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Optimization of Parameters for GUS Gene Transformation of *Porphyra yezoensis* by Particle Bombardment

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We optimized the biological and physical parameters for DNA delivery into thalli of the red alga *Porphyra yezoensis* using a particle bombardment device. The efficiency of transformation was determined using the β-glucuronidase (GUS) assay. The optimal helium pressure, distance of tungsten particle flight, and ratio of DNA to tungsten particles were 23 kgf/cm^2, 8 cm, and 5 μg/mg tungsten, respectively. During bombardment, osmotic treatment with a mixture of 0.6 M mannitol and sorbitol increased the efficiency of GUS transformation. After 2 days, the blue color indicating GUS activity was observed using a histochemical assay.

Key words: GUS gene, Particle bombardment, *Porphyra yezoensis*, Transformation

Introduction

The past decade has seen the genetic modification of various seaweeds, including the red algae *Porphyra*, *Gracilaria*, *Grateloupia*, *Kappaphycus*, and *Ceramium* (Kuang and Wang, 1998) and the green alga *Ulva* (Huang et al., 1996). Among these, red seaweeds have received attention for biotechnological purposes because they synthesize unique cell walls containing sulfated polysaccharides, photosynthetic pigments, and unsaturated fatty acids. The red alga *Porphyra* is one of the most important edible seaweeds cultivated commercially in Korea, Japan, and China. In 2005, aquaculture production of *Porphyra* in Korea was estimated at 181,000 tons (wet weight) or US $138 million (http://fs.fips.go.kr).

Particle bombardment is a powerful method for the genetic transformation of various organisms and tissues (Finer et al., 1992; Halford and Shewry, 2000). The main benefit of this method is that intact plant tissues can serve as targets. Additionally, no antibiotics are needed to remove bacteria, as in *Agrobacterium*-mediated plant transformation, and the transformed tissues can be visualized easily by the presence of the green fluorescent protein after β-glucuronidase (GUS) staining (Jefferson et al., 1987; Cho et al., 1999). The principle of particle bombardment is the acceleration of small DNA-coated particles toward intact cells and tissues, resulting in the penetration of the protoplasm by the particles and the subsequent expression of the introduced DNA. Due to the physical nature of the process, there is no biological limitation on the actual DNA delivery. The genotype is not a limiting factor. Therefore, the particle bombardment method has achieved successful transformation in a number of tissues of a wide range of plant species, including those that are otherwise impossible or very difficult to transform (Sunagawa and Magae, 2002). Transformed plant tissues include cell suspensions, calli, immature embryos, mature embryo parts, meristems, leaf pieces, microspores, and pollen (Athmaram et al., 2006). We investigated the optimal bombardment conditions for foreign gene transformation in *Porphyra yezoensis* and the factors influencing the transformation efficiency as a basic technique for the stable production of transformed seaweed.

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Materials and Methods

Plant materials and tissue culture

Young blades of *Porphyra yezoensis*, 1-3 cm in length, were kindly provided by the National Fisheries Research and Development Institute of Korea and were preserved at -80°C until use. Upon use, the

cryopreserved blades were placed in 1/10 PES medium (Provasoli, 1968) for 1 day, and then the blades were sterilized by sonicating for 30 s twice, soaking in 0.1% betadine in sterilized sea water for 30 s, and washing with sterilized sea water at least three times. Finally, they were transferred to an antibiotics solution containing 10 mg/L kanamycin, 10 mg/L penicillin, 20 mg/L streptomycin, 2 mg/L neomycin, and 0.15 mg/L nystatin in 1/10 PES for 1 day (Choi et al., 2002). Prior to particle bombardment, the viability of young thalli was assessed using a triphenyltetrazolium chloride (TTC) assay (Nam et al., 1998). Thalli showing healthy absorbance values of 0.7 or higher at OD_{545} were used.

