Factors influencing efficiency of transient gene expression in the red macrophyte *Porphyra yezoensis*

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Keywords: *Porphyra yezoensis*, Codon usage, β-Glucuronidase (EC 3.2.1.21), Particle bombardment, Transient gene expression.

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The marine red alga *Porphyra yezoensis* has been proposed as a model plant for physiological and genetic studies in seaweeds because of its biological and economical importance. However, the progress of molecular biological studies using gene transfection and genetic transformation systems has been hindered by difficulties in the expression of foreign genes in *P. yezoensis* cells. To overcome this situation, we developed a transient gene expression system to monitor gene expression in *P. yezoensis* cells. An artificial β-glucuronidase (GUS) coding region was synthesized to adapt it to the codon usage of *P. yezoensis* (PyGUS) and then evaluated for efficiency as a reporter of transient gene expression by particle bombardment. We also demonstrated the importance of using the promoter of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from *P. yezoensis* for efficient expression of PyGUS, because the cauliflower mosaic virus (CaMV) 35S promoter, which has been successfully used for monitoring gene expression in nuclei and chloroplasts of higher plants, was less active in *P. yezoensis* cells. Therefore, the lack of knowledge about differences in the regulatory machinery of gene expression between *P. yezoensis* and terrestrial plants seems to be why experimental systems for monitoring gene expression were previously not developed in *P. yezoensis*. Establishment of the transient gene expression system in *P. yezoensis* could facilitate biotechnological developments in this organism.

1. Introduction

Both Rhodophyta (red alga) and Chlorophyta (green alga) are thought to have been established by the primary endosymbiosis of an ancient cyanobacterium into a certain host cell [1,2]. However, red and green algae differentially adapted to survive mainly in the sea and in fresh water, and ancient Charophyceae evolved from the latter is thought to have emerged from a water habitat to aerated land [3,4]. Thus, there are characteristic differences between Rhodophyta and Chlorophyta living today. For example, the pigments for light harvesting in Rhodophyta are phycoerythrin, phycocyanin and allophycocyanin in phycobilisomes as in cyanobacteria [5,6], although Chlorophyta lack phycobilisomes. Since phycoerythrin absorbs blue light, which penetrates water to a greater depth than other wavelengths, Rhodophyta tend to live at greater depths of water than Chlorophyta. Therefore, characterization of responses to light quality and temperature of sea water could lead to new insight into physiological regulation in red algae.

Red algae like *Porphyra yezoensis* (*susabinori*), *P. tenera* (*asakusanori*) and *P. haitanensis* are important sources of food (*nori*), especially in Asia [7]. In addition, Rhodophyta is used for medical purposes. The most typical example is porphyran, a sulfated polysaccharide from the cell wall of *P. yezoensis* and dried *nori*, whose physiological functions in humans are antitumor activity, antihypertensive and antihyperlipidemic activities, and macrophage stimulating activity [8-10]. Despite such economical and medical importance, our understanding of the molecular bases of development and physiological regulation in Rhodophyta is quite poor, which prevents progress in breeding of seaweeds.

We have developed a laboratory culture system and freeze-stock method for *P. yezoensis* [11], which was recently recognized as a model seaweed for fundamental and applied studies of marine plants [12,13]. Based on the large-scale culture of *P. yezoensis* in the laboratory, collection and analyses of expressed sequence tags (ESTs) have also been performed [14,15], which enabled us to isolate genes of interest. However, since experimental systems for analyzing the regulation and function of genes have not yet been established in *P. yezoensis* or in other seaweeds, functional analysis of genes from multicellular algae is not yet possible.

Considerable progress has been made in the genetic engineering of unicellular eukaryotic algae. Green fluorescent protein (GFP) reporter and transformation systems of nuclear and chloroplast genomes have already been established in *Chlamydomonas reinhardtii* [16-18]. Nuclear transformation system was also developed for *Volvox carteri* [19] and the diatom *Phaeodactylum tricornutum* [20]. This progress in unicellular algae led us to try establishment of gene expression and disruption systems in seaweeds. In fact, transient expression of SV40 promoter-GUS and SV40 promoter-LacZ constructs in Rhodophyta and Phaeophyceae and of a cauliflower mosaic virus (CaMV) 35S promoter-GUS construct in *P. miniata* and *P. yezoensis* has been reported [21-24]. However, there have been problems repeating these experiments in other laboratories. Thus, reproducible gene expression systems in multicellular algae need to be established.

In the present study, we report the establishment of a transient gene expression system in *P. yezoensis* by microparticle bombardment. Our results indicated that optimization of codon usage in the coding sequence and appropriate selection of promoters are important for reproducible and efficient expression of the reporter gene.

2. Materials and Methods

2.1. Cultivation of P. yezoensis

Leafy gametophytes of *P. yezoensis* (strain TU-1) [11] grown to 2-3 mm long were further cultured for 2 weeks in 1.8 L of enriched sea life (ESL) medium [25], to which 15 polyvinyl alcohol (PVA) monofilaments (ca. 4 cm long; Kinoshita Fishing Net Mfg. Co. Ltd, Japan) had been added, at 15^{o}C with a cycle of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance for 10 h and darkness for 14 h. The ESL medium was continuously bubbled with filter-sterilized air and renewed weekly. After release from gametophytes, the monospores attached to the PVA monofilaments were moved to a new culture vessel and grown for 6 or 7 weeks under the same conditions. Gametophytes of ca. 1.5 cm wide were used for particle bombardment. The rest of gametophytes on the PVA monofilaments were cultured again for another 3 weeks to grow them to ca. 2-3 mm for monospore release.

2.2. Synthesis of artificial GUS coding region and construction of p35S-PyGUS

To optimize the GUS coding region to the codon usage of *P. yezoensis*, the overlap extension PCR method [26] was employed to synthesize an artificial GUS coding sequence, designated PyGUS (Fig. 1), using the 30 oligonucleotide primers

listed in Table 1. Combination of primers and step-by-step PCR were done according to Rouwendal et al. [26] with *Pyrobest* DNA polymerase (TaKaRa). In a series of PCR reactions, unreacted oligomers were removed by a Microcon-100 (TaKaRa). Since primers 1F and 15R contain *Bam*HI and *Sac*I sites, respectively (Table 1), the GUS coding sequence in pBI221 was replaced with PyGUS after digestion of pBI221 and the PCR fragment with *Bam*HI and *Sac*I, which resulted in the production of p35S-PyGUS.

