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# Efficient *gusA* Transient Expression in *Porphyra yezoensis* Protoplasts Mediated by Endogenous Beta-tubulin Flanking Sequences

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**Abstract** Endogenous tubulin promoter has been widely used for expressing foreign genes in green algae, but the efficiency and feasibility of endogenous tubulin promoter in the economically important *Porphyra yezoensis* (Rhodophyta) are unknown. In this study, the flanking sequences of beta-tubulin gene from *P. yezoensis* were amplified and two transient expression vectors were constructed to determine their transcription promoting feasibility for foreign gene *gusA*. The testing vector pATubGUS was constructed by inserting 5'- and 3'-flanking regions (*Tub5'* and *Tub3'*) up- and down-stream of β-glucuronidase (GUS) gene (*gusA*), respectively, into pA, a derivative of pCAT®3-enhancer vector. The control construct, pAGUSTub3, contains only *gusA* and *Tub3'*. These constructs were electroporated into *P. yezoensis* protoplasts and the GUS activities were quantitatively analyzed by spectrometry. The results demonstrated that *gusA* gene was efficiently expressed in *P. yezoensis* protoplasts under the regulation of 5'-flanking sequence of the beta-tubulin gene. More interestingly, the pATubGUS produced stronger GUS activity in *P. yezoensis* protoplasts when compared to the result from pBI221, in which the *gusA* gene was directed by a constitutive CaMV 35 S promoter. The data suggest that the integration of *P. yezoensis* protoplast and its endogenous beta-tubulin flanking sequences is a potential novel system for foreign gene expression.

**Key words** *Porphyra yezoensis*; transient expression; protoplasts; beta-tubulin; promoter; β-glucuronidase (GUS)

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## 1 Introduction

*Porphyra yezoensis* is one of the most widely consumed and cultivated seaweeds in the world. Over the past decades, much effort has been made in improving the cultured stocks and developing breeding techniques of alga. At present, nuclear transformation systems of eukaryotic algae such as green unicellular alga *Chlamydomonas reinhardii* (Rochaix and Dillewijn, 1982), diatom *Phseodactylum tricornutum* (Lioudmila *et al.*, 2000) and simple multicellular green alga *Volvox carteri* (Hallmann *et al.*, 1997) have been developed. Considerable progress has also been made in the genetic engineering of commercially important macroalgae. For example, hepatitis B surface antigen gene (HbsAg) was expressed in *Laminaria japonica* (Jiang *et al.*, 2002). However, techniques concerning efficient foreign gene expression in *P. yezoensis* are still limited (Mizukami *et al.*, 2004). Transient expressions of GUS gene were reported in *P. miniata* (Kubler *et al.*, 1994) and *P. yezoensis* (Kuang *et al.*, 1998) under the control of viral CaMV 35 S promoter. A portion of 18 S rDNA sequence from *P. yezoensis* was attempted to improve expression level (Liu *et al.*, 2003). Although 35 S promoter has a wide host range and is widely used in many plant cells (Odell *et al.*, 1985; Jefferson *et al.*, 1987), this virus-derived constitutive promoter is not always the best choice for gene expression due to its tissue specificity or possible toxicity of accumulated target gene product to host cells. Therefore, in order to establish a reliable gene expression system in *P. yezoensis*, it is important to investigate the usefulness of endogenous promoter sequences (Mizukami *et al.*, 2004). Recently, the promoter sequence of a chloroplast ribuose-bisphosphate-carboxylase/oxygenase (Rubisco) gene has been used for the transient expression in *P. yezoensis* protoplasts (Mizukami *et al.*, 2004).

Beta-tubulin genes are evolutionarily important and expressed abundantly in cells. As one of the cytoskeletal proteins, beta-tubulin is expressed steadily in *Porphyra* life cycle. In green alga such as *C. reinhardtii* and *V. carteri*, endogenous beta-tubulin promoters have been utilized for foreign gene expression (Davies *et al.*, 1992;

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Hallmann *et al.*, 1997). On the basis of our report on the flanking sequences of *P. yezoensis* beta-tubulin gene (Gong *et al.*, 2005), here we further investigate their potential as an efficient regulatory element for foreign gene expression in *P. yezoensis*.