Plasmid

The plasmid pBI121 contains the neomycin phosphotransferase II (NPTII) gene as a selection marker and the bacterial GUS gene as a reporter (Jefferson et al., 1987). The NPT II gene is flanked by a nopaline synthase (nos) promoter and a nos terminator, and the GUS gene is flanked by a CaMV 35S promoter and a nos terminator. Plasmid DNA isolated from *Agrobacterium tumefaciens* strain LBA4404 using Qiagen columns (Hamburg, Germany) was used in all particle bombardment experiments.

Particle preparation and bombardment

A 1-mL aliquot of 70% ethanol was added to 60 mg of tungsten particles, and the suspension was vortexed vigorously for 3-5 min to remove aggregated lumps. The suspension was spun for 1 min at 10,000 rpm, and the supernatant was discarded. The pellet was resuspended in 50% glycerol solution to produce a final concentration of 60 mg/mL, and 25 μL aliquots of the suspension were placed in microtubes while vortexing the suspension. A 2.5 μL aliquot of 1 μg/μL DNA solution, 25 μL of 2.5 M $CaCl_2$, and 10 μL of 0.1 M spermidine (free base form) were added in succession to the 25 μL of particle suspen-sion. The mixture was vortexed for 3 min, spun for 10 s at 10,000 rpm, and the supernatant was discarded. The pellet was washed with 70% and then absolute ethanol. The final pellet was resuspended in 25 μL of absolute ethanol. A 6 μL aliquot of the sample was loaded onto the center of the macrocarrier, air dried, and bombarded. The biolistic particle bombardment device, namely the Gene Gun II (Ver. 1.0; Biotec Inc., Korea) is a modified type of the PDS-100/He (Bio-Rad Inc., Hercules, CA, USA) device. In the Gene Gun II system, the acceleration distances are fixed to 4, 6, 8, and 10 cm, and the pressure in the chamber can be raised to 30 kgf/cm^2.

Osmotic treatment

The effect of osmotic treatments on transformation was measured by adding various concentrations of sorbitol and/or mannitol solutions immediately after bombardment. The osmoticum was adjusted from 0.2 to 1.0 M in sterilized seawater. The osmotic treatment was carried out in the dark for 1 h after bombardment. The bombarded tissues were then transferred to PES medium containing 100 mg/L kanamycin and incubated for 2 days at 18°C in the dark prior to the GUS assay.

Thallus preparation for protein and GUS assays

Thallus tissue (ca. 100 mg) was chopped to small pieces on a tile cooled on ice, transferred to a microtube, and homogenized with Tissue Tearor (BioSpec Products Inc., Bartlesville, OK, USA) in 1 mL of GUS assay buffer (50 mM Tris-HCl [pH 7.0], 0.1% sarcosyl, and 0.1% Triton X-100) for 30 s (Jefferson et al., 1987). The homogenate was centrifuged at 12,500 rpm for 10 min at 4°C. A 600 μL aliquot of the supernatant, which was dark red and contained high amounts of crude protein, was transferred to a new tube. To determine the total amount of protein, 20 μL of supernatant were used for quantification by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard. GUS activity was determined using a fluorometer.

Determination of GUS activity and the GUS gene

A 20 μL aliquot of supernatant was mixed with 200 μL of 2 mM fluorometric substrate 4-methylumbelliferyl β-D-glucuronide solution (M-9130; Sigma, St. Louis, MO, USA) and incubated for 1 h at 37°C. The reaction was terminated by the addition of 800 μL of 0.2 M Na_2CO_3. Fluorescence was then measured with excitation at 365 nm and emission at 460 nm on a Mini-Fluorometer TKO100 (Hoeffer Scientific Instruments, San Francisco, CA, USA). The data were expressed in pmole/h/mg of total protein.