2.3. Isolation of the GAPDH *promoter*

Genomic DNA of *P. yezoensis* was extracted using a DNeasy plant mini kit (Qiagen) and digested separately with *Apa*LI, *Ngo*MIV and *Sal*I; the corresponding restriction sites are located in the 5' untranslated region of a *P. yezoensis* cDNA encoding GAPDH (GenBank accession no. AY273820). After cleanup with the QIAquick gel extraction kit (Qiagen) and self-ligation of digested genomic DNA, the 5' upstream region of the *GAPDH* gene was amplified by an inverse PCR (IPCR) method [27] using primers GAPDH-IPCR-F1 and GAPDH-IPCR-R1 (Table 2 and Fig. 2). The amplification protocol comprised an initial denaturation at 94^{o}C for 2 min followed by 35 cycles of 94^{o}C for 30 sec, 62^{o}C for 1 min and 72^{o}C for 4 min, and final extension at 72^{o}C for 15 min with *LA Taq* polymerase (TaKaRa). The IPCR products of 1.5 kb, 3.3 kb and 2.5 kb from *Apa*LI-, *Ngo*MIV- and *Sal*I-digested DNA were cloned into pT7Blue (Invitrogen) and sequenced, which confirmed that the 2.8 kb and 2.3 kb fragments contain the *GAPDH* promoter region. Since the IPCR fragment does not contain a translation start codon, primers GAPDH-PCR-F1 and GAPDH-PCR-R1 (Table 2 and Fig. 2) were used for PCR, carried out with an initial incubation at 94^{o}C for 1 min

followed by 30 cycles of 98°C for 10 sec and 68°C for 4 min, and final incubation at 72°C for 15 min with *LA Taq*. The amplified PCR fragment was cloned into pT7Blue and confirmed by sequencing. The nucleotide sequence of the isolated promoter region has been submitted to the GenBank under accession no. AB303420.

2.4. Plasmid construction with the GAPDH *promoter*

The *CaMV 35S* promoter (835 bp) was replaced with the 5' upstream region of the *GAPDH* gene (2.3 kb) to construct pGAPDH-GUS and pGAPDH-PyGUS (Fig. 3A). The *P. yezoensis GAPDH* upstream region was amplified again using primers HindIII-GAPDH-F1 and BamHI-GAPDH-R1 (Table 2) with the following program: 98°C for 10 sec followed by 35 cycles of 94°C for 10 sec, 60°C for 30 sec and 72°C for 4 min, and final incubation at 72°C for 7 min with *Ex Taq* polymerase (TaKaRa). Since the 5' upstream region of the *GAPDH* gene contains an internal *Bam*HI site, the PCR fragment was divided into two parts by digestion with *Xba*I, which recognizes a site that is in the first intron (see Fig. 2). To replace the *CaMV 35S* promoter with the 5'upstream region of the *GAPDH* gene, the *Xba*I-*Bam*HI fragment was inserted into *Xba*I-*Bam*HI-digested p35S-GUS (pBI221) and p35S-PyGUS and the resulting plasmids were digested with *Hind*III and *Xba*I. The *Hin*dIII-*Xba*I fragment from the amplified DNA was then cloned into the digested plasmids. Although the final constructs contain a GAPDH coding sequence corresponding to the first 7 amino acids (Fig. 2), in-frame fusion occurred between the small GAPDH and reporter coding sequences.

2.5. Particle bombardment

After alkaline lysis, the bacteria transformed with plasmids used for transient expression analysis (p35S-GUS, p35S-PyGUS, pGAPDH-GUS and pGAPDH-PyGUS) were precipitated with isopropanol and purified 3 times by CsCl-density gradient centrifugation or directly by QIAquick spin columns (Qiagen). Gold particles (5, 10, 15, 20 and 60 mg, all 0.6 μm diameter, Bio-Rad) were suspended in 1 ml of 70% ethanol, mixed by vortexing for 5 min and soaked in the same solution for 15 min at room temperature. After centrifugation at 800 *g* for 5 sec, the supernatant was discarded. The particles were then washed 3 times by soaking in 1 ml of sterile water, vortexed for 1 min and centrifuged at 800 *g* for 2 sec. After the supernatant was discarded, the particles were suspended in 1 ml of 50% sterile glycerol solution and then divided into 25 μl each of aliquots. To coat the particles with plasmids, an aliquot containing gold particles was sequentially mixed with 25 μl of DNA solution (1, 5, 10 or 20 μg) with 20 sec vortexing, 50 μl of 2.5 M $CaCl_2$ with 1 min vortexing and 10 μl of 0.1 M spermidine with 2 min vortexing. The supernatant was removed by centrifugation at 800 *g* for 2 sec. The resulting pellet was washed with 140 μl of 70% ethanol, washed with 140 μl of pure ethanol and resuspended in 48 μl of pure ethanol.

Leafy gametophytes of *P. yezoensis* were put on 2.5 cm glass microfiber filters (GF/B; Whatman), cut into discs of 1 cm diameter (approximately 3.5 x 10^5 cells) by a cork borer, and placed in the center of 9 cm Petri dishes. DNA transfer was carried out using the IDERA particle acceleration device, type GTE-III (Tanaka Inc., Japan) according to the manufacturer's instructions. Using 6 μl of coated particles on the

bombarding cartridge of the machine, particle bombardment was carried out under various conditions including 0.2, 0.4 or 0.6 MPa of helium pressure, 400, 500, 600 or 700 mmHg of vacuum pressure and 3, 6 or 9 cm from cartridge to materials as indicated in the text and Table 3. When scaly leaves of onion bulb were used, the epidermis was cut into 2 x 2 cm squares and put onto glass filters. These materials were bombarded with 0.6 MPa of helium pressure, 600 mmHg of vacuum pressure and a 7-cm target distance on 1/2 Murashige-Skoog (MS)-0.5% agar plates. After bombardment, plant materials were replaced on ESL medium plates for *P. yezoensis* or 1/2 MS medium plates for onion epidermis and incubated at 15^{o}C for 48 h in the dark prior to GUS histochemical assay.

2.6. GUS histochemical assay

Staining of bombarded materials with 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) was performed essentially according to the method of Jefferson et al. [28]. After bombardment, materials were incubated with a staining solution, which consisted of 2 mM X-gluc, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.5% Triton X-100, 1.5 M sorbitol and 50 mM sodium phosphate, pH 7.0, at 37^{o}C for 12 h before observation with a microscope.

3. Results

3.1. Expression of genes for 35S-GUS and 35S-PycGUS in P. yezoensis *gametophytes*

Since Kuang et al. [22] reported the availability of the *CaMV 35S* promoter for the expression of foreign genes in *P. yezoensis*, we first tested the expression of a 35S-GUS transcriptional fusion gene (Fig. 3A) in gametophytes of laboratory cultured *P. yezoensis*. The conditions for particle bombardment were 600 mmHg of vacuum pressure, 62.5 μg of particles/shot and 3.3 μg of DNA/shot with various helium pressures and target distances (see Table 3). When p 35S-GUS was bombarded, no GUS expression was observed (Table 3 and Fig. 3B). Thus, we were not able to confirm the results of Kuang et al. [22].

