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Development of a transient gene expression system in the red macroalga, *Porphyra tenera*

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Abstract

Porphyra is a commercially valuable source of food and drugs, and represents an important model organism for algal research. However, genetic research on *P. tenera* has been limited due to lack of a heterologous gene expression system. In the present study, we isolated a native promoter, the *PtHSP70* promoter, for efficient expression of foreign genes in this organism. This promoter lies approximately 1 kb upstream of the coding sequence for Heat Shock Protein 70 (*HSP70*) and was isolated using adapter-mediated genomic PCR. Promoter activity was evaluated using the synthetic GUS gene (PyGUS) with optimized codons for *Porphyra yezoensis*. Interestingly, the *PtHSP70* promoter allowed equivalent expression of PyGUS in both *P. tenera* and *P. yezoensis*, whereas the *GAPDH* promoter from *P. yezoensis* was not fully functional in *P. tenera*. These data suggest that the *PtHSP70* promoter has a more conserved regulatory mechanism than the *PyGAPDH* promoter between these species. We also established an efficient transient transformation system for *P. tenera* by evaluating various transformation parameters such as quantity of gold particles, pressure of helium and vacuum, developmental stages of leafy gametophytes, and target distance. Under the optimal conditions of transient transformation, the frequency of GUS expression was determined by histochemical staining to be 30-50 cells per bombardment. Therefore, the new transient transformation system using the *PtHSP70* promoter can be used for foreign gene expression in *P. tenera*, which may advance the development of *P. tenera* as a model organism.

Introduction

Porphyra, a genus of marine red macroalgae, is considered a commercially valuable source for foods, fertilizers, medicines, and chemicals (Harada et al. 1997; Oohusa 1993; Yoshizawa et al. 1995). More than 130 species of *Porphyra* have been reported worldwide (Zhang et al. 2005) and several species, such as *P. yezoensis*, *P. tenera*, *P. seriata*, and *P. dentate* have been cultivated in East Asia (Oohusa 1993). These species are considered model seaweeds for marine biotechnology (Fukuda et al. 2008; Oohusa 1993) because of their biological and economical importance, as well as small genome size. The haploid genomes of *Porphyra* are estimated to be approximately 260-500 Mbp (Kapaun et al. 1991; Le Gall et al. 1991; Matsuyama-Serisawa et al. 2007). However, heterologous gene expression to manipulate molecular pathways has been hindered by the difficulty of genetic transformation in *Porphyra* (Fukuda et al. 2008).

Transient expression of GUS under control of the heterologous SV40 or CaMV 35S promoter has been reported in *Porphyra* and *Gracilaria* (Gan et al. 2003; Kübler et al. 1994; Kuang et al. 1998); however, the CaMV 35S promoter offered no substantial expression of GUS in *P. yezoensis* (Fukuda et al. 2008). Similarly, all attempts using the CaMV 35S promoter to express foreign genes in the green alga, *Chlamydomonas reinhardtii*, had failed (Blankenship and Kindle 1992). Possible reasons for the failure to express foreign genes using heterologous promoters in these algae may be due to the lack of the necessary regulatory elements for these promoters or poor codon-usage of the transgene. These problems were overcome by using both native promoters and codon optimization of the transgene in *Chlamydomonas* (Ahn et al. 2010; Franklin et al. 2002; Kozminski et al. 1993; Shao and Bock 2008). Furthermore, a recent study has reported that both codon modification and use of a native promoter enabled expression of foreign genes in *P. yezoensis* (Fukuda et al. 2008; Takahashi et al. 2010);

synthetic GUS (PyGUS) was successfully expressed using the native promoters of both the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin genes in *P. yezoensis*.

