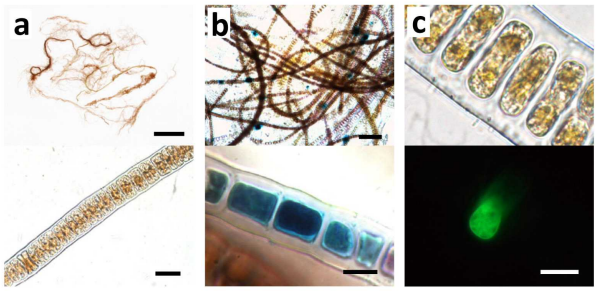


Fig. 4

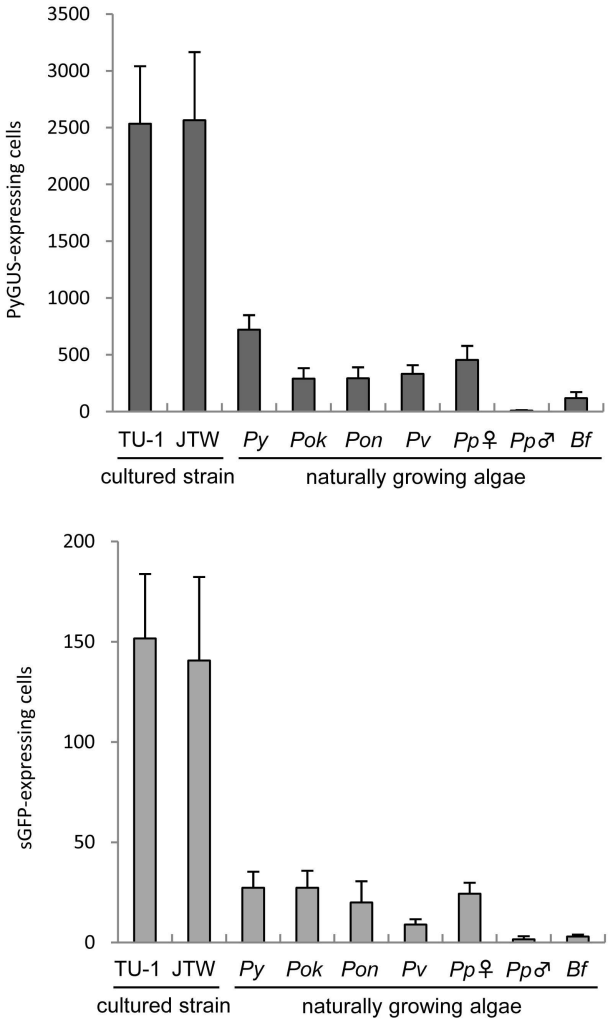

PyGUS-expressing cells
3500
3000
2500
2000
1500
1000
500
0
TU-1
JTW
Py
Pok
Pon
Pv
Pp♀
Pp♂
Bf
cultured strain
naturally growing algae
sGFP-expressing cells
200
150
100
50
0
TU-1
JTW
Py
Pok
Pon
Pv
Pp♀
Pp♂
Bf
cultured strain
naturally growing algae

Fig. 5

Table 1 List of red algal species used in this study

Species	Class	Location of collection	Date of collection
Porphyra yezoensis (TU-1)	Bangiophyceae	Laboratory cultured strain (Kuwano et al. 1996)	
Porphyra tenera (JTW)	Bangiophyceae	Laboratory cultured strain (Park et al. 2008)	
Porphyra yezoensis	Bangiophyceae	Nanaehama, Hokuto, Hokkaido	8 March 2010
Porphyra okamurae	Bangiophyceae	Usujiri, Hakodate, Hokkaido	20 January 2010
Porphyra onoi	Bangiophyceae	Nanaehama, Hokuto, Hokkaido	8 March 2010
Porphyra variegata	Bangiophyceae	Mori, Hokkaido	17 April 2010
Porphyra pseudolinearis	Bangiophyceae	Mori, Hokkaido	17 April 2010
Bangia fuscopurpurea	Bangiophyceae	Esashi, Hokkaido	14 May 2010
Chondrus ocellatus	Florideophyceae	Nanaehama, Hokuto, Hokkaido	25 January 2010
Gloiopeltis furcata	Florideophyceae	Nanaehama, Hokuto, Hokkaido	25 January 2010
Gracilaria vermiculophylla	Florideophyceae	Nanaehama, Hokuto, Hokkaido	25 January 2010
Mazzaella japonica	Florideophyceae	Nanaehama, Hokuto, Hokkaido	25 January 2010