## 2 Materials and Methods

### 2.1 Bacterial Strains and *P. yezoensis*

*E. coli* DH5α was used for transformation. *P. yezoensis* Qingdao-1 is a pure line established in the Genetic Laboratory, Ocean University of China (Liu *et al.*, 2004).

### 2.2 Construction of Transient Expression Vector

Genomic DNA of *P. yezoensis* was extracted according to the manual of NucleoSpin® Plant Kit (Clontech). *P. yezeonsis* beta-tubulin 5' flanking sequence (*Tub5'*) was amplified with the method previously described (Gong *et al.*, 2005) with primers Tub5f (5'CCTCGAGATTCCTCCCCTCTCAGC3') and Tub5r (5'GGAAGATCTGGTGGCAGGCAGCAGAGGA3'), containing *Xho* I and *Bgl* II sites (underlined). The PCR product was cloned into pBluescript KS II T-vector to construct the plasmid pBSTub5. The 3' flanking sequence (*Tub3'*) was amplified with primers Tub3f (5'CGAGCTCGGCGTCATCCGCCGCAATT3') and Tub3r (5'CGGTACCGAGACTACAAAACGTATG-3'), and then cloned into the *Sac* I and *Kpn* I sites of pBluescript KS II vector, resulting in plasmid pBSTub3. Vector pA was constructed by the self-ligation of the large fragment of pCAT®3-enhancer vector (Promega) digested with *Bam*H I and *Bgl* II (Fig.1). Gene *gusA* was obtained from the *Xba* I-*Sac* I fragment of vector pBI 221. *Tub5'-gusA-Tub3'* chimera was inserted into the *Xho* I and *Kpn* I site of pA, and the resultant plasmid pATubGUS (Fig.1) was confirmed by restriction enzyme digestion. The control plasmid pAGUSTub3 contained only *gusA-Tub3'* fragment (Fig.1).

### 2.3 Protoplast Isolation and Culturing

Freshly collected or previously stored (−20℃) thalli were used for isolation of protoplast of *P. yezoensis* (Liu *et al.*, 2003). Protoplast was cultured in darkness at 18℃ for 12 h before transformation. The culture medium was prepared with autoclaved seawater containing 10 ppm $NaNO_3$ and 1 ppm $KH_2PO_4$ (Dai *et al.*, 1993).

### 2.4 Protoplast Transformation

The plasmid for electroporation was purified with polyethylene glycol (Sambrook *et al.*, 2001). The protoplast of *P. yezoensis* was harvested by centrifugation (Liu *et al.*, 2003). The pellet was washed once with ice-cold electroporation buffer (30 mmol $L^{-1}$ Hepes, 5 mmol $L^{-1}$ $CaCl_2$, 427 mmol $L^{-1}$ mannite, and 150 mmol $L^{-1}$ NaCl), and then diluted to $5\times10^6$ cells $mL^{-1}$. Resuspended *Porphyra* protoplast was mixed with plasmid DNA (20 μg $mL^{-1}$) and transferred to a 0.4 cm cuvette for electroporation (Eppendorf multiporator). In the standard protocol, electric pulse voltage was set at 2 kV $cm^{-1}$. Once the electric pulse was delivered, the mixture was transferred to a culture dish containing 20 mL of culture medium and incubated in darkness at 20℃ for 24 h, 36 h and 48 h, respectively.