Because *Porphyra* is an important species in marine biotechnology as a model organism, it is important to develop gene manipulation systems for it. A recent study indicated success using the *PyGAPDH* promoter for foreign gene expression in *P. yezoensis* (Fukuda et al. 2008). However, it is possible that differences in regulatory elements for gene expression might exist between *Porphyra* species and identification of specific and universal promoters will be required for development of a foreign gene expression system in *Porphyra*. In the present study, we isolated the *PtHSP70* promoter from *P. tenera* and confirmed its ability to express foreign genes in *P. tenera* and *P. yezoensis*. Our results suggest that the *PtHSP70* promoter is effective for heterologous gene expression and that an optimized system for transient transformation may help promote molecular biological studies in *P. tenera*.

Materials and Methods

Culture conditions of *P. tenera* and *P. yezoensis*

Leafy gametophytes of *P. tenera* (strain TJH-1R, Seaweed Research Center, NFRDI, Korea) grown to 2–3 mm in length were cultured further in 1 L of enriched sea life (ESL) medium at 12°C under a 10-h light/14-h dark cycle. The cultivation of leafy gametophytes of *P. yezoensis* strain TU-1 was also performed as described by Fukuda et al (2008). These cultures were continuously aerated with filter-sterilized air and renewed weekly.

Isolation of the *PtHSP70* promoter from *P. tenera*

Genomic DNA was extracted from *P. tenera* using a DNeasy plant mini kit (Qiagen, USA) and polysaccharide contamination was removed using the CTAB method (Ausubel et al. 1994). Adapter ligation-mediated PCR (O'Malley et al. 2007) was adopted to amplify the promoter region of *PtHSP70*. Genomic DNA was digested by *Xho*I and then ligated with *Xho*I-adapter primer that had 5'-phosphorylation and 3'-amino C7. PCR was performed with adapter-ligated genomic DNA, 1.5 units of LA Taq DNA polymerase (Takara, Japan), 100 μ M of each dNTP, 5 pmol of gene-specific primers (PtHSP70p R2 and R3), and adapter-specific primers (AP1 and AP2) using a T1 thermal cycler (Biometra, Germany). The gene-specific primers were designed using a consensus sequence from *P. seriata* and *P. yezoensis* ESTs. Amplification was performed with 35 cycles at 94°C for 30 s, 62°C for 1 min, and 72°C for 4 min. Primers used for isolation of the *PtHSP70* promoter are listed in Table 1. For isolation of the 5'-promoter region of *PtHSP70*, genomic DNA was digested using *Bam*HI and adapter ligation, and a second PCR amplification was performed using promoter-specific primers (PtHSP70p R4 and R5) and adapter-specific primers (Table 1). Finally, the *PtHSP70* promoter (1030-bp) was amplified by PCR with the Pt-HSP70-P-1kF and PtHSP70p R3 primers designed from the combined sequences of two clones obtained from the first and second PCRs. The nucleotide sequence of *PtHSP70* promoter was shown in Fig. 1.

Plasmid construction for transient PyGUS expression

The vector backbone used in the present study was obtained from the GAPDH-PyGUS construct (Fukuda et al. 2008). The *PyGAPDH* promoter was replaced with the *PtHSP70* promoter and three constructs were generated to evaluate expression levels of PyGUS. Plasmid PtHSP70-PyGUS1 (Fig. 2) contained the *PtHSP70* promoter upstream of *PyGUS*. For PtHSP70-PyGUS2, a 3'-modification of the *PtHSP70* promoter was performed by inserting a 5'-coding sequence (18-bp) of *PtHSP70*. The 5'-coding sequence (21-bp) of

PyGAPDH was inserted 3' of the *PtHSP70* promoter to construct PtHSP70-PyGUS3. Restriction enzyme sites used for the three constructs are displayed in Fig. 2. The following primers were used for generating these constructs: PtHSP70-GUS1, 5'-AAG GAT CCC ATC GTC GGG TGC ACA-3' and 5'-AAG GAT CCC ATC GTC GGG TGC ACA-3'; PtHSP70-GUS2, 5'-TTG GAT CCG CTC ACT GCA GAC GCC AT-3' and 5'-TTG GAT CCG CTC ACT GCA GAC GCC AT-3'; PtHSP70- GUS3, 5'-AGA CGC CAT GGT CGG GTG CAC A-3' and 5'-AGA CGC CAT GGT CGG GTG CAC A-3'.