Supplemental Figure legends

Fig. S1

Morphological characteristics of gametophytic blades of the cultured strains *P. yezoensis* TU-1 and *P. tenera* JTW. (**a-e**) *P. yezoensis* TU-1. (**f-j**) *P. tenera* JTW. (**a** and **f**) A gametophytic blades. (**b** and **g**) Surface view of vegetative cells. (**c** and **h**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**d** and **i**) Surface view of reproductive cells. The pale yellow region corresponds to the spermatangial sorus (black arrowhead) and the other regions correspond to the carpogonial and zygotosporangial sorus (white arrowhead). (**e** and **j**) Fluorescent image of chromosomes in a gametophytic cell (haploid stage) that was fixed with Bouin's fluid and then stained with SYBR Gold solution for 3 minutes. Both of these algal cells have 3 chromosomes per cell. Scale bar: 1 cm in **a** and **f**, 50 μm in **b**, **d**, **g** and **i**, 20 μm in **c** and **h** and 10 μm in **e** and **j.**

Fig. S2

Morphological characteristics of gametophytic blades of naturally growing *Porphyra* species having an asexual life cycle via monospores. (**a-g**) *P. yezoensis*. (**h-l**) *P. okamurae*. (**m-q**) *P. onoi*. (**a**) A gametophytic blade of *P. yezoensis*. (**b**) Surface view of vegetative cells. (**c**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**d**) Surface view of reproductive cells. The relatively pale region corresponds to the spermatangial sorus (black arrowhead) and the rest of the image indicates the carpogonial and zygotosporangial sorus (white arrowhead). (**e**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. (**f**) Section of the zygotosporangial sorus. (**g**) Section of the spermatangial sorus. (**h**) A gametophytic blade of *P. okamurae*. (**i**) Surface view of a marginal portion of a blade having small serrations. (**j**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**k**) Surface view of reproductive cells. The pale yellow region corresponds to the spermatangial sorus and the other regions correspond to the carpogonial and zygotosporangial sorus. (**l**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 4 chromosomes per cell. (**m**) Gametophytic blades of *P. onoi* adhering to *Chondrus ocellatus*. (**n**) Surface view of vegetative cells. (**o**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells, containing 2 chloroplasts per cell. (**p**) Surface view of reproductive cells. The colorless region corresponds to the spermatangial sorus (black arrowhead) and the other regions correspond to the

carpogonial and zygotosporangial sorus (white arrowhead). (**q**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. Scale bar: 1 cm in **a**, **h** and **m**, 50 μm in **b**, **d**, **j**, **k**, **n** and **p**, 20 μm in **c**, **f**, **g**, **j** and **o** and 10 μm in **e**, **l** and **q**.

