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Research Note

Heterologous activation of the *Porphyra tenera* HSP70 promoter in Bangiophyceean algal cells

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Bangiophyceae, *Porphyra tenera*, *Porphyra yezoensis*, HSP promoter, PyGUS, transient gene expression, particle bombardment

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Note to:

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Son SH, Ahn JW, Uji T, Choi DW, Park EJ, Hwang MS, Liu JR, Choi D, Mikami K, Jeong WJ. Development of a transient gene expression system in the red macroalga, *Porphyra tenera*. J. Appl. Phycol. 2011; in press; DOI: 10.1007/s10811-011-9652-9.

Abstract

Porphyra has attracted significant attention for its biological and industrial importance. However, establishment of a stable nuclear transformation has not yet been achieved in these organisms, which impedes the molecular biological study and the development of a molecular breeding method for them. Toward establishing the stable transformation, we have recently developed an efficient transient gene expression system in Bangiophyceae algae, in which the *HSP70* promoter from *P. tenera* (*PtHSP70* promoter) was activated heterologously in *P. yezoensis* cells. Since heterologous promoters are required for homologous recombination-based stable transformation, the identification of heterologously activated promoters is important in establishing a stable transformation system in individual Bangiophyceae alga. We here examined the activation of the *PtHSP70* promoter using the GC-rich PyGUS reporter system in additional *Porphyra* and *Bangia* species. The results indicated that this promoter drove expression of the *PyGUS* gene efficiently in all examined algae, whereas there was quite low expression of PyGUS by the cauliflower mosaic virus 35S promoter that is widely used as a heterologous promoter in the transformation of green land plants. Therefore, heterologous activation of the *PtHSP70* promoter could promote the establishment of the stable transformation system in various kinds of Bangiophyceae algae.

Introduction

Bangiophyceae are marine red macroalgae (seaweeds) containing many economically important species, including *P. yezoensis* and *P. tenera*, both of which are cultivated industrially in the coastal area of Japan, Korea, and China.¹ Recently, global warming has caused critical problems such as a decreased growth rate, discoloration due to deficiency of nutrition, and disease by fungal or bacterial infection.²⁻⁴ Development of beneficial strains such as thermo-tolerant and disease-resistant red algae is therefore eagerly anticipated.

To develop beneficial red algal strains, it is necessary to establish a genetic transformation system, which is an essential tool not only for elucidating the molecular mechanisms of cellular processes but also for producing organisms carrying beneficial characteristics such as a high growth rate, thermo- or cold tolerance, and disease resistance. Although genetic transformation is routinely used for molecular biological study in a variety of organisms, such as bacteria, yeast, higher plants, animals, and even some microalgae, it has not yet been developed in marine macroalgae. Toward establishing a genetic transformation system in red macroalgae, we have developed a transient gene expression system in *P. yezoensis*,^{5,6} by which efficient gene transfer and gene expression became available in Bangiophycean algae.⁷ The next step is to develop a system to insert a foreign gene into the genome via DNA recombination, for which it is important to use the heterologous promoter to drive the expression of a reporter or selection marker gene. If an endogenous promoter is used, recombination will occur efficiently at the corresponding site in the genome. In addition to our previous report on some heterologously employable promoters in Bangiophycean algae,^{7,8} here we evaluate the *P. tenera* *HSP70* promoter as an additional candidate for the heterologous promoter in red algal cells.

Establishment of the transient gene expression system in *P. yezoensis*: A brief summary

There are four essential steps to establishing a stable transformation system: transfection of foreign genes, efficient expression of foreign gene, integration of a foreign gene into the genome, and selection and isolation of transformed cells. Since the first two factors could become available by the establishment of a transient gene expression system, we have tried to develop such a system in the red macroalga *P. yezoensis*. In this context, we previously found that the particle bombardment method enables transfection of foreign genes into *P. yezoensis* cells, enabling us to develop a foreign gene expression system.

There are two critical factors in the establishment of the transient gene expression system in *P. yezoensis*, codon usage and promoter strength to direct the reporter gene expression. It was reported that β -glucuronidase (GUS) reporter and *CaMV* 35S promoter, which are widely used in plant transformation, are functional in *Porphyra* cells;⁹⁻¹¹ however, we have found incompatibility of these elements in *P. yezoensis* cells.^{5,6} Since coding regions of red macroalgal genes are GC-rich, especially for the 3rd letter in codons, it is possible that codon bias would prevent the expression of a foreign gene such as the bacterial

uidA gene (encoding GUS). Thus, we artificially synthesized the gene encoding GC-rich GUS, designated as *PyGUS*, by introducing base substitutions from AT to GC. When the *PyGUS* coding region was fused with the *CaMV* 35S promoter, it was expressed very weakly but significantly in *P. yezoensis* cells,^{5,6} suggesting the importance of the adaptation of codon usage and also the defect of the *CaMV* 35S promoter in *P. yezoensis* cells. The latter was resolved by employing the 5' upstream regions of endogenous genes such as the *PyGAPDH* and *PyAct1* promoters, showing strong expression of the *PyGUS* reporter gene.^{5,6,12} The *PyAct1* promoter has also enabled the efficient expression of commercially released humanized fluorescent protein genes whose codon usage is similar to that in *P. yezoensis*.^{13,14} Taken together, the optimization of codon usage and employment of endogenous promoters are essential factors for establishing a transient gene expression system in *P. yezoensis* cells.