Transient gene expression using particle bombardment

The expression constructs recovered from *E. coli* cells using a Plasmid® midi kit (Qiagen, USA). Transient transformation was performed by particle bombardment using 60 mg gold particles (0.6 µm diameter). Particles were washed with 1 ml of 70% ethanol by vortexing, rinsed three times with sterile water, and then resuspended in a 50% glycerol solution. Different amounts of gold particles (25, 50, 75, 100, 250, and 500 µg) coated with 20 µg plasmid were used to evaluate efficiency of transient transformation, according to the method of Fukuda et al (2008). Leafy gametophyte samples on microfiber filter (25-mm diameter, GF/B; Whatman, Germany) were placed in 10 cm Petri dishes filled with ESL 0.6% agar medium. Particle bombardment was performed using a particle delivery system (PDS, 1000/He; BioRad, USA) under various pressure conditions (900, 1000, and 1300 psi of helium, 28 in Hg of vacuum). Different target distances (3, 6, and 9 cm) were also tested. After bombardment, samples were incubated in liquid ELS media at 12°C for 2 days in the dark. Transient transformation of *P. yezoensis* was performed as previously described (Fukuda et al. 2008), other than the particle bombardment. DNA transfer was carried out using a PDS 1000/He under these conditions: 900 psi of helium, 28 in Hg of vacuum, 3 cm of target distance, and 150 µg gold particles/shot.

Histochemical and fluorometric GUS assays

GUS histochemical staining using 5-bromo-4-chloro-3-indolylglucuronide (X-gluc; Sigma, USA) was carried out according to Fukuda et al (2008). Quantitative fluorometric assays for GUS activity were performed on gametophytes 48h after bombardment according to Jefferson, et al. (1987), using p-nitrophenyl glucuronide (PNPG; Sigma, USA) for *P. tenera* and 4-methylumbelliferyl-D-glucuronide (4-MU; Calbiochem, Germany) for *P. yezoensis*. For *P. yezoensis*, GUS values were expressed as pmoles of 4-MU per minute per milligram protein. The fluorescence was measured with a spectrofluorometer (Picofluor; Turner Designs, USA). Protein concentrations were determined by the method of Bradford (1976). Because GUS activity in transiently transformed *P. tenera* was too low to measure fluorometrically, it was evaluated as the differences in OD values.

Results

Isolation of the *PtHSP70* promoter and evaluation of its activity

We isolated the putative promoter region of *PtHSP70* using adapter-mediated genomic PCR (Fig. 1) and then generated three constructs to evaluate promoter activity (Fig. 2a). These constructs allowed substantial expression of PyGUS in *P. tenera* (Fig. 2b), indicating that the *PtHSP70* promoter directs PyGUS expression.

Optimization of transformation conditions for transient PyGUS expression

To develop optimal conditions for transient transformation of *Porphyra*, we tested various parameters for particle bombardment such as target distance, developmental stage of thallus, and amount of gold particle using the *PtHSP70*-PyGUS1 construct. All bombardments were

performed under 900 psi of helium and 28 in Hg of vacuum pressure; optimizations of the vacuum and helium pressure were also carried out (data not shown). To optimize distance of sample to rupture disk, 3, 6, or 9 cm target distances were tested and the highest efficiency of transient transformation was detected at the target distance of 3 cm (Fig. 3a). In higher plants, transformation was affected by developmental stage. Therefore, different developmental stages of thallus (1-3 cm in length) were subjected to the bombardment test and higher levels of transient transformation were obtained in younger thallus (Fig. 3b). Various amounts of gold particles (50 to 500 µg) were also used to check efficiency of transient transformation, and 250 µg of gold particles (Fig. 3c) resulted in the largest number of GUS-expressing cells.

