Optimization of Particle Bombardment Conditions by Monitoring of Transient sGFP(S65T) Expression in Transformed Soybean

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In an attempt to increase the efficiency of soybean transformation by particle bombardment, we examined the effects of bombardment parameters such as gold particle size, target distance, acceleration pressure, amount of DNA per bombardment, and number of bombardments. Transgene delivery to embryogenic tissue grown in suspension culture was evaluated by monitoring the transient expression of a gene for a modified form of jellyfish green fluorescent protein [sGFP(S65T)]. Optimal transient expression of sGFP(S65T) was obtained when the tissue was bombarded twice at an acceleration pressure of 7.6 MPa (1,100 psi) and a distance of 6 cm with gold particles that were 0.6 µm in diameter and coated with 0.8 µg of DNA. Application of these optimized conditions proved effective for the generation of stable transgenic soybean plants. Stable transgene integration in the transformants was confirmed by Southern blot analysis. The average transformation efficiency achieved with the optimized protocol was significantly higher than that with the conventional protocol.

Key Words: Glycine max (L.) Merrill, particle bombardment, sGFP(S65T), transient gene expression.

Introduction

Gene transfer by particle bombardment (biolistics) is a widely accepted technique with broad applications in plant molecular biology. As with other physical methods, particle bombardment readily enables to overcome the physical and genetic barriers to gene transfer in plants. It is a versatile technique that has been applied successfully for both transient and stable transformation in several plant species (Christou 1993, Sanford et al. 1993).

Particle bombardment is commonly used to generate transgenic plants in soybean [Glycine max (L.) Merrill]. Specifically, this method involves the delivery of microprojectiles coated with DNA molecules into primary explants, including shoot meristems (McCabe et al. 1988, Aragao et al. 2000) and embryogenic suspension tissue (Finer and Mcmullen 1991), that are capable of plant regeneration. However, the utility of this approach is limited by its low transformation efficiency and by the integration of multiple copies or reconstitution of transgenes that results in their cosuppression (Kinney et al. 2001, Reddy et al. 2003, El-Shemy et al. 2004). There are several possible factors for the low transformation efficiency, some of which can be overcome by refinement of the DNA delivery parameters (Jain et al. 1996). The effects of various conditions that influence DNA delivery in soybean have not been previously explored systematically, however.

Transient expression of marker genes such as uidA (which encodes β-glucuronidase) is often used to optimize transformation protocols (Li et al. 1992, Liu et al. 1992, Gallo-Meagher and Irvine 1993, Eady et al. 1996, Jain et al. 1996, Kapila et al. 1997, Maximova et al. 1998). The gene for the green fluorescent protein (GFP) of jellyfish (Aequorea victoria) is a popular reporter that has been used widely and successfully to assess the transient expression of the transgenes introduced through either Agrobacterium- or particle
bombardment-based transformation systems (Stewart 2001). A modified form of the gene for GFP, encoding a mutant [sGFP(S65T)] in which serine-65 is replaced by threonine, was designed to increase the fluorescence intensity and to reduce the rate of photobleaching at an excitation wavelength of 490 nm for expression studies in higher organisms (Chiu et al. 1996). The increased brightness enables individual fluorescing cells or small multicellular structures to be readily detected. Elliot et al. (1999) used sGFP(S65T) to observe early transformation events and to improve the efficiency of stable transformation in sugarcane. We have now used the transient sGFP(S65T) expression as a tool to monitor the effects of various conditions on the efficiency of particle bombardment-mediated transformation in embryogenic suspension tissue of soybean. We also examined the extent to which the results obtained can be applied to improve the efficiency of stable soybean transformation.

Materials and Methods

Plasmid construction

The pUHG(SK) plasmid (6.5 kb) was a derivative of pUC19 and included a multiple cloning site from pBlue-Script SK+ (Stratagene, La Jolla, CA, USA) that is located between the sGFP(S65T) reporter gene and hpt, which encodes hygromycin phosphotransferase and confers hygromycin resistance (Fig. 1). Both genes are under the regulatory control of the 35S promoter of cauliflower mosaic virus and the polyadenylation region of the nopaline synthase gene. The construction of pUHG(SK) has been described in detail previously (Rahman et al. submitted for publication).