Fig. S3

Morphological characteristics of gametophytic blades of naturally growing *Porphyra* species having no asexual life cycle. (**a-g**) *P. variegate*. (**h-l**) A female gametophyte of *P. pseudolinearis*. (**m-f**) A male gametophyte of *P. pseudolinearis*. (**a**) A gametophytic blade of *P. variegata* adhering to a blade of the seagrass *Phyllospadix iwatensis*. (**b**) Surface view of vegetative cells. (**c**) Section of a gametophytic blade, showing the double-layer arrangement of vegetative cells. (**d**) Surface view of reproductive cells. The pale yellow right region corresponds to the spermatangial sorus (black arrowhead) and the left region corresponds to the carpogonial sorus (white arrowhead). (**e**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. (**f**) Section of the carpogonial sorus. (**g**) Section of the spermatangial sorus. (**h**) A female gametophytic blade of *P. pseudolinearis*. (**i**) Surface view of vegetative cells. (**j**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**k**) Surface view of the zygotosporangial sorus. (**l**) Section of the zygotosporangial sorus. (**m**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 4 chromosomes per cell. (**n**) A male gametophytic blade of *P. pseudolinearis*. (**o**) Surface view of vegetative cells. (**p**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**q**) Surface view of the spermatangial sorus. (**r**) Section of the spermatangial sorus. (**s**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 4 chromosomes per cell. Scale bar: 1 cm in **a**, **h** and **n**, 50 μm in **b**, **d**, **i**, **k**, **o** and **q**, 20 μm in **c**, **f**, **g**, **j**, **l**, **p** and **r** and 10 μm in **e**, **m** and **s**.

Fig. S4

Morphological characteristics of gametophytes of *B. fuscopurpurea*. (**a**) Macroscopic view of gametophytes. (**b**) Microscopic view of a gametophyte having a uniseriate filamentous shape. (**c**) Monosporangial sorus. (**d**) Carpogonial sorus. (**e**) Spermatangial sorus. (**f**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. Scale bar: 1 cm in **a**, 20 μm in **b-e** and 5 μm in **f.**