Efficiency of PyGUS expression under the control of *PtHSP70* promoter

The efficiency of *PyGUS* expression by each *PtHSP70* construct was determined under conditions of optimal bombardment (1 cm of thallus, 3 cm target distance, and 250 µg of gold particles). High levels of GUS-expression were detected in the samples bombarded with *PtHSP70*-*PyGUS*1 and *PtHSP70*-*PyGUS*2 (Fig. 4a), suggesting that the 1-kb upstream region of the *PtHSP70* gene benefits transcription of a foreign gene in *P. tenera*. However, the *PtHSP70*-*PyGUS*3 construct resulted in low *PyGUS* expression relative to the other *PtHSP70*-GUS constructs (Fig. 4a), indicating that the 3'-modification of the *PtHSP70* promoter with the seven amino acids of *PyGAPDH* protein was not effective. The *PyGAPDH* promoter derived from *P. yezoensis* allowed both gene expression of *PyGUS* and GUS protein activity in *P. tenera*, but both were lower than obtained with the *PtHSP70* promoter (Figs. 4a and 4b, respectively).

Activity of *PtHSP70* promoter in heterologous host *P. yezoensis*

In a separate experiment, PtHSP70-PyGUS1 was transformed into the heterologous host, *P. yezoensis*, to determine its activity. Substantial expression of PyGUS driven by the *PtHSP70* promoter was detected (Fig. 5a) and the promoter showed almost the same efficiency of PyGUS expression as with the *PyGAPDH* promoter in *P. yezoensis* (Fig. 5b). GUS protein activity was measured with fluorometric GUS assays. Both samples transformed with PtHSP70-PyGUS1 and PyGAPDH-PyGUS displayed almost the same GUS activity in *P. yezoensis* (Fig. 5c). However, different efficiencies of transient transformation were detected in *P. tenera* and *P. yezoensis* (Figs. 4a and 5b); the number of PyGUS-expressing cells in *P. yezoensis* was approximately 20-fold higher than that in *P. tenera*.

Discussion

Development of an efficient system of transient transformation for *Porphyra* is worthwhile because of its importance as a model organism. *Porphyra* has an important advantage for genetic engineering; it develops from single monospore to whole plant, and many monospores are released from the leafy (gametophytic) thalli. This suggests that the tissue culture and regeneration systems required for genetic engineering in higher plants may not be necessary in *Porphyra*. In general, various promoters, including species specific and universal promoters, are essential for foreign gene expression. For example, in the monocot plant rice, the native ubiquitin promoter is more efficient than the heterologous 35S promoter, widely used in eudicots (Wang and Oard 2003). The regulatory region of the HSP70 gene is an excellent candidate for development of a promoter in *Porphyra*. *HSP70* promoters, which provide both constitutive and stress-inducible expression, are widely utilized to express heterologous and homologous genes in eukaryotic organisms (Medford et al. 1989; Schroda et al. 2000). In the green alga, *Chlamydomonas*, the *HSP70* promoter allows efficient

transgene expression due to its constitutive expression and suppression of transgene silencing by sequence elements in the promoter (Schroda et al. 2002).

In the present study, we isolated the *PtHSP70* promoter (Fig. 1) and demonstrated that this promoter can drive PyGUS expression in *P. tenera* (Fig. 2b). Optimal conditions for transient transformation of *P. tenera* by particle bombardment were also established using *PtHSP70*-PyGUS vectors (Fig. 3). Transient PyGUS expression driven by the *PtHSP70* promoter was confirmed using histochemical staining and enzyme assays in *P. tenera* (Figs. 2b and 4), and suggested that the *PtHSP70* promoter has a regulatory function in gene expression.