Optimization of codon preference of jellyfish GFP to reflect that of *Chlamydomonas*, yeast, humans and tobacco was successful in increasing efficiency of GFP expression [17,18,26,29,30]. Since codons of the GUS coding sequence from *E. coli* are A/T biased, it was possible that artificial synthesis of a modified GUS utilizing the codon preference of *P. yezoensis* would improve the expression efficiency. Indeed, sequencing data from *P. yezoensis* ESTs [14,15] indicate a bias for G and C in the third position of codons for the protein coding sequences (see http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Porphyra+yezoensis+[gbpln]). Thus, we synthesized the coding sequence of GUS to adapt it to the codon usage of *P. yezoensis* according to the method described by Rouwendal et al. [26], using the oligonucleotides shown in Table 1, in which an A or T nucleotide at the third position of codons was replaced with the more favorable nucleotides, G or C. The GC content of the resulting coding sequence was increased from 51.8% to 66.6% (Fig. 1).

An artificial GUS coding sequence, designated PyGUS, was used for making the expression construct p35S-PyGUS (Fig. 3A). Following bombardment with p35S-PyGUS, significant but low GUS expression was observed, especially under

conditions of 0.6 MPa of helium pressure and a 6 cm target distance (Table 3 and Fig. 3C). Thus, optimization of codon preference is one of the important factors for efficient GUS expression in *P. yezoensis* gametophyte cells, although the activity of the *CaMV 35S* promoter was very weak in *P. yezoensis* cells.

3.2. Expression of genes for GAPDH-GUS and GAPDH-PyGUS in P. yezoensis *gametophytes*

Our preliminary data from DNA microarray experiments indicated that expression of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which catalyzes the sixth step of glycolysis and also participates in several nonmetabolic processes such as activation of transcription, initiation of apoptosis and vesicle shuttling from ER to Golgi [31-34], was very strong in gametophyte cells (unpublished results). Since the strength of the promoter may also influence the expression level of a reporter gene, the *CaMV 35S* promoter of p35S-GUS and p35S-PyGUS was replaced by the promoter of the *GAPDH* gene to produce the expression constructs pGAPDH-GUS and pGAPDH-PyGUS (Fig. 3A).

Table 3 and Fig. 3D and E show the highly expression of pGAPDH-PyGUS and lack of expression of pGAPDH-GUS. For pGAPDH-PyGUS, the number of blue cells did not change significantly under conditions of 0.4 and 0.6 MPa helium pressure and 3 and 6 cm target distance, although no expression was observed under conditions of 0.2 MPa and 9 cm. These results demonstrated the importance of promoter strength in the expression of the GUS reporter in addition to adaptation of codon usage.

Since differences in the promoter and codon usage do not influence the activity

of GUS enzymatic activity itself, we proposed that the number of GUS-expressing cells reflects the efficiency of the reporter gene-expression in *P. yezoensis* cells under the constant experimental conditions.

3.3. Determination of optimum bombardment conditions

We next examined the effects of vacuum pressure, amount of particles and plasmid DNA on the efficiency of expression of pGAPDH-PyGUS. Vacuum pressure was the most important factor, since the expression efficiency for 700 mmHg was 3 times higher than that for 600 mmHg (Fig. 4A). Figure 4B and C show that the optimum amounts of particles and plasmids were 62.5 μg/shot and 3.3 μg/shot, respectively, when the helium pressure was 0.6 MPa. There was no difference in the expression efficiency when plasmids were prepared by CsCl density gradient centrifugation or using QIAquick spin columns (Qiagen) (Fig. 4C). The optimal conditions for the transient expression of pGAPDH-PycGUS were 700 mmHg of vacuum pressure, 0.6 MPa of helium pressure, 62.5 μg of particles/shot, 3.3 μg of plasmids/shot and 6 cm of target distance for leafy gametophytes of *P. yezoensis*.

3.4. Expression of the codon-adapted GUS reporter in land plant cells

We further addressed whether the PyGUS with the coding sequence adapted to the codon usage of *P. yezoensis* is translatable in cells of green terrestrial plants. Scaly leaves of onion bulb were employed to examine the expression of four fusion genes (Fig. 3A). Both 35S-GUS and 35S-PyGUS were expressed efficiently in onion

epidermis cells (Fig. 5), although no expression was observed for GAPDH-GUS or GAPDH-PyGUS (data not shown).

4. Discussion

The primary goal of our study was to establish a transient gene expression system in the marine red alga *P. yezoensis*. The situation about the expression of foreign genes and genetic transformation in marine multicellular algae is currently very confusing, because previous reports about usability of SV40-LacZ and CaMV 35S-GUS fusion genes for the transient expression in multicellular algal cells [21-24] could not be successfully confirmed in our (Fig. 3B) and other laboratories. To clarify why earlier results were not reproducible, we engineered the coding region of GUS to fit the codon usage of protein coding sequences from *P. yezoensis* ESTs [14,15] and compared the expression efficiency of the native and synthetic GUS coding regions under the control of the *CaMV 35S* and *GAPDH* promoters. The results demonstrated the importance of optimization of codon usage by increasing the GC content in the GUS coding sequence in combination with use of the strong *GAPDH* promoter for the increase in the efficient expression of the GUS reporter gene in *P. yezoensis* cells (Figs. 3 and 4 and Table 3). PyGUS is the first synthetic reporter that confers efficient expression in marine multicellular algae. We believe that the system described here will provide a new trustworthy tool for studying the functions of genes in *P. yezoensis* as a model marine plant.

The *CaMV 35S* promoter was not used by *P. yezoensis* cells (Fig. 3B and Table 3). The *CaMV 35S* promoter is well utilized for gene expression in green algal cells

[35-37], *E. coli* [38] and terrestrial plants. Although core promoters of protein coding genes from terrestrial plants and green algae usually have the TATA box in common, the TATA box and other known motifs were not found in the *GAPDH* promoter (Fig. 2) and other promoters of *P. yezoensis* genes (unpublished results). These findings suggest that the regulatory mechanisms of gene expression in red algae might be different from those in green algae and land plants.

Overall, our study demonstrated the utility of the codon-optimized PyGUS as a reporter conferring efficient expression in *P. yezoensis*. This also suggests that the adaptation of codon usage in other reporter sequences could enhance their expression efficiency. Indeed, our preliminary experiments showed that when the coding sequence of GFP was optimized to the codon usage in *P. yezoensis*, the level of fluorescence signal increased (data not shown). Using codon-optimized GUS and GFP reporters, we plan to analyze the machinery regulating gene expression and subcellular localization of proteins in the response to cues for development and stress responses in *P. yezoensis*.