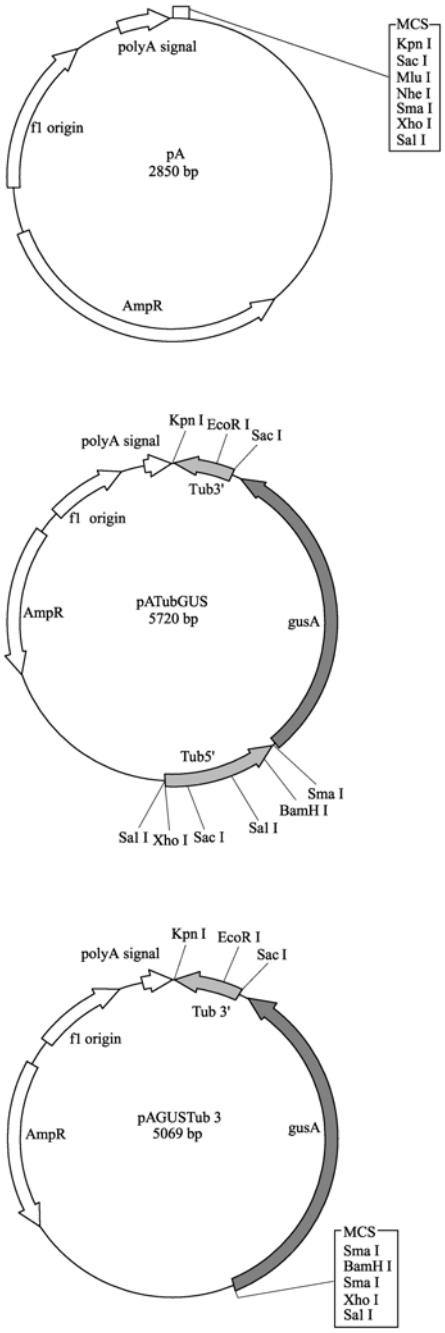


Fig.1 Maps of the testing vectors.

### 2.5 Quantitative Analysis of GUS Activity

Protoplasts transformed were harvested after incubation and washed once, and then resuspended in GUS extraction buffer (50 mmol $L^{-1}$ $Na_3PO_4$, pH7.0, 10 mmol $L^{-1}$ β-mercaptoethanol, 10 mmol $L^{-1}$ EDTA, 0.1% sodium dodecyl sarcosinate, 0.1% Triton X-100). After ultrasonic treatment of protoplasts, the solution was centrifuged at

15 000 g and 4℃ for 3 min with supernatant collected for GUS activity assaying. Protein concentrations of protoplast extracts were determined with the method of Bradford (Bradford, 1976) using micro-Bradford assay kit (Beyotime Institute of Biotechnology, Haimen, China). The β-glucuronidase activity in protoplast extracts was measured through the hydrolysis of *para*-nitrophenyl-β-D-glucuronide (pNPG). One unit of β-glucuronidase activity is defined as 1 pmol of *p*-nitrophenol (pNP) liberated per min per milligram of protein (Russell and Klaenhammer, 2001). One-way ANOVA was performed to determine the effects on GUS activities. Significant differences were determined by Newman-Keuls test. All statistical analyses were performed using the SPSS package program version 10.0.1. Differences were considered as being significant if $P<0.05$ level.

## 3 Results

### 3.1 Construction and Confirmation of Vector

The 678 bp *Tub5'* and 351 bp *Tub3'* fragments were obtained by PCR, according to 5'- and 3'- flanking sequence of *P. yezoensis* beta-tubulin gene, respectively (Fig.2). As shown in Fig.1, the vector pATubGUS was constructed by inserting the *Tub5'-gusA-Tub3'* fragment into the *Xho* I and *Kpn* I sites in the multiple cloning site region of pA, and the control plasmid pAGUSTub3 contained only *gusA-Tub3'* fragment. The recombinant plasmids pATubGUS and pAGUSTub3 were identified by restriction enzymes analyses (Fig.2).