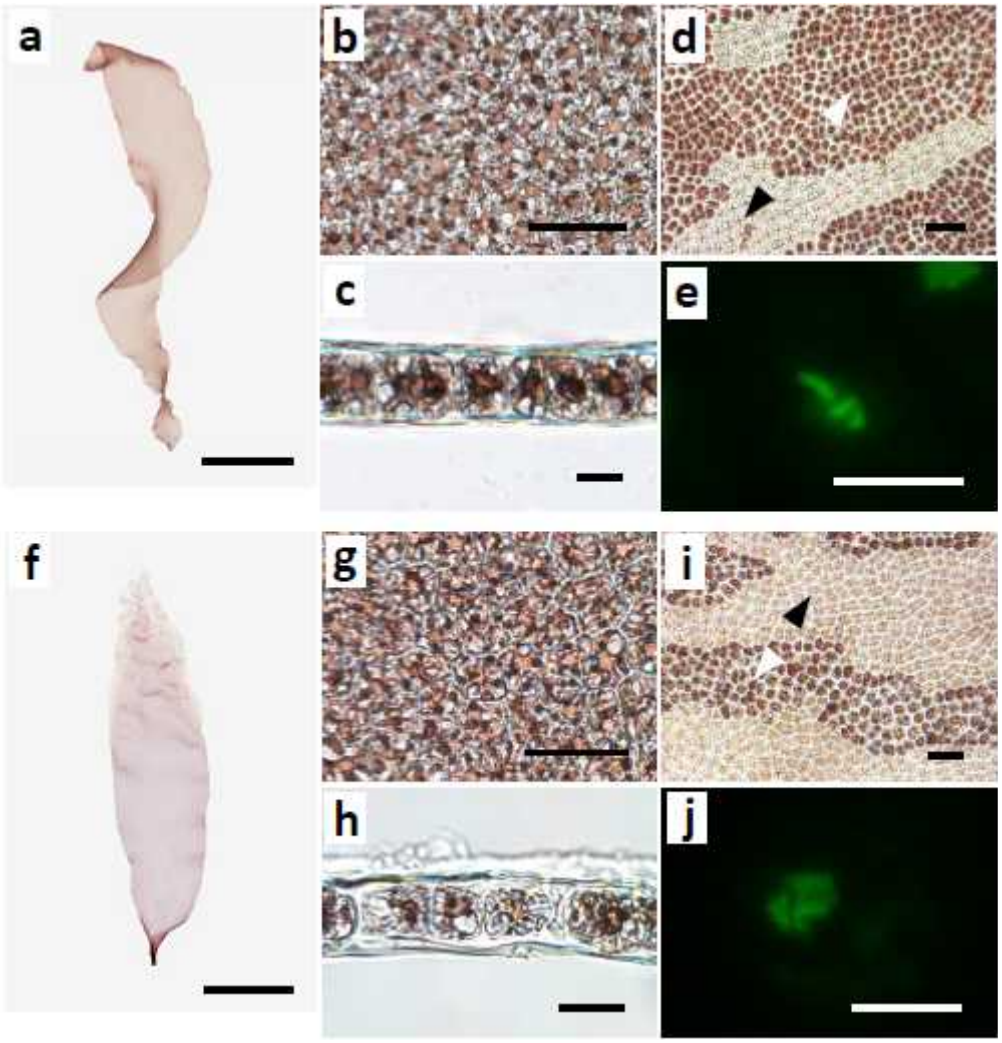


Fig. S1 Morphological characteristics of gametophytic blades of the cultured strains *P. yezoensis* TU-1 and *P. tenera* JTW. (**a**-**e**) *P. yezoensis* TU-1. (**f**-**j**) *P. tenera* JTW. (**a** and **f**) A gametophytic blades. (**b** and **g**) Surface view of vegetative cells. (**c** and **h**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**d** and **i**) Surface view of reproductive cells. The pale yellow region corresponds to the spermatangial sorus (black arrowhead) and the other regions correspond to the carpogonial and zygotosporangial sorus (white arrowhead). (**e** and **j**) Fluorescent image of chromosomes in a gametophytic cell (haploid stage) that was fixed with Bouin's fluid and then stained with SYBR Gold solution for 3 minutes. Both of these algal cells have 3 chromosomes per cell. Scale bar: 1 cm in **a** and **f**, 50 μm in **b**, **d**, **g** and **i**, 20 μm in **c** and **h** and 10 μm in **e** and **j.**