Acknowledgements

We thank Miho Sasaki for technical assistance in algal cultivation.

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Figure legends

Fig. 1. Comparison of the native and synthetic GUS coding regions. The differences between native and synthetic GUS, PyGUS, are noted below the native GUS sequence by letters, while unchanged nucleotides are indicated by dots. The optimized codons were refined according to the codon usage indicated in http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Porphyra+yezoensis+[gbpln]. The amino acid sequence is shown below the nucleotide sequence by the single letter code. The stop codon is indicated by an asterisk. The nucleotide and amino acid numbers are indicated on both the left- and right-hand sides.

Fig. 2. Nucleotide sequence of the 5' upstream region of the *P. yezoensis GAPDH* gene. The nucleotide sequence of an *Ngo*MIV fragment amplified by IPCR is shown, for which the nucleotide position is related to the first nucleotide of the initiation codon (boxed) as +1. Bold uppercase letters indicate the 5'-upstream and coding regions and lowercase letters indicate intron sequences. Positions of the *Apa*LI, *Bam*HI, *Ngo*MIV, *Sal*I and *Xba*I sites that were used in IPCR and construction of expression plasmids are underlined. PCR primers for amplification of the 5' flanking region and construction of expression plasmids are underlined with the names for the sequences shown in Table 2.

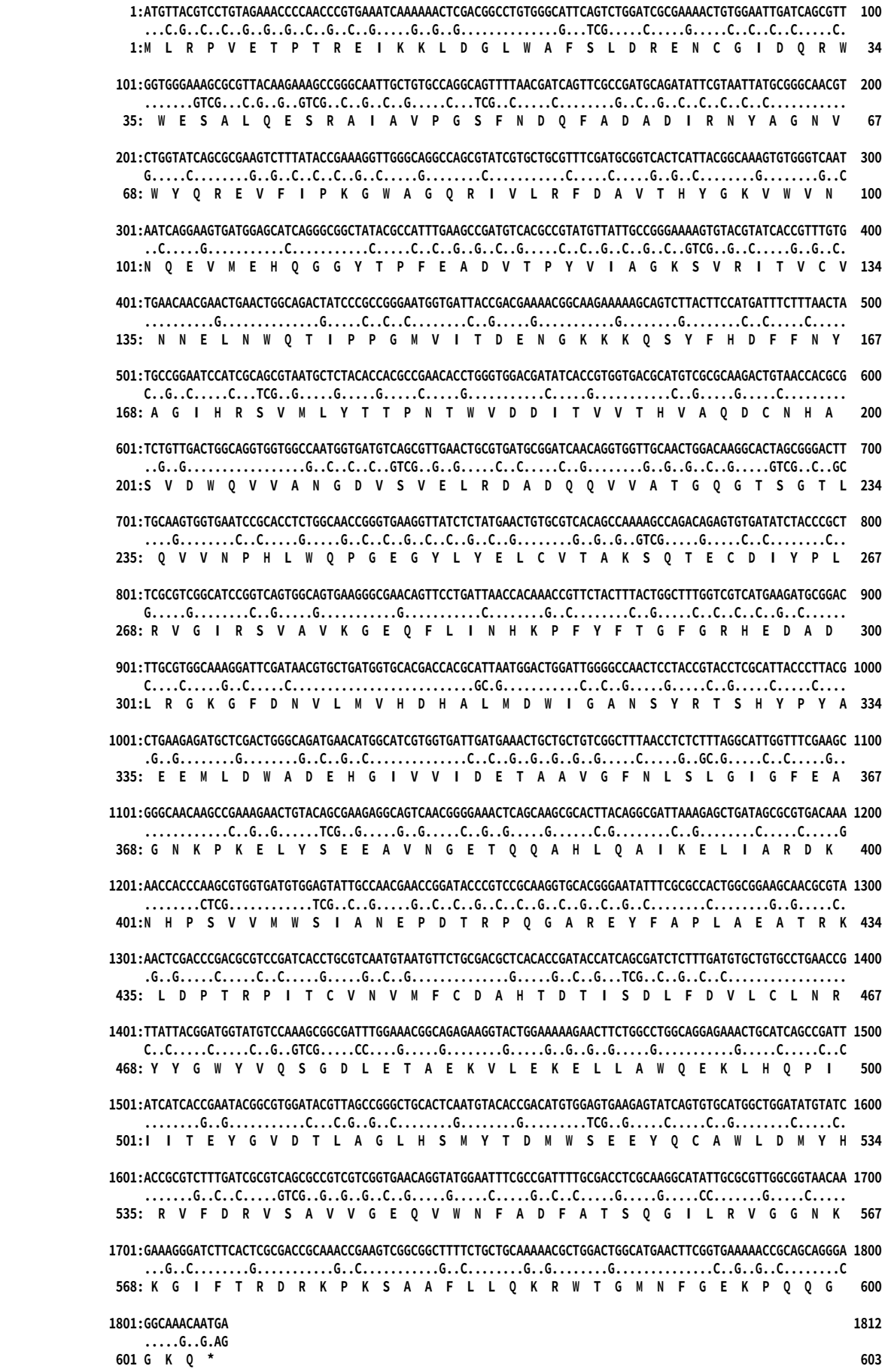
Fig. 3. Expression of PyGUS under the direction of the *GAPDH* promoter in gametophyte cells of *P. yezoensis*. (A) Schematic representation of the structure of the fusion genes used in particle bombardment experiments. The *CaMV 35S* (green) and *GAPDH* (yellow) promoters were fused to native and synthetic GUS coding sequences (light blue and blue), which resulted in the construction of 35S-PyGUS, GAPDH-GUS and GAPDH-PyGUS (35S-GUS was already available in pBI221). The *CaMV 35S* and *GAPDH* promoters are 835 bp and 2.2 kb, respectively. NOSt, the *nos* gene terminator (253 bp). (B-E) GUS expression analysis of 35S-GUS (B), 35S-PyGUS (C), GAPDH-GUS (D) and GAPDH-PyGUS (E) in gametophyte cells of *P. yezoensis* following particle bombardment. Only GAPDH-PyGUS was expressed efficiently (E). The bombardment conditions were 700 mmHg of vacuum pressure, 62.5 μg of gold particles/shot, 3.3 μg of plasmids/shot, 6 cm target distance and 0.4 MPa of helium pressure. Typical GUS staining images were taken from three independent experiments (Table 1). Scale bars = 100 μm.