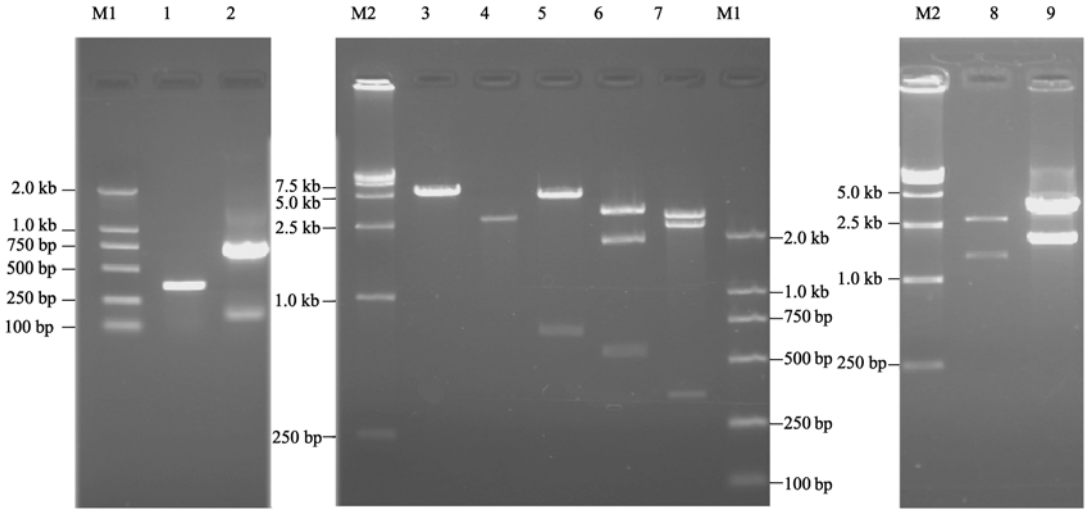


Fig.2 Amplification of beta-tubulin flanking sequence and confirmation of pATubGUS and pAGUSTub3 constructs by digestion of restriction enzymes. M1. DL-2 000 Marker (TaKaRa); M2. DL-15 000 Marker (TaKaRa); 1. Amplification of *Tub3'*; 2. Amplification of *Tub5'*; 3. pATubGUS/*Kpn* I; 4. pATubGUS/*Xho* I+*Kpn* I; 5. pATubGUS/*Xho* I+*Sma* I; 6. pATubGUS/*Sma* I+*Sac* I; 7. pATubGUS/*Sac* I+*Kpn* I; 8. pAGUSTub3/*Sma* I+*Sac* I; 9. pAGUSTub3/*Sac* I+*Kpn* I. *Tub5'-gusA-Tub3'* (2.9 kb), *Tub5'* (678 bp), *gusA* (1.8 kb) and *Tub3'* (351 bp) could be obtained from pATubGUS by digestion of restriction enzymes respectively (lanes 4, 5, 6, 7). And *gusA* (1.8 kb) and *Tub3'* (351 bp) could be obtained from pAGUSTub3 by digestion of restriction enzymes respectively (lanes 8 and 9).

### 3.2 Transient Expression of GUS

Pulse length during electroporation affected GUS expression significantly. In our experiment, three pulse lengths (0.2, 0.3 and 0.4 ms) were initially selected. After incubation for 36 h, GUS activities were determined by the spectrophotometric evaluation. The data demonstrated that the GUS activities were the highest in the 0.3 ms group (Fig.3), and the lowest in the 0.2 ms group. Therefore pulse length was optimized at 0.3 ms for all the later assays.

We also observed that incubation time after transformation influenced the GUS activity (Fig.4). The GUS activity in 24 h incubation group was the highest compared to the incubation times of 36 h or 48 h, indicating the expression and turnover pattern of GUS protein in this system. At shorter incubation times (*e.g.* 12 h), GUS activity was much lower than that at 24 h (data not shown), which was probably associated with the lower expression of GUS protein.

After 24 h incubation, protoplasts of *P. yezoensis* electroporated with construct pATubGUS, a substantial GUS activity of about 12.42 pmol pNP $min^{-1}$ (mg protein)$^{-1}$, was observed, which was significantly different from the transformations with control plasmid pAGUSTub3 (2.29 pmol pNP $min^{-1}$ (mg protein)$^{-1}$) (Fig.5). The activity observed in the control might be the reflection of leaky background GUS activity, which is consistent with the observations in other reports (Rosfjord *et al.*, 1994). The mock protoplasts only showed a minor background noise. Interestingly, the protoplasts transformed with pATubGUS showed stronger GUS activity ($P<0.05$) than that with pBI221 (9.94 pmol pNP $min^{-1}$ (mg protein)$^{-1}$) (Fig.5), a *gusA* expression vector mediated by constitutive CaMV 35S promoter, indicating a potential advantage using Tub flanking sequences in *P. yezoensis*.

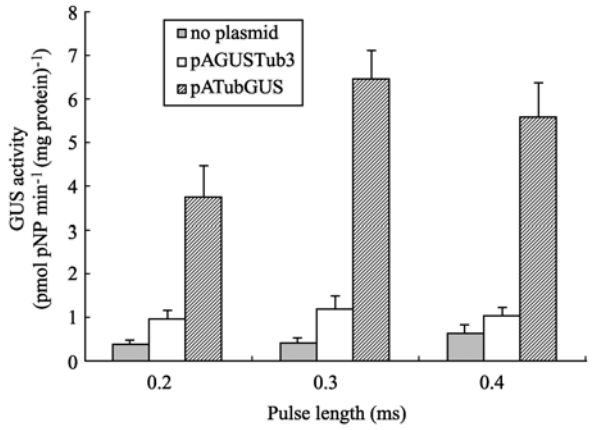


Fig.3 Effect of pulse length on the GUS activity in *P. yezoensis* protoplasts after 36 h's incubation. Each value presented is the average of results from six independent experiments.