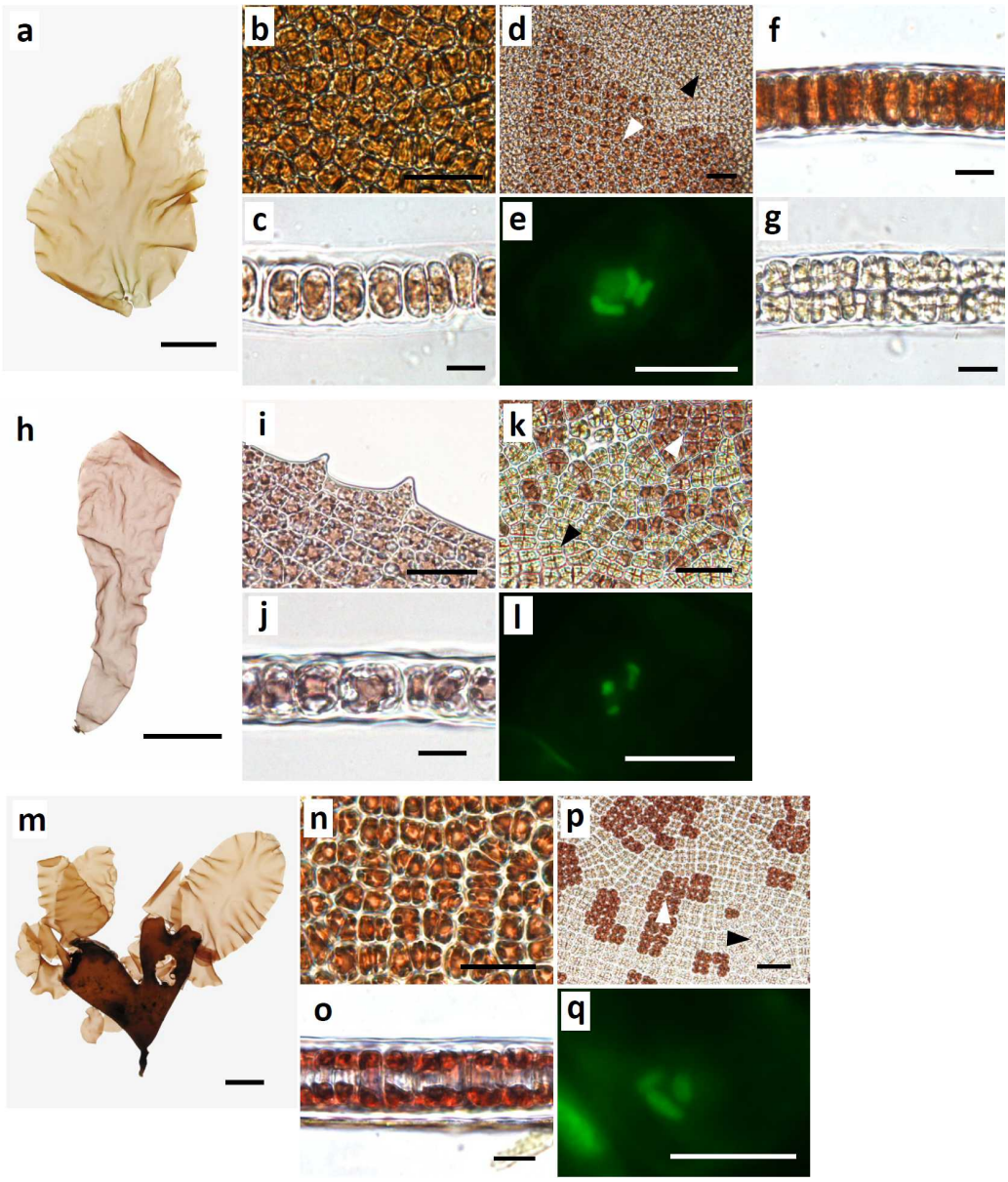


Fig. S2 Morphological characteristics of gametophytic blades of naturally growing *Porphyra* species having an asexual life cycle via monospores. (**a-g**) *P. yezoensis*. (**h-l**) *P. okamurae*. (**m-q**) *P. onoi*. (**a**) A gametophytic blade of *P. yezoensis*. (**b**) Surface view of vegetative cells. (**c**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**d**) Surface view of reproductive cells. The relatively pale region corresponds to the spermatangial sorus (black arrowhead) and the rest of the image indicates the carpogonial and zygotosporangial sorus (white arrowhead). (**e**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. (**f**) Section of the zygotosporangial sorus. (**g**) Section of the spermatangial sorus. (**h**) A gametophytic blade of *P. okamurae*. (**i**) Surface view of a marginal portion of a blade having small serrations. (**j**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**k**) Surface view of reproductive cells. The pale yellow region corresponds to the spermatangial sorus and the other regions correspond to the carpogonial and zygotosporangial sorus. (**l**)

Fluorescent image of chromosomes stained with SYBR Gold solution, containing 4 chromosomes per cell. (**m**) Gametophytic blades of *P. onoi* adhering to *Chondrus ocellatus*. (**n**) Surface view of vegetative cells. (**o**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells, containing 2 chloroplasts per cell. (**p**) Surface view of reproductive cells. The colorless region corresponds to the spermatangial sorus (black arrowhead) and the other regions correspond to the carpogonial and zygotosporangial sorus (white arrowhead). (**q**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. Scale bar: 1 cm in **a**, **h** and **m**, 50 μm in **b**, **d**, **j**, **k**, **n** and **p**, 20 μm in **c**, **f**, **g**, **j** and **o** and 10 μm in **e**, **l** and **q**.