Fig. 4. Effects of changing experimental conditions on the expression of GAPDH-PyGUS fusion genes used for particle bombardment. Basic conditions were 700 mmHg of vacuum pressure, 62.5 μg of gold particles/shot, 3.3 μg of plasmids/shot, 6 cm target distance and 0.4 or 0.6 MPa of helium pressure. The effects of factors such as strength of vacuum pressure (A), amount of gold particles (B) and quantity of plasmid (C) on the efficiency of expression of the 35S-PyGUS fusion gene was examined in thalli of *P. yezoensis*. In (C), the expression efficiency using plasmids purified with QIAquick spin columns (3.3C) and linearized with *Hin*dIII (3.3CL) was also compared. The data shown are the mean values ± SD from three independent

experiments.

Fig. 5. Expression of GUS and PyGUS under the direction of the *CaMV 35S* promoter in onion epidermal cells. All fusion genes, 35S-GUS, 35S-PyGUS, GAPDH-GUS and GAPDH-PyGUS (Fig. 3A), were introduced into the cells by particle bombardment. Efficient expression of the 35S-GUS (A) and 35S-PyGUS (B) was observed, although GAPDH-GUS and GAPDH-PyGUS were not expressed (data not shown). The bombardment conditions were 600 mmHg of vacuum pressure, 62.5 μg of gold particles/shot, 3.3 μg of plasmids/shot, 8 cm target distance and 0.4 MPa of helium pressure. Typical GUS staining images were taken from three independent experiments. Scale bars = 500 μm.

Figure 1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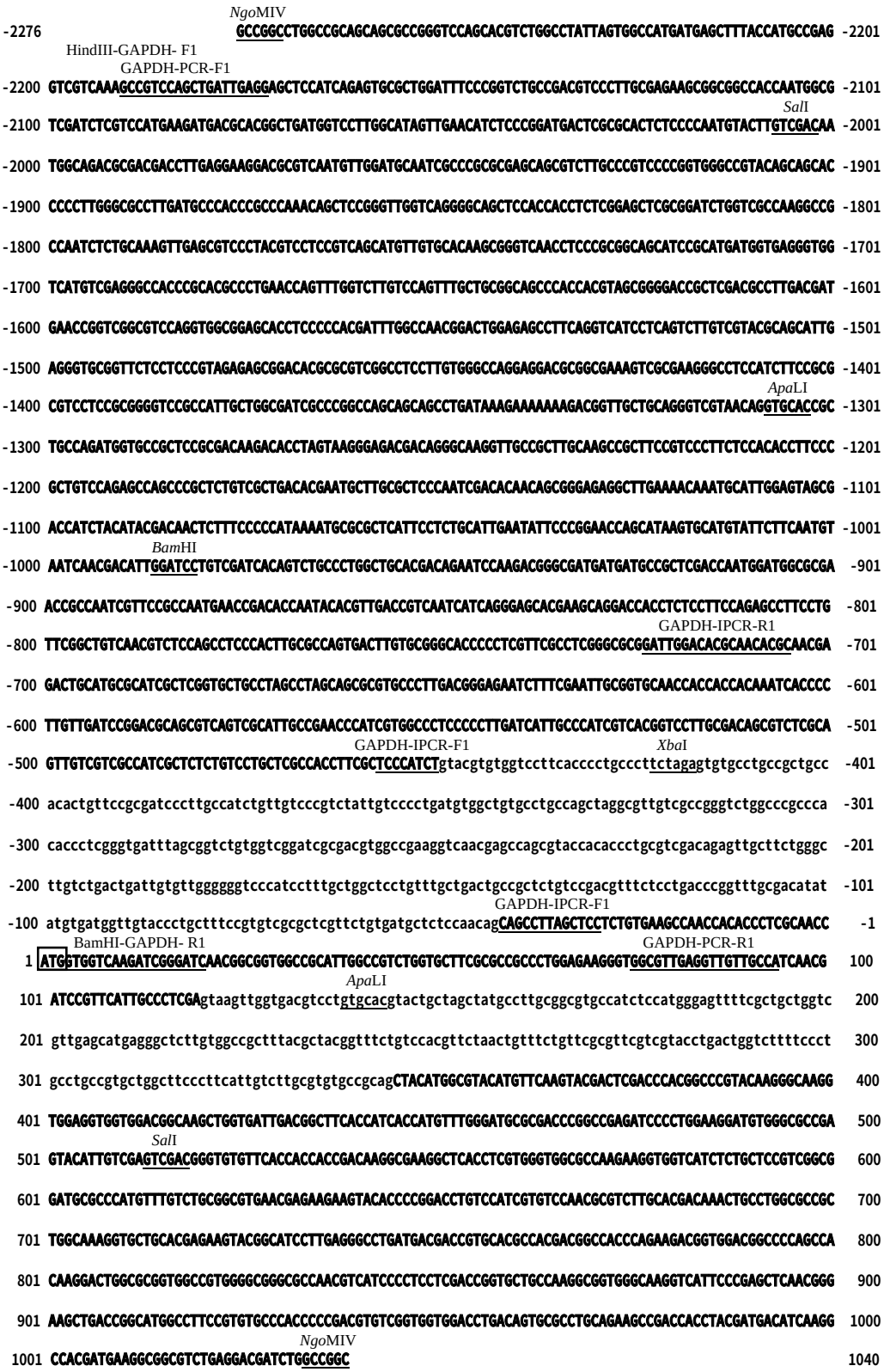
35: W E S A L Q E S R A I A V P G S F N D Q F A D A D I R N Y A G N V 67
201:CTGGTATCAGCGCGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAAT 300
68: W Y Q R E V F I P K G W A G Q R I V L R F D A V T H Y G K V W V N 100
301:AATCAGGAAGTGATGGAGCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTG 400
101:N Q E V M E H Q G G Y T P F E A D V T P Y V I A G K S V R I T V C V 134
401:TGAACAACGAACTGAACTGGCAGACTATCCCGCCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAACTA 500
135: N N E L N W Q T I P P G M V I T D E N G K K K Q S Y F H D F F N Y 167
501:TGCCGGAATCCATCGCAGCGTAATGCTCTACACCACGCCGAACACCTGGGTGGACGATATCACCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCG 600
168: A G I H R S V M L Y T T P N T W V D D I T V V T H V A Q D C N H A 200
601:TCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCAACTGGACAAGGCACTAGCGGGACTT 700
201:S V D W Q V V A N G D V S V E L R D A D Q Q V V A T G Q G T S G T L 234
701:TGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCTATGAACTGTGCGTCACAGCCAAAAGCCAGACAGAGTGTGATATCTACCCGCT 800
235: Q V V N P H L W Q P G E G Y L Y E L C V T A K S Q T E C D I Y P L 267
801:TCGCGTCGGCATCCGGTCAGTGGCAGTGAAGGGCGAACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATGAAGATGCGGAC 900
268: R V G I R S V A V K G E Q F L I N H K P F Y F T G F G R H E D A D 300
901:TTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGCATTACCCTTACG 1000
301:L R G K G F D N V L M V H D H A L M D W I G A N S Y R T S H Y P Y A 334
1001:CTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTGGTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTTTCGAAGC 1100
335: E E M L D W A D E H G I V V I D E T A A V G F N L S L G I G F E A 367
1101:GGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAACGGGGAAACTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAA 1200
368: G N K P K E L Y S E E A V N G E T Q Q A H L Q A I K E L I A R D K 400
1201:AACCACCCAAGCGTGGTGATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGGTGCACGGGAATATTTCGCGCCACTGGCGGAAGCAACGCGTA 1300
401:N H P S V V M W S I A N E P D T R P Q G A R E Y F A P L A E A T R K 434
1301:AACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAACCG 1400
435: L D P T R P I T C V N V M F C D A H T D T I S D L F D V L C L N R 467
1401:TTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATT 1500
468: Y Y G W Y V Q S G D L E T A E K V L E K E L L A W Q E K L H Q P I 500
1501:ATCATCACCGAATACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATGTATC 1600
501:I I T E Y G V D T L A G L H S M Y T D M W S E E Y Q C A W L D M Y H 534
1601:ACCGCGTCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAA 1700
535: R V F D R V S A V V G E Q V W N F A D F A T S Q G I L R V G G N K 567
1701:GAAAGGGATCTTCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTGCAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGA 1800
568: K G I F T R D R K P K S A A F L L Q K R W T G M N F G E K P Q Q G 600
1801:GGCAAACAATGA 1812
601 G K Q * 603