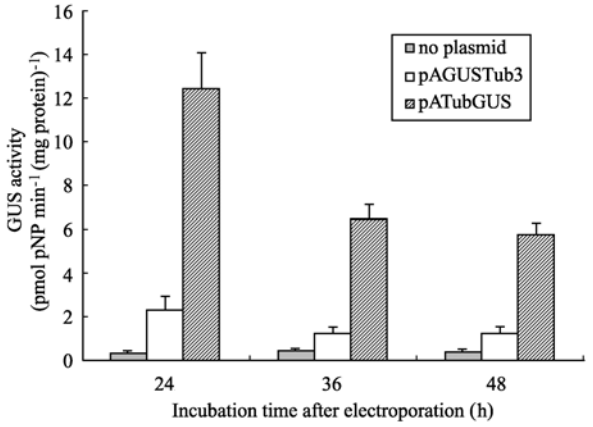


Fig.4 Effect of incubation time on the GUS activety in *P. yezoensis* protoplasts electroporated using 30 ms pulse length. Each value presented is the average of results from six independent experiments.

## 4 Discussion

Some electroporation parameters that affect the transformation efficiency of *P. yezoensis* protoplasts were examined in our lab (Liu *et al.*, 2003). In this study, the pulse voltage was optimized to be 2 kV cm$^{-1}$ for all assays. The effect of incubation time after electroporation on GUS transient expression was found to be interesting. GUS activities were detected in *Porphyra* protoplasts after 48 h incubation (Mizukami *et al.*, 2004; Liu *et al.*, 2003). In contrast, using electroporating method in this experiment, the highest GUS activities were detected at 24 h after transformation, and slightly decreased from 36 h to 48 h. Further experiments would be required to clarify whether the decrease of GUS activity is at transcriptional or posttranscriptional level.

In transient expression vector pATubGUS, 337 bp long *Tub3'* was fused downstream of *gusA* gene. *Tub3'* flanking region contained a putative polyA signal like motif (AAGAAA) (Gong *et al.*, 2005), so efficient termination function could be expected. However, it is not known if this 3'-flanking sequence also contains other regulatory elements for the proper or stable expression of the beta-tubulin gene of *P. yezoensis*.

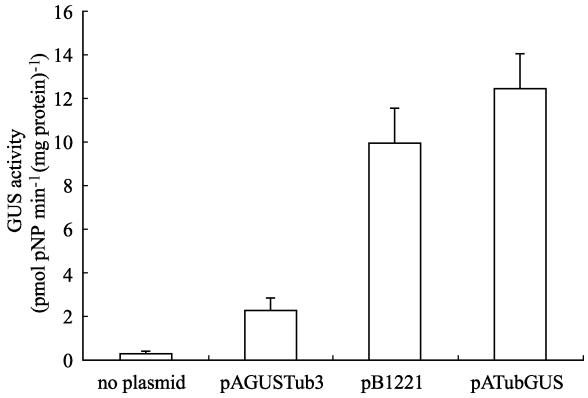


Fig.5 Quantitative analysis of GUS activity in *P. yezoensis* protoplasts electroporated with different plasmids. Each value presented is the average of results from six independent experiments.

In the testing vector pATubGUS, 663 bp long upstream sequence of *P. yezoensis* beta-tubulin was incorporated. One of the obvious features of this sequence was that the GC content (66.42%) was much higher than that of other commonly used promoters in high plants and other algae. However, typical promoter cis-elements such as TATA box and CAAT box were not found in this region (Gong *et al.*, 2005). It seems that the 5' flanking region of *P. yezoensis* beta-tubulin was not a normal promoter, but the transient expression assay demonstrated that *gusA* gene expression was drastic and was even higher than that of CaMV 35 S promoter. The results not only indicate that *Porphyra* might have unique transcriptional regulation mechanism that is different from other organisms, but also suggest that the 5' upstream sequence of beta-tubulin of *P. yezoensis* could function as promoter for foreign gene expression, and might be better suited for the genetic manipulation in *Porphyra.*

## Acknowledgements

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