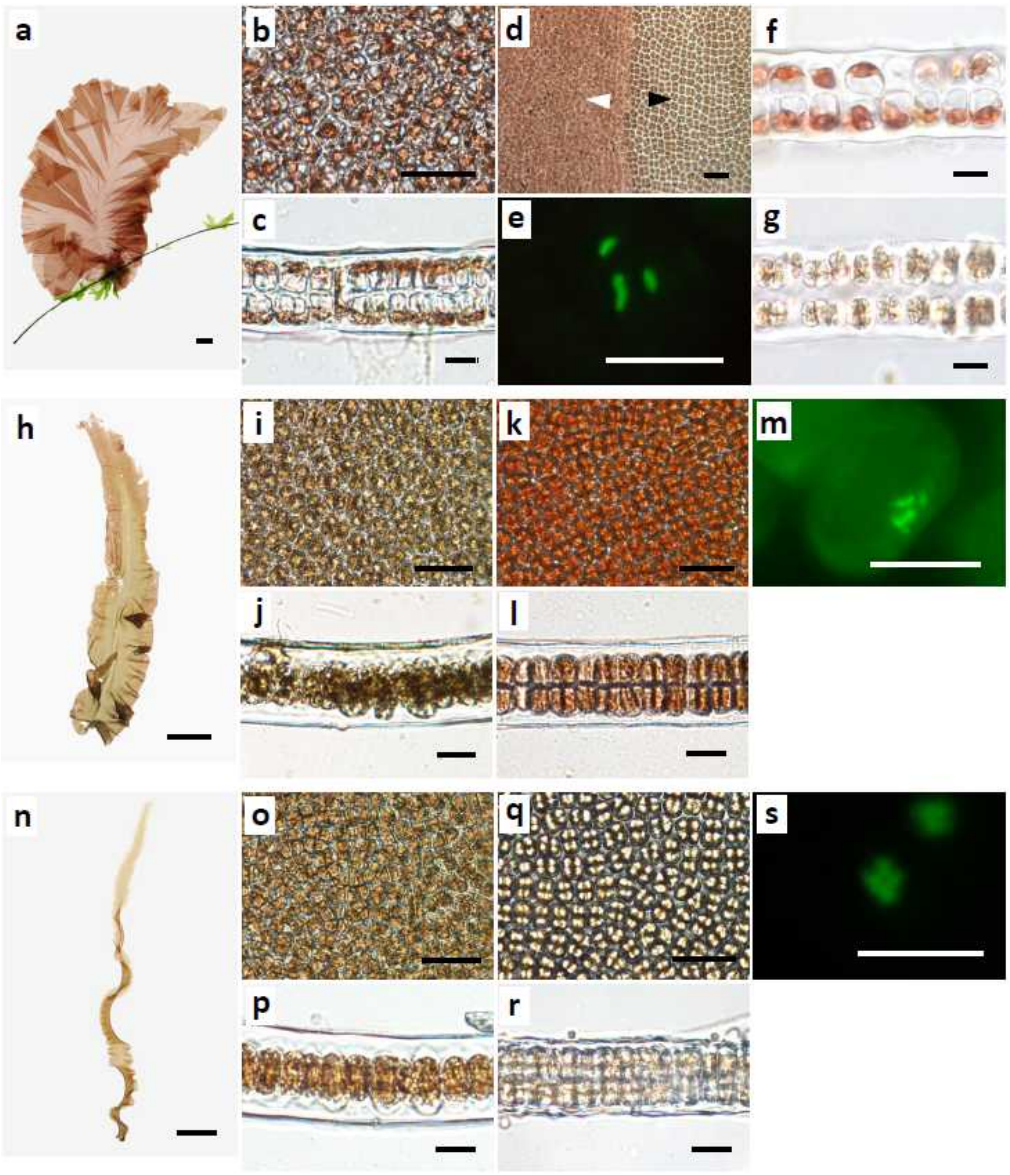


Fig. S3 Morphological characteristics of gametophytic blades of naturally growing *Porphyra* species having no asexual life cycle. (**a-g**) *P. variegate*. (**h-l**) A female gametophyte of *P. pseudolinearis*. (**m-f**) A male gametophyte of *P. pseudolinearis*. (**a**) A gametophytic blade of *P. variegata* adhering to a blade of the seagrass *Phyllospadix iwatensis*. (**b**) Surface view of vegetative cells. (**c**) Section of a gametophytic blade, showing the double-layer arrangement of vegetative cells. (**d**) Surface view of reproductive cells. The pale yellow right region corresponds to the spermatangial sorus (black arrowhead) and the left region corresponds to the carpogonial sorus (white arrowhead). (**e**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. (**f**) Section of the carpogonial sorus. (**g**) Section of the spermatangial sorus. (**h**) A female gametophytic blade of *P. pseudolinearis*. (**i**)