For detection of the GUS gene using the polymerase chain reaction (PCR), total DNA was extracted from the thalli of wild and transgenic *Porphyra* species (Hong et al., 1995). DNA (100 ng) was used for PCR analysis with the upstream primer 5'-TCCTGTAGAAACCCCAACC-3' and the downstream primer 5'-GCTAGCCTTGTCCAGTTG-3', reflecting nucleotide positions 17-35 and 674-691 of the GUS gene, using *Taq* polymerase. The amplification conditions consisted of an initial denaturation step at 94°C for 5 min; followed by 30 cycles at 94°C for 1 min for melting, 55°C for 2 min for annealing, and 72°C for 3 min for synthesis; and a final exten-

sion at 72°C for 10 min. The PCR products were separated by electrophoresis on 2% agarose gel.

Histochemical assay

The procedure described by Jefferson et al. (1987) was followed, with some modification. Following gene transfer, tissues were incubated at 18°C in the dark for 1 day. For the histochemical assay of GUS activity, a staining solution containing the following components was added to the tissues: 100 mM Tris-HCl buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1.0 mM 5-bromo-4-chloro-3-indoryl-β-D-glucuronic acid (X-gluc: B-4527; Sigma, St. Louis, MO, USA), and 0.1% triton X-100 in sterile sea water. When present, GUS cleaves glucuronic acid from X-gluc to produce an insoluble indigo dye following oxidative dimerization. After incubation at 37°C for 2 days, blue foci on the surface of GUS transformants were observed under a microscope.

Statistical analysis

The experiments were repeated at least six times for each independent assay. The mean values of the treatments were compared to those of the control using Student's *t*-tests. Data are reported as mean ± standard error.

Results

Helium pressure and acceleration distance

To optimize the conditions of the Gene Gun II system, the helium pressure in the chamber was first set to 23 kgf/cm^2. Of the available particle flight distances (4-10 cm), 8 cm showed the highest specific activity of the GUS enzyme (data not shown). Thus, we examined various helium pressures of 19 to 25 kgf/cm^2 at a distance of 8 cm (Fig. 1). Because GUS activity appeared high at 23 and 21 kgf/cm^2, we fixed the acceleration distance and helium pressure at 8 cm and 23 kgf/cm^2, respectively.

Relative concentration of DNA and particles

To efficiently coat DNA with tungsten particles, we evaluated the relative concentration of DNA. We tested different amounts of DNA from 1 to 7 μg for each 1 mg of tungsten particles for bombardment (Fig. 2). GUS activity was increased at higher amounts of DNA and reached a maximum at 5 μg of DNA per 1 mg of tungsten particles.

Effect of osmotic treatment

To prevent the release of cytoplasmic solution from particle-bombarded tissues, osmotic treatment with sorbitol and/or mannitol was conducted for 1 h after bombardment. We compared GUS activity derived from the osmotic effects of sorbitol, mannitol, or an equimolar mixture of sorbitol and mannitol (Fig. 3). The highest GUS enzyme activity was obtained by osmotic treatment with a 0.6 M sorbitol/mannitol mixture.

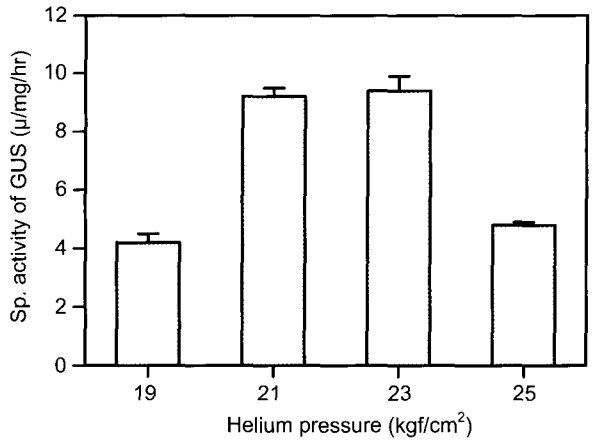

Fig. 1. Comparison of GUS activity expressed after particle bombardment under various helium pressures at an acceleration distance of 8 cm. Data are mean ± SD (n > 6).