The *HSP70* promoter from *P. tenera* functions as a heterologous strong promoter in Bangiophycean algae

It is necessary to employ heterologous promoters for stable nuclear transformation, since it is possible that endogenous promoters in expression vectors might cause unpredictable gene silencing. Indeed, promoters derived from the green microalga *Chlamydomonas reinhardtii* or *Volvox carteri* are available in some volvocine algae and used for driving the foreign gene expression.^{15,16} A recent study showed that the *PyAct1* promoter is active not only in *P. yezoensis* but also in five other *Porphyra* species, including *P. tenera*, and one *Bangia* species.⁷ In addition, the promoter region of the *HSP70* gene derived from *P. tenera* (*PtHSP70* promoter) was shown to be active in *P. yezoensis* cells.⁸ However, it remains to be determined whether the *PtHSP70* promoter is available as a heterologous promoter in other Bangiophycean algal cells. Thus, we evaluated the ability of the *PtHSP70* promoter to drive the transient expression of the *PyGUS* reporter gene in *P. okamurae*, *P. pseudolinearis*, and *Bangia fuscopurpurea* cells.

We introduced expression plasmids p*PyAct1*-*PyGUS*, p*PtHSP70*-*PyGUS*, and p*CaMV35S*-*PyGUS* (see Fig. 1) into five Bangiophycean algae (*P. tenera*, *P. yezoensis*, *P. okamurae*, *P. pseudolinearis*, and *B. fuscopurpurea*) by the particle bombardment method using a PDS-1000/He (Bio-Rad, USA) under the conditions shown in Table 1. As shown in Fig. 2, cells expressing *PyGUS* were observed in all species when p*PyAct1*-*PyGUS* or p*PtHSP70*-*PyGUS* was introduced, while only a few cells expressed *PyGUS* following the introduction of p*CaMV35S*-*PyGUS*. These observations were confirmed by quantitative analysis of the *PyGUS* enzymatic activity by a fluorometric GUS assay and the number of *PyGUS*-expressing cells (Fig. 3). Results indicated that the *PtHSP70* promoter is widely applicable for high-level expression of foreign genes in Bangiophycean algae, as is the *PyAct1* promoter, although the *CaMV* 35S promoter was less functional in these algae. Therefore, the transient gene expression system using heterologous *PtHSP70* promoter could enable molecular biological analyses in Bangiophycean algae, such as subcellular localization of proteins using fluorescent protein reporter and silencing desired genes

by RNA interference.

In addition, it is noteworthy that the PyGUS expression was high in *P. tenera* and *P. yezoensis* compared to expression in other algae used in this study (Fig. 3). This may reflect not only the difference in the promoter activity but also the introduction efficiency of the expression plasmid by particle bombardment among the algal species, which may have been affected by the differences in cell wall strength among these species. Thus, besides the use of another strong promoter, it seems worth trying to improve the gene transfer methods such as modifying the conditions of particle bombardment or loosening the cell walls by treating with cell wall-degrading enzymes available in *P. okamurai*, *P. pseudolinearis*, and *B. fuscopurpurea*.

The *PtHSP70* promoter and the *PyAct1* promoter are available as heterologous promoters in Bangiophyceae algae, indicating their availability not only for transient gene expression but also for stable transformation in these algae. Since our major goal is to establish a stable transformation in red macroalgae, the next step is to establish methods of integration of a foreign gene into the genome, and selection and isolation of transformed cells. These methodological improvements could lead us to identify genes critically involved in development and stress responses, which will help us determine how to accomplish molecular breeding.

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Figure legends

Figure 1. Schematic representation of reporter constructs used in this study. The *CaMV 35S* (green), *PyAct1* (red), and *PtHSP70* (orange) promoters were fused to PyGUS. *tNOS*, nopaline synthase terminator.