The *PtHSP70* promoter allowed relatively high expression of PyGUS in *P. tenera* compared to the *PyGAPDH* promoter (Fig. 4), indicating that the *PtHSP70* promoter has useful regulatory elements for expression of foreign genes in *P. tenera*. We also determined the efficiency of the *PtHSP70* promoter in *P. yezoensis*; the *PtHSP70* promoter derived from *P. tenera* allowed the same amount of expression of PyGUS in this host as the *PyGAPDH* promoter in *P. tenera* (Fig. 5). These results suggest that the regulatory components of the *PtHSP70* promoter may be conserved among these species and that the *PtHSP70* promoter may be applied as a universal promoter to other *Porphyra* species. Previous studies have reported that HSP70 proteins are highly conserved and that its promoter can be active in heterologous hosts (Seok et al. 2006; Spena et al. 1985; Whitley et al. 1999). Thus, these previous studies support our results showing the ability of the *PtHSP70* promoter to drive expression similarly in both *P. tenera* and *P. yezoensis*. We detected different efficiencies of transient transformation between *P. tenera* and *P. yezoensis* (Fig. 4a and 5b), possibly due to differences in cell wall strength between these species. Such differences might exist naturally

and/or be caused by long-term laboratory gametophyte culture via repeated asexual propagation from monospore to gametophytes.

In conclusion, the *PtHSP70* promoter may be used for constitutive expression of a foreign gene and as a universal promoter in the genus, *Porphyra*. The optimization of a transient transformation system and the practical application of the *PtHSP70* promoter may contribute to development of *P. tenera* as a model organism for algal research.

Figure legends

Fig. 1 Nucleotide sequence of the *PtHSP70* promoter. Underlining indicates primers used for isolation of the promoter.

Fig. 2 Schematic diagrams of plasmid constructs and confirmation of expression. **(a)** *PyGAPDH* (pink) and *PtHSP70* promoters (red) were fused to PyGUS. Modifications of 3'-*PtHSP70* promoter (blue) were performed for PtHSP70-PyGUS2 and PtHSP70-PyGUS3. PyGUS, synthetic GUS; NosT, the nopaline synthase terminator. **(b)** Histochemical staining to determine GUS expression in *P. tenera*.

Fig. 3 Optimization of transient transformation conditions by particle bombardment in *P. tenera*. **(a)** Efficiency of transient transformation between various target distances. **(b)** Comparison of transient transformation rate under different developmental stages of the leafy gametophyte *P. tenera*. **(c)** Efficiency of transient transformation by different amounts of gold particles. **(a-c)** Data are expressed as mean $\pm$ SD ($n=9$).

Fig. 4 Expression efficiency of PyGUS directed by the *PtHSP70* promoter in *P. tenera*. **(a)** Efficiency of PyGUS expression between *PtHSP70*-PyGUS constructs. **(b)** Measurement of GUS activity in *P. tenera*. **(a, b)** Data are expressed as mean $\pm$ SD ($n=9$).

Fig. 5 Expression of PyGUS driven by the *PtHSP70* promoter in the heterologous host, *P. yezoensis*. **(a)** Histochemical staining to detect PyGUS expression in *P. yezoensis*. **(b)** Comparison of the expression efficiency between the *PtHSP70* and *PyGAPDH* promoters in *P. yezoensis*. **(c)** Measurement of GUS activity in *P. yezoensis*. **(b, c)** Data are expressed as mean $\pm$ SD ($n=3$).