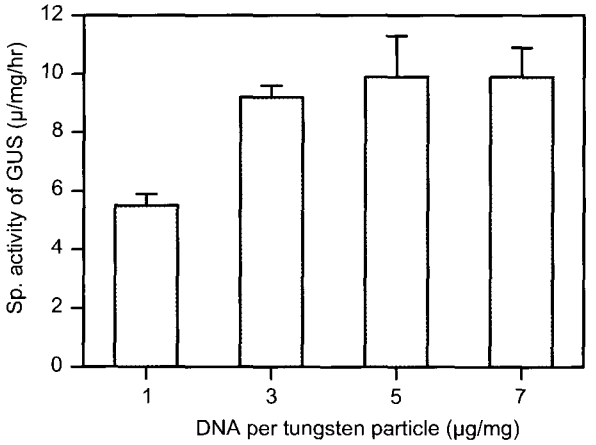

Fig. 2. Comparison of GUS activity expressed after particle bombardment with tungsten particles coated with various amounts of DNA. Data are mean ± SD (n > 6).

Intrinsic GUS-like activity in *Porphyra* tissue

To survey the presence of intrinsic GUS genes in wild-type *Porphyra* species, the total DNA from each species was amplified using GUS-specific primers. No PCR products (675 bp) corresponding to the GUS gene were detected from *P. dentata, P. perforata, P. seriata, P. suborbiculata, P. tenera*, or *P. yezoensis* (data not shown). By increasing the amount of protein

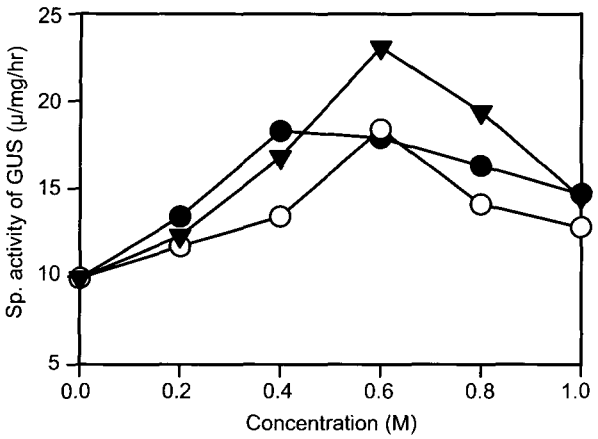

Fig. 3. Effect of osmotic treatment using sorbitol and/or mannitol on particle-bombarded tissues. ○, sorbitol. ●, mannitol. ▼, sorbitol and mannitol mixture.

as for GUS activity, the fluorescence value also increased. The addition of 20% methanol in the fluorometric assay mixture resulted in complete suppression of the intrinsic GUS-like activity.

GUS activity in transformants

At 2 days after GUS bombardment, the development of blue color was detected using a histochemical assay and observed under a microscope. The presence of blue loci indicated transient GUS activity in putative transformant cells of *P. yezoensis* after 2 days of culture (Fig. 4A). Some rhizoids that developed from tissues from near the holdfast after 5 days were stained light blue. Vegetative cells along the edges of thalli divided after 1 week and showed the distinct blue color of GUS activity via the expression of the transformed GUS gene (Fig. 4B). Monospores were produced and generated young plantlets after 1 month; the putative transformants exhibited the blue color indicating GUS activity (Fig. 4C).