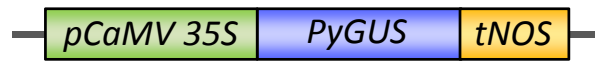
Figure 2. Comparison of PyGUS expression directed by three different promoters. For each species, the left panel shows a gametophytic blade and the right three panels correspond to histochemical detection of PyGUS expressing gametophytic cells transiently transformed by pCaMV35S-PyGUS, pPyAct1-PyGUS, and pPtHSP70-PyGUS, respectively. Scale bars correspond to 1 cm in images of gametophytic blades and 100 μm in images of PyGUS expression. In these experiments, gametophytes of culture strain *P. yezoensis* TU-1 and *P. tenera* T-8 were grown in ESL medium at 15°C under irradiation of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps with a photo-period of 10 h light: 14 h dark. Gametophytes of *P. okamurae*, *P. pseudolinearis*, and *B. fuscopurpurea*, whose morphological and cytological characteristics were described in ref. 7, were grown in the same conditions until using for transient transformation experiments. For transient transformation, gametophytic blades of *Porphyra* species were cut into 1.0-cm squares and placed on a filter paper and filamentous *B. fuscopurpurea* were densely aligned on filter paper. After removal of excess fluid by an aspirator, expression plasmids pCaMV35S-PyGUS,^{5,6} pPyAct1-PyGUS,^{6,7,12} and pPtHSP70-PyGUS⁸ were introduced in algal pieces by the particle bombardment method under conditions shown in Table 1. Then, bombarded materials were cultured in ESL medium for 48 h and subjected to stain with 2 mM 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) solution as described previously.⁷

Figure 3. Comparison of the PyGUS enzymatic activity (left) and PyGUS-expressing cells (right) in Bangiophyceean algae. The PyGUS enzymatic activity was quantified by the fluorometric GUS assay as described previously⁷ and the number of PyGUS-expressing cells stained by X-Gluc per shot was counted under microscope. Values are the mean $\pm$ SD (n=3). Pt, *P. tenera*; Py, *P. yezoensis*; Po, *P. okamurae*; Pp, *P. pseudolinearis*; Bf, *B. fuscopurpurea*.

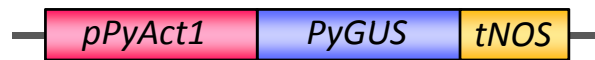
Table 1. Conditions of particle bombardment

Parameter	Value
Size of gold particles	0.6 μm
Amount of gold particles	150 μg /shot
Amount of plasmid DNA	5 μg /shot
Burst pressure of rupture disk	650 psi (for <i>Porphyra</i>), 1,100 psi (for <i>B. fuscopurpurea</i>)
Vacuum pressure	28 inches Hg
Target distance	3 cm