Acknowledgments

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Figure 2

a

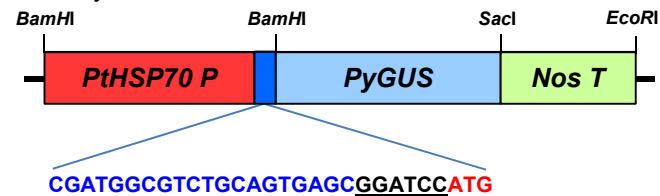
PyGAPDH-PyGUS



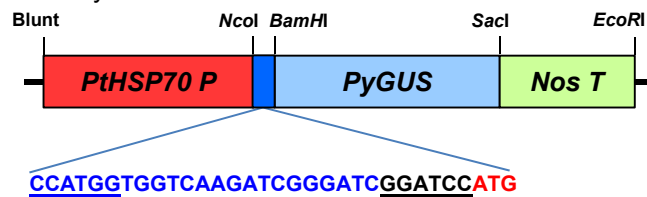
PtHSP70-PyGUS1



PtHSP70-PyGUS2

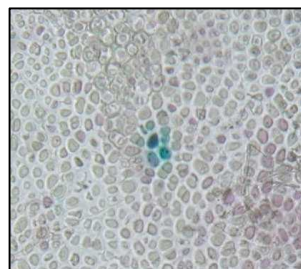


PtHSP70-PyGUS3

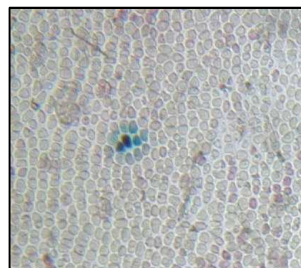
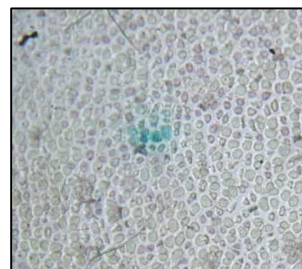


b

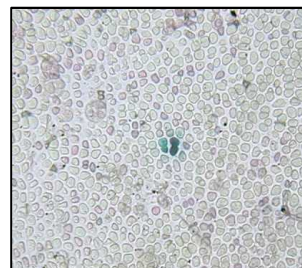
PyGAPDH-PyGUS



PtHSP70-PyGUS1



PtHSP70-PyGUS2



PtHSP70-PyGUS3

Figure 3

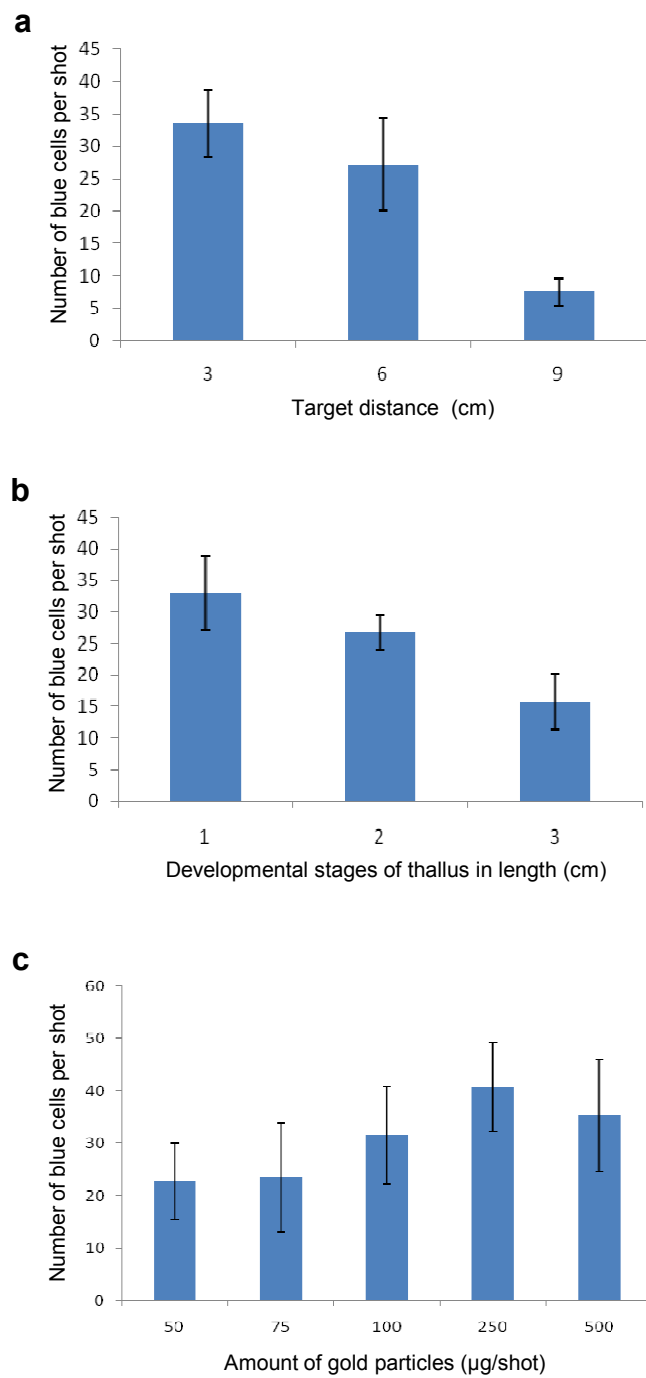
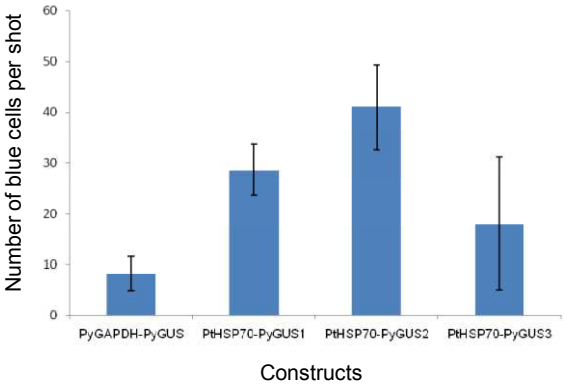