Figure 2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HindIII-GAPDH- F1
GAPDH-PCR-F1
SalI
ApaLI
BamHI
GAPDH-IPCR-R1
GAPDH-IPCR-F1
XbaI
GAPDH-IPCR-F1
BamHI-GAPDH- R1
GAPDH-PCR-R1
ApaLI
SalI
NgoMIV

Figure 3

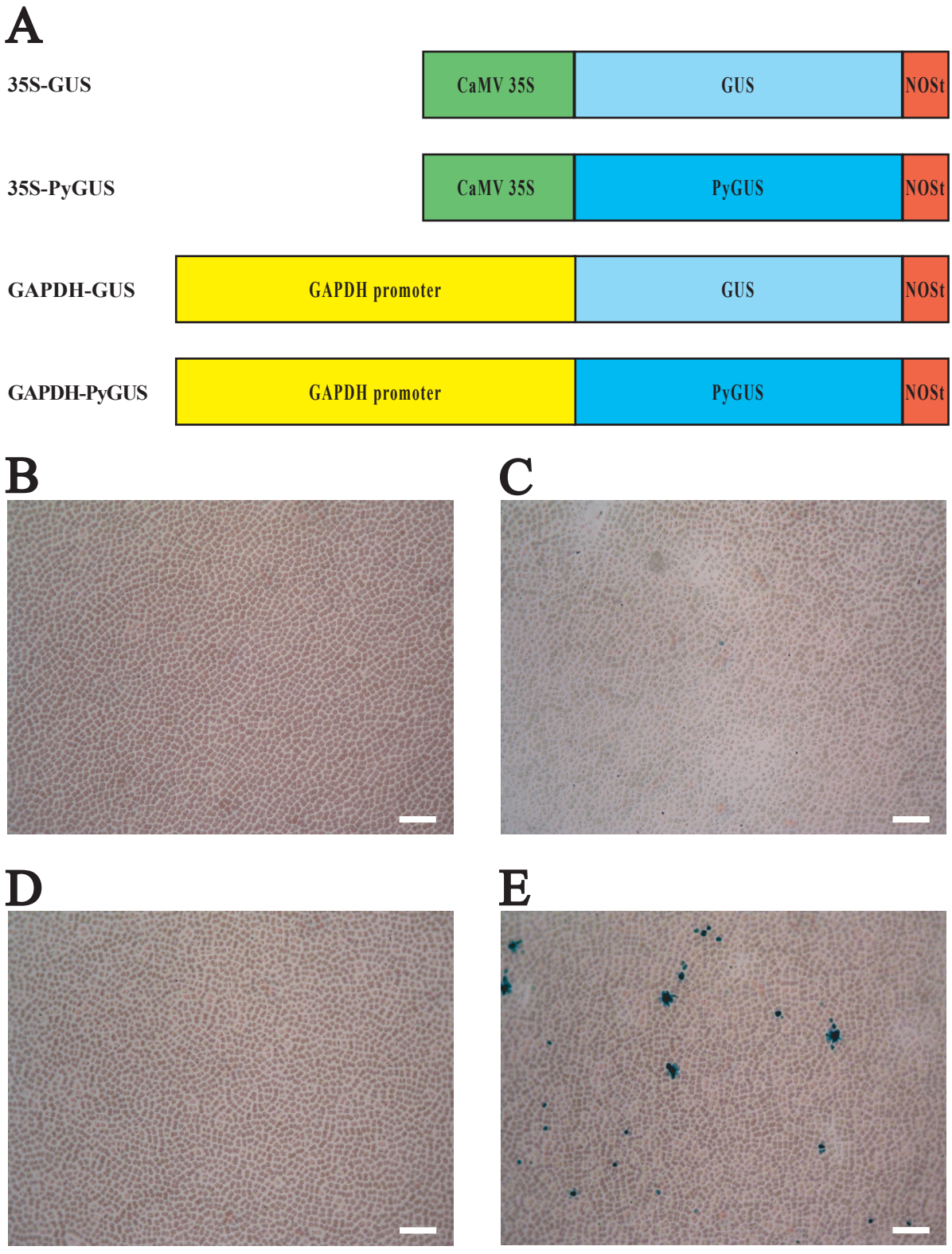
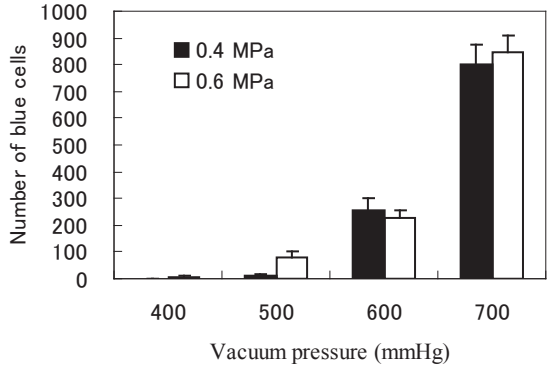
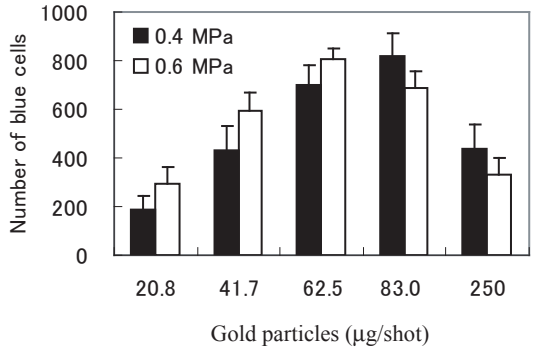