pCaMV35S-PyGUS



pPyAct1-PyGUS



pPtHSP70-PyGUS

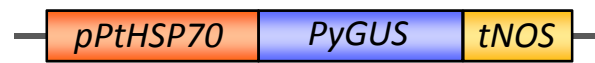


Figure 1

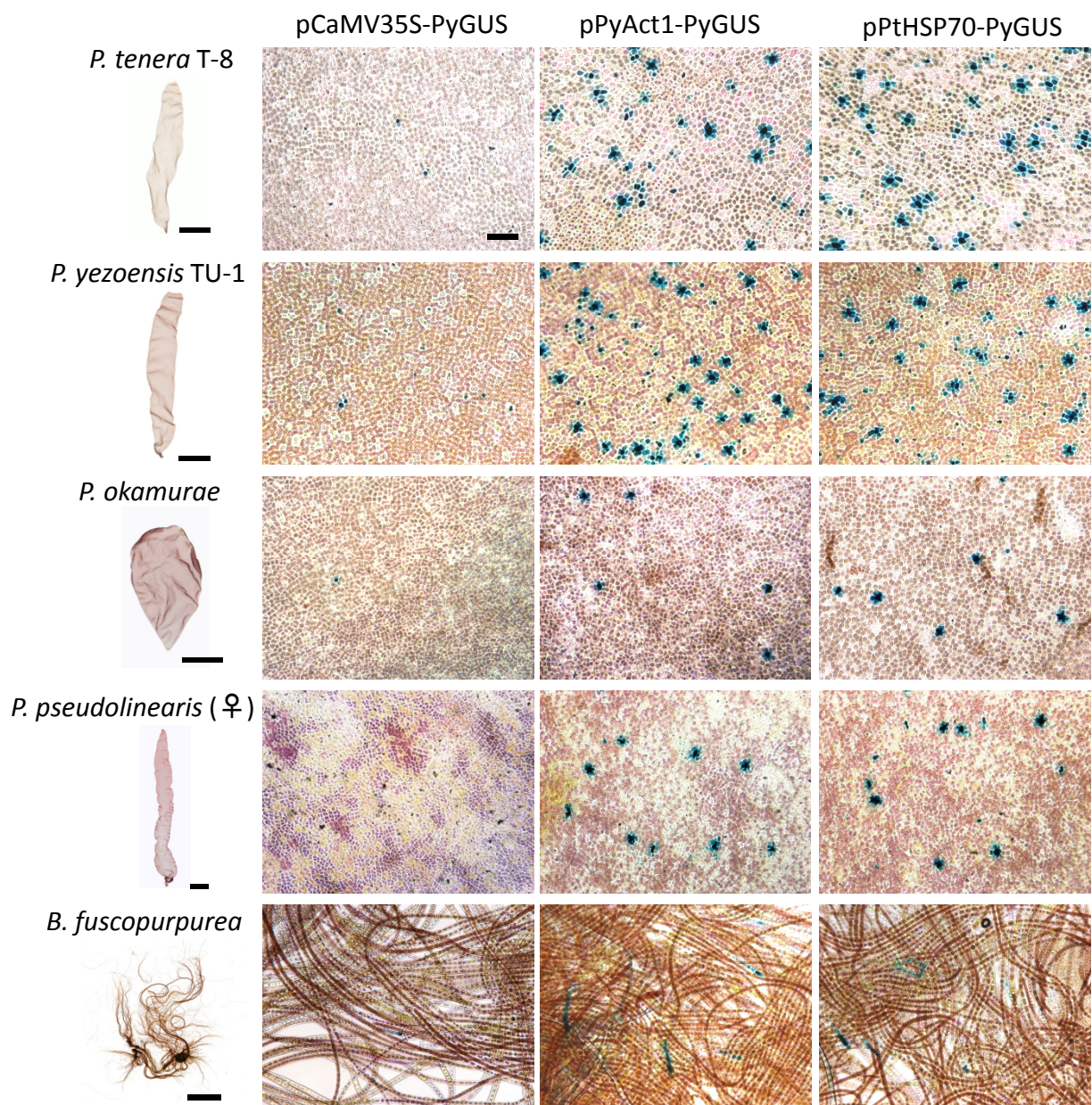


Figure 2

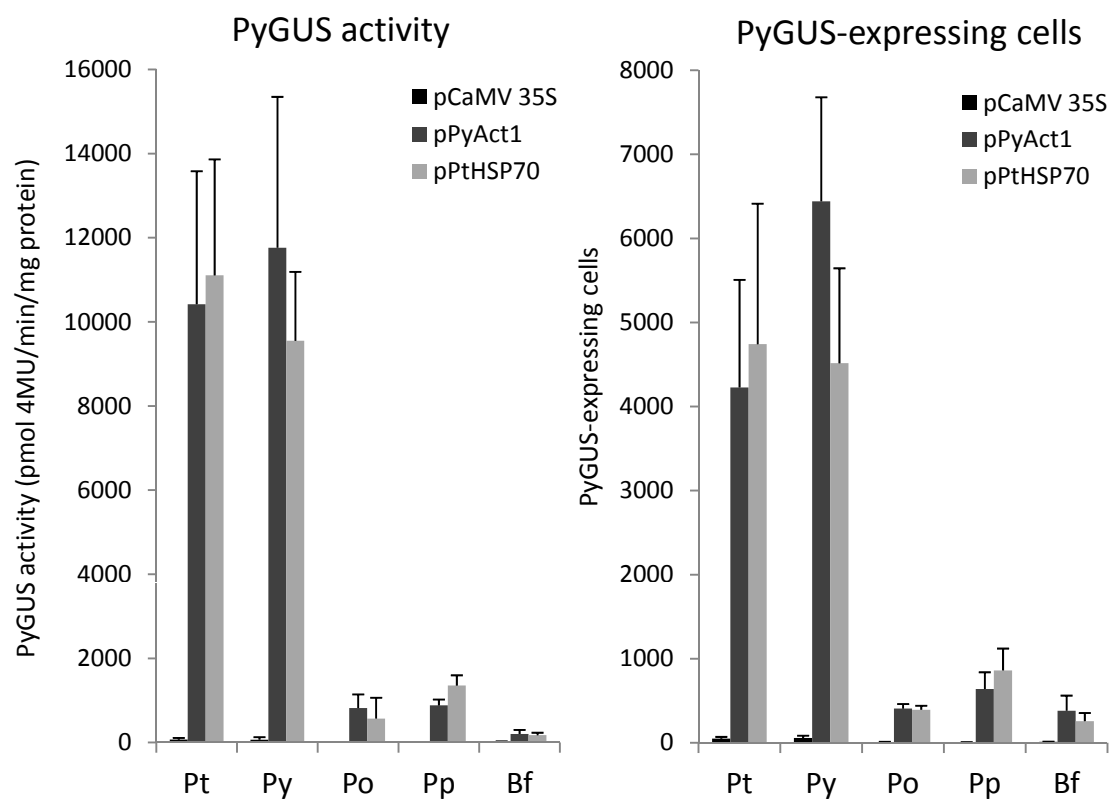


Figure 3