Figure 4

a



b

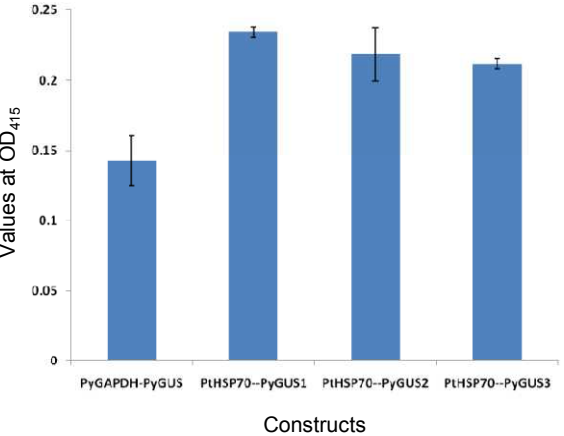
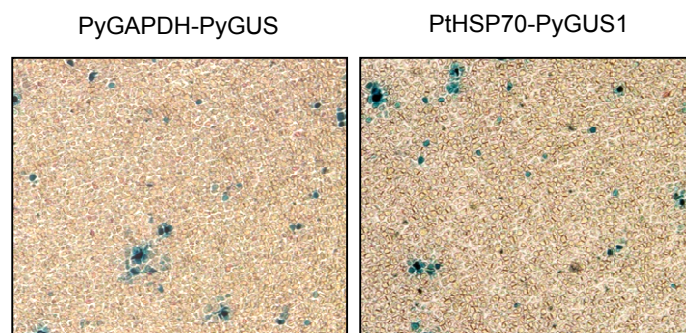
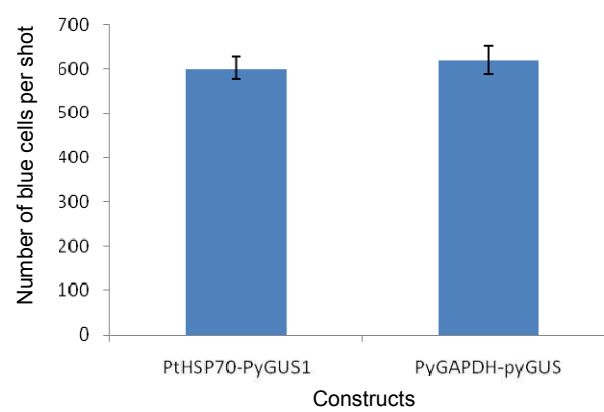


Figure 5

a



b



c