A
35S-GUS
CaMV 35S
GUS
NOSt
35S-PyGUS
CaMV 35S
PyGUS
NOSt
GAPDH-GUS
GAPDH promoter
GUS
NOSt
GAPDH-PyGUS
GAPDH promoter
PyGUS
NOSt
B
C
D
E

Figure 4

A


1000
900
800
700
600
500
400
300
200
100
0
Number of blue cells
0.4 MPa
0.6 MPa
400
500
600
700
Vacuum pressure (mmHg)

B


1000
800
600
400
200
0
Number of blue cells
0.4 MPa
0.6 MPa
20.8
41.7
62.5
83.0
250
Gold particles (μg/shot)

C

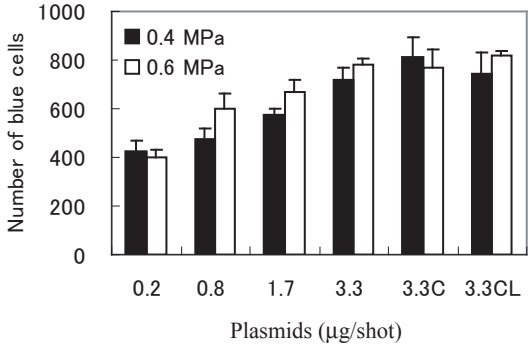

1000
800
600
400
200
0
Number of blue cells
0.4 MPa
0.6 MPa
0.2
0.8
1.7
3.3
3.3C
3.3CL
Plasmids (μg/shot)

Figure 5

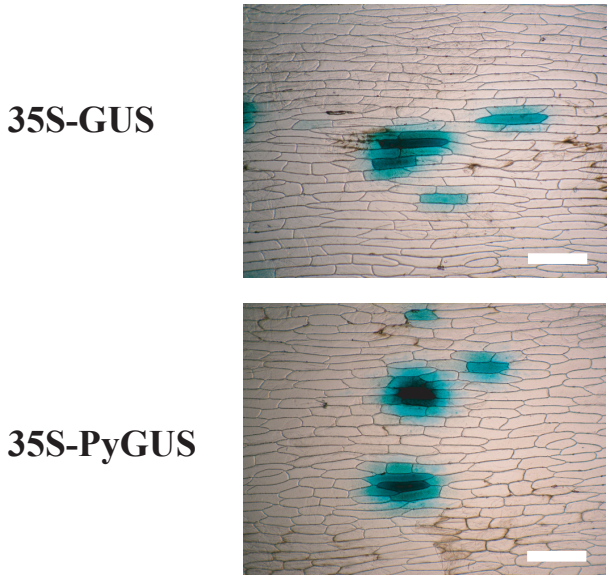


Table 1

Table 1
List of synthetic oligonucleotide primers used for artificial synthesis of PyGUS