Surface view of vegetative cells. (**j**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**k**) Surface view of the zygotosporangial sorus. (**l**) Section of the zygotosporangial sorus. (**m**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 4 chromosomes per cell. (**n**) A male gametophytic blade of *P. pseudolinearis*. (**o**) Surface view of vegetative cells. (**p**) Section of a gametophytic blade, showing the single-layer arrangement of vegetative cells. (**q**) Surface view of the spermatangial sorus. (**r**) Section of the spermatangial sorus. (**s**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 4 chromosomes per cell. Scale bar: 1 cm in **a**, **h** and **n**, 50 μm in **b**, **d**, **i**, **k**, **o** and **q**, 20 μm in **c**, **f**, **g**, **j**, **l**, **p** and **r** and 10 μm in **e**, **m** and **s**.

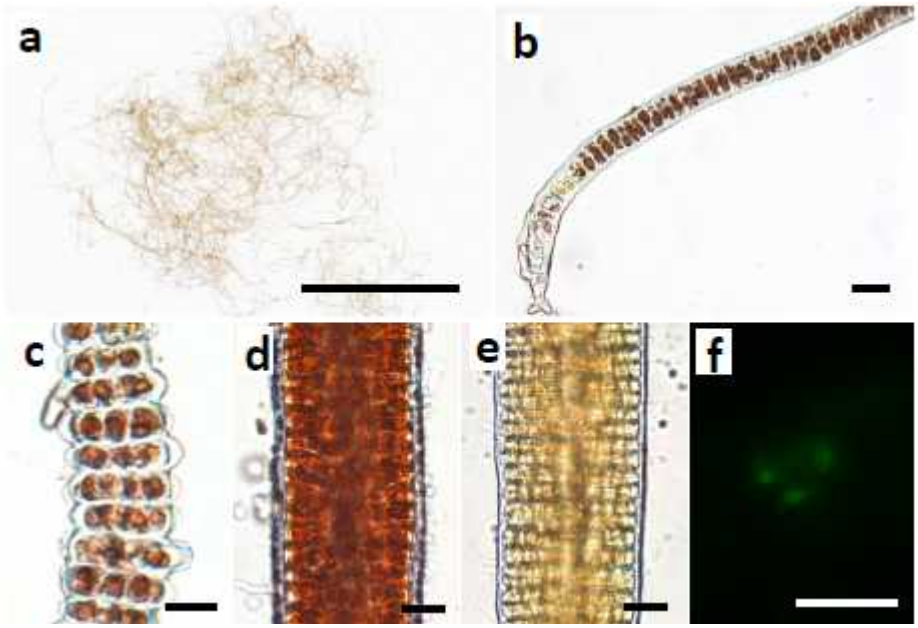


Fig. S4 Morphological characteristics of gametophytes of *B. fuscopurpurea*. (**a**) Macroscopic view of gametophytes. (**b**) Microscopic view of a gametophyte having a uniseriate filamentous shape. (**c**) Monosporangial sorus. (**d**) Carpogonial sorus. (**e**) Spermatangial sorus. (**f**) Fluorescent image of chromosomes stained with SYBR Gold solution, containing 3 chromosomes per cell. Scale bar: 1 cm in **a**, 20 μm in **b**-**e** and 5 μm in **f.**

Table S1 List of primers used in PCR

Primer	Sequence
PyGUS-F1	5'-AACGTGCTGATGGTGCAC-3'
PyGUS-R1	5'-AAGTTCATGCCCGTCCAG-3'
sGFP-F1	5'-AGGGCGAGGAGCTGTTCAC-3'
sGFP-R1	5'-TTGTACAGCTCGTCCATGCC-3'