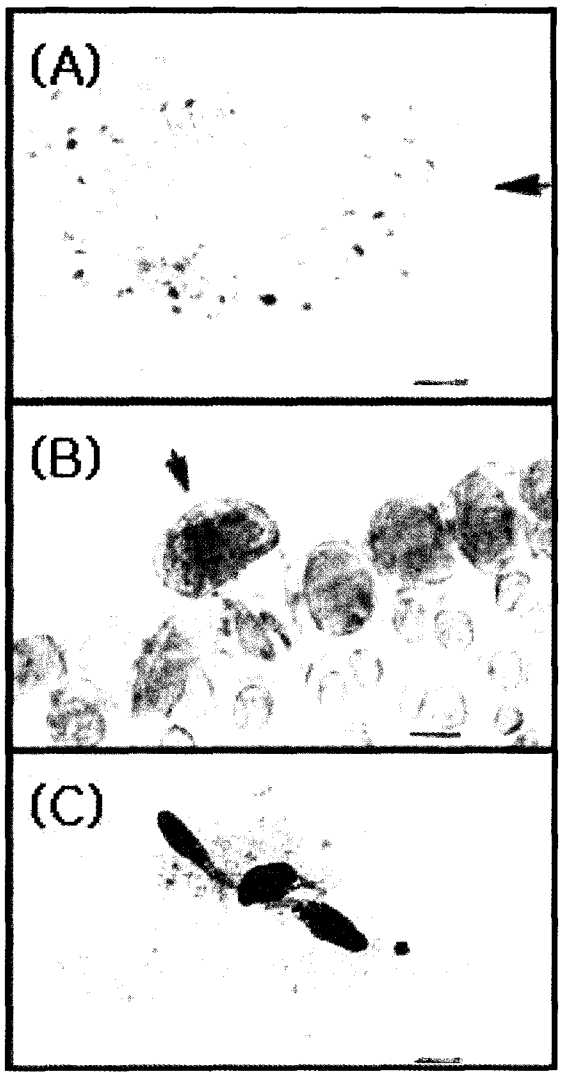

Fig. 4. Histochemical assay of transient GUS activity in vegetative cells of *Porphyra yezoensis* thalli. (A) The blue color indicative of GUS activity developed after 2 days. The arrow indicates transient GUS activity. Scale bar represents 100 μm. (B) The arrow indicates transient GUS activity in newly dividing cells along edge of the tissue after 1 week. Scale bar represents 25 μm. (C) Young plantlets regenerated from transformed monospores show the blue color indicative of GUS activity after 1 month. Scale bar represents 200 μm.

Discussion

Although gene transfer by particle bombardment is theoretically applicable to all types of cells and tissues, the method requires careful optimization of the biological and physical parameters involved in bombardment for each plant material (Charest et al., 1993). Under the optimal conditions determined in our study, foreign genes could be delivered to intact young *Porphyra* thalli using the particle bombardment device. This method can circumvent the difficulties of regenerating plants from protoplasts, which are required for polyethylene glycol or electroporation-mediated transformation procedures.

Although most plant species lack GUS, several reports have noted endogenous GUS-like activity in non-transformed plants (Plegt and Bion, 1989; Hu et al., 1990). We used PCR amplification to check for the presence of the GUS gene in various *Porphyra* species, but did not detect any corresponding PCR products. However, we did observe endogenous

GUS-like activity in *P. yezoensis*. Thus, we always assayed GUS activity in comparison to control, non-transgenic *Porphyra* tissues. Additionally, endogenous GUS activity can be eliminated by elevating the pH of the reaction mixture to 9.0 (Hodal et al., 1992) and by the addition of methanol to the reaction mixture (Kosugi et al., 1990).

The GUS reporter gene was convenient for evaluating the gene transfer efficiency (Jefferson et al., 1987). Although the chloramphenicol acetyltransferase gene is also a useful reporter gene and encodes an enzyme not normally found in plant tissues, the assay for this reporter is relatively difficult and expensive. The β-galactosidase gene is somewhat inconvenient for the quantification of gene expression because of the presence of high endogenous β-Galactosidase activity in plant cells. However, it offers the advantages of accurate fluorometric assays and precise histochemical localization, even in tissues of the seaweed *P. yezoensis*. CaMV35S is a promoter widely used to express foreign genes in land plants (Jefferson et al., 1987). This promoter also seems to achieve a high level of transcription in the seaweed *P. yezoensis*.

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