Primer	Position [a]	Sequence
GUS-Py1F	1- 48	5'-CGGGATCCATGCTGCGCCCCGTGGAGACGCCCACGCGCGAGATCAAGAAGCTGGAC-3' [b]
GUS-Py1R	93- 29	5'-GTCGATGCCGCAGTTCTCGCGGTCCAGCGAGAACGCCCACAGGCCGTCCAGCTTCTTGATCTCGC-3'
GUS-Py2F	76- 166	5'-GAGAACTGCGGCATCGACCAGCGCTGGTGGGAGTCGGCGCTGCAGGAGTCGCGCGCGATCGCGGTGCCCGGCTCGTTCAACGACCAGTTCG-3'
GUS-Py2R	225- 149	5'-GATGAACACCTCGCGCTGGTACCACACGTTGCCCGCGTAGTTGCGGATGTCCGCGTCCGCGAACTGGTCGTTGAACG-3'
GUS-Py3F	208- 300	5'-CAGCGCGAGGTGTTCATCCCCAAGGGCTGGGCGGGCCAGCGCATCGTGCTGCGCTTCGACGCGGTGACGCACTACGGCAAGGTGTGGGTGAAC-3'
GUS-Py3R	363- 283	5'-GTAGGGCGTCACGTCCGCCTCGAAGGGCGTGTAGCCGCCCTGGTGCTCCATCACCTCCTGGTTGTTCACCCACACCTTGCC-3'
GUS-Py4F	347- 421	5'-CGGACGTGACGCCCTACGTGATCGCGGGCAAGTCGGTGCGCATCACGGTGTGCGTGAACAACGAGCTGAACTGGC-3'
GUS-Py4R	480- 402	5'-GTACGACTGCTTCTTCTTGCCGTTCTCGTCCGTGATCACCATGCCGGGGGGGATCGTCTGCCAGTTCAGCTCGTTGTTC-3'
GUS-Py5F	460- 545	5'-GGCAAGAAGAAGCAGTCGTACTTCCACGACTTCTTCAACTACGCGGGCATCCACCGCTCGGTGATGCTGTACACGACGCCCAACAC-3'
GUS-Py5R	598- 528	5'-CGTGGTTGCAGTCCTGCGCCACGTGCGTCACCACCGTGATGTCGTCCACCCACGTGTTGGGCGTCGTGTAC-3'
GUS-Py6F	582- 646	5'-GCAGGACTGCAACCACGCGTCGGTGGACTGGCAGGTGGTGGCGAACGGCGACGTGTCGGTGGAGC-3'
GUS-Py6R	715- 630	5'-GGTTCACCACCTGCAGCGTGCCCGACGTGCCCTGGCCCGTCGCCACCACCTGCTGGTCCGCGTCGCGCAGCTCCACCGACACGTCG-3'
GUS-Py7F	699- 783	5'-GCTGCAGGTGGTGAACCCCCACCTGTGGCAGCCCGGCGAGGGCTACCTGTACGAGCTGTGCGTGACGGCGAAGTCGCAGACGGAG-3'
GUS-Py7R	849- 767	5'-GATCAGGAACTGCTCGCCCTTCACCGCCACCGAGCGGATGCCCACGCGCAGGGGGTAGATGTCGCACTCCGTCTGCGACTTCG-3'
GUS-Py8F	832- 925	5'-GGCGAGCAGTTCCTGATCAACCACAAGCCCTTCTACTTCACGGGCTTCGGCCGCCACGAGGACGCGGACCTGCGCGGCAAGGGCTTCGACAACG-3'
GUS-Py8R	994- 909	5'-GGTAGTGCGACGTGCGGTACGAGTTCGCGCCGATCCAGTCCATCAGCGCGTGGTCGTGCACCATCAGCACGTTGTCGAAGCCCTTG-3'
GUS-Py9F	978-1055	5'-CCGCACGTCGCACTACCCCTACGCGGAGGAGATGCTGGACTGGGCGGACGAGCACGGCATCGTGGTGATCGACGAGAC-3'
GUS-Py9R	1120-1038	5'-GCTCCTTGGGCTTGTTGCCCGCCTCGAAGCCGATGCCCAGCGACAGGTTGAAGCCCACCGCCGCCGTCTCGTCGATCACCACG-3'
GUS-Py10F	1103-1189	5'-GCAACAAGCCCAAGGAGCTGTACTCGGAGGAGGCGGTGAACGGCGAGACGCAGCAGGCGCACCTGCAGGCGATCAAGGAGCTGATCG-3'
GUS-Py10R	1240-1172	5'-GCTCGTTCGCGATCGACCACATCACCACCGAGGGGTGGTTCTTGTCGCGCGCGATCAGCTCCTTGATCG-3'
GUS-Py11F	1224-1284	5'-GTCGATCGCGAACGAGCCCGACACGCGCCCCCAGGGCGCGCGCGAGTACTTCGCGCCCCTG-3'
GUS-Py11R	1348-1267	5'-CGCAGAACATCACGTTCACGCACGTGATGGGGCGCGTGGGGTCCAGCTTGCGCGTCGCCTCCGCCAGGGGCGCGAAGTACTC-3'
GUS-Py12F	1330-1404	5'-GTGAACGTGATGTTCTGCGACGCGCACACGGACACGATCTCGGACCTGTTCGACGTGCTGTGCCTGAACCGCTAC-3'
GUS-Py12R	1464-1387	5'-CTCCTTCTCCAGCACCTTCTCCGCCGTCTCCAGGTCGCCCGACTGCACGTACCAGCCGTAGTAGCGGTTCAGGCACAG-3'
GUS-Py13F	1446-1518	5'-GAAGGTGCTGGAGAAGGAGCTGCTGGCGTGGCAGGAGAAGCTGCACCAGCCCATCATCATCACGGAGTACGGC-3'
GUS-Py13R	1582-1500	5'-CGCACTGGTACTCCTCCGACCACATGTCCGTGTACATCGAGTGCAGGCCCGCCAGCGTGTCCACGCCGTACTCCGTGATGATG-3'
GUS-Py14F	1565-1650	5'-CGGAGGAGTACCAGTGCGCGTGGCTGGACATGTACCACCGCGTGTTCGACCGCGTGTCGGCGGTGGTGGGCGAGCAGGTGTGGAAC-3'
GUS-Py14R	1710-1634	5'-GATGCCCTTCTTGTTGCCGCCCACGCGCAGGATGCCCTGCGACGTCGCGAAGTCCGCGAAGTTCCACACCTGCTCGC-3'
GUS-Py15F	1693-1766	5'-GGCAACAAGAAGGGCATCTTCACGCGCGACCGCAAGCCCAAGTCGGCGGCGTTCCTGCTGCAGAAGCGCTGGAC-3'
GUS-Py15R	1812-1750	5'-CGAGCTCCCTACTGCTTGCCGCCCTGCTGGGGCTTCTCGCCGAAGTTCATGCCCGTCCAGCGCTTCTGCAG-3' [b]

[a] The number of each position is based on assignment of the first nucleotide of the initiation codon as +1.

[b] *Bam*HI (GGATCC) and *Sac*I (GAGCTC) site are underlined.

Table 2

Table 2
List of primers used in PCR

Primer	Position [a]	Sequence
GAPDH-IPCR-F1	-458 - -451, -42 - -30	5'-TCCCATCTCAGCCTTAGCTCC-3'
GAPDH-IPCR-R1	-706 - -724	5'-GCGTGTTGCGTGTCCAATC-3'
GAPDH-PCR-F1	-2191 - -2173	5'-GCCGTCCAGCTGATTGAGG-3'
GAPDH-PCR-R1	94 - 76	5'-TGGCAACAACCTCAACGCC-3'
HindIII-GAPDH-F1	-2191- -2173	5'-CCCAAGCTTGCCGTCCAGCTGATTGAGG-3' [b]
BamHI-GAPDH-R1	21- 1	5'-CGGGATCCGATCCCGATCTTGACCACCAT-3' [b]

[a] The number of each position is based on assignment of the first nucleotide of the initiation codon as +1.

[b] *Hin*dIII (AAGCTT) and *Bam*HI (GGATCC) sites are underlined.

Table 3

Table 3
Comparison of expression efficiency of GUS and PyGUS expression under the direction of *CaMV 35S* and *GAPDH* promoters in gametophyte cells of *P. yezoensis* [a]

Promoter	Reporter	Helium pressure (MPa)	Target distance (cm)	Number of blue-stand cells Exp. 1	Exp. 2	Exp. 3	mean±SD
CaMV 35S	GUS	0.2	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
		0.4	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
		0.6	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
CaMV 35S	PyGUS	0.2	3	3	1	1	2± 2
			6	0	0	0	
			9	0	0	0	
		0.4	3	0	7	5	4± 6
			6	4	1	4	3± 3
			9	0	0	0	
		0.6	3	1	4	3	3± 3
			6	4	6	5	5± 2
			9	0	2	3	2± 3
GAPDH	GUS	0.2	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
		0.4	3	0	0	0	
			6	1	0	0	
			9	0	0	0	
		0.6	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
GAPDH	PyGUS	0.2	3	192	106	139	146±73
			6	11	39	59	36±41
			9	0	0	0	
		0.4	3	273	244	208	242±55
			6	325	334	261	307±67
			9	3	9	11	8± 7
		0.6	3	321	269	305	298±45
			6	281	322	265	289±50
			9	48	59	61	56±12
None		0.2	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
		0.4	3	0	0	0	
			6	0	0	0	
			9	0	0	0	
		0.6	3	0	0	0	
			6	0	0	0	
			9	0	0	0	

[a] The experimental conditions for all bombardments included constant values of 62.5 μg gold particles/shot, 3.3 μg plasmid/shot and 600 mmHg.