Particle bombardment and evaluation of transient sGFP(S65T) expression in embryogenic tissue

Plants of the soybean cv. Jack, a highly competent variety for somatic embryo induction and regeneration from immature cotyledons (Tomlin et al. 2002), were grown in soil under natural light conditions and at a controlled temperature of 25°C in a glasshouse, and embryogenic tissue was induced from immature cotyledons as previously described (El-Shemy et al. 2004). The embryogenic tissue was maintained and allowed to proliferate by culture at 25°C under cool white fluorescent light (23-h-light, 1-h-dark cycle, 5 to 10 µmol m⁻² s⁻¹) in 100-ml flasks containing 25 ml of fresh FN Lite liquid medium [FN Lite macro-salts, MS micro-salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), asparagine (1 g/l), 2,4-dichlorophenoxyacetic acid (2,4-D) (5 mg/l), and 1% sucrose, adjusted to pH 5.8] (Finer and Nagasawa 1988). The tissue was subcultured weekly.

Sets of bombardment parameters were evaluated according to a multifactorial design in two independent experiments using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Richmond, CA, USA). In the first experiment, the effects of the gold particle size (0.6, 1.0 or 1.6 µm), acceleration pressure (6.2, 7.6 or 9.3 MPa, corresponding to 900, 1100 or 1350 psi), and bombardment target distance (the distance between the stopping screen and the target plate: 6, 9 or 12 cm) were evaluated. The number of bombardments and the DNA load per bombardment were fixed at two bombardments each with 0.8 µg of DNA. The most effective value of each parameter determined in the first experiment was fixed in the second experiment, in which the effects of the number of bombardments (1, 2 or 3) and DNA load per bombardment (0.4, 0.8, 1.6 or 3.2 µg) were assessed.

Plasmid DNA for particle bombardment was isolated from Escherichia coli DH5α harboring pUHG(SK) by using a Plasmid Midi kit (Qiagen, Valencia, CA, USA). Coating of gold particles with DNA and particle bombardment were performed according to a modified version of a previously described procedure (Finer et al. 1992). Briefly, 50 µl of the gold particle suspension (60 mg/ml in 50% glycerol) was mixed with 50 µl of plasmid DNA at various concentrations, 50 µl of 2.5 M CaCl₂, and 20 µl of 0.1 M spermidine in 1.5-ml plastic microtubes. The mixture was incubated for 3 min at room temperature and centrifuged to isolate the gold particles, which were then washed with 70% ethanol and resuspended in 300 µl of 95.5% ethanol. About 0.8 g of embryogenic suspension tissue was arranged to form a circle 2.5 cm in diameter in the center of a 9-cm petri dish containing MSD20 medium [MS salts, B5 vitamins, 3% sucrose, asparagine (1 g/l), 2,4-D (20 mg/l), and 0.2% Gelrite (Wako, Osaka, Japan), adjusted to pH 5.8]. Embryogenic tissue on each of three plates was then bombarded with the DNA-coated gold particles under the various test conditions. Twenty-four hours after bombardment, the expression of sGFP(S65T) was monitored by excitation with blue light (Chiu et al. 1996). Fluorescence in both embryogenic tissue and developing embryos was detected with a fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a filter set for excitation at 455 to 490 nm and emission at >515 nm. Transient sGFP(S65T) expression was evaluated based on the intensity of blue light spots in the observation field of the stereomicroscope. The fluorescence intensity was scored according to five levels: 0, 1, 2, 3 and 4 (Fig. 2). The mean value was determined from the analysis of six observation fields.
Stable transformation by particle bombardment and recovery of transgenic plants

Embryogenic tissue on each of six plates was bombarded with pUHG(SK) under the optimal conditions defined in the transient expression experiments. For comparison, the same quantity of embryogenic tissue was transformed according to the conditions previously described (El-Shemy et al. 2004), bombardment twice at an acceleration pressure of 9.3 MPa and a target distance of 6 cm with 1.0-µm-diameter gold particles coated with 0.8 µg of DNA. Embryogenic tissue was returned to FN Lite medium after bombardment. After 1 week, the suspension cultures were incubated for 2 weeks in fresh FN Lite medium supplemented with hygromycin B (15 mg/l) (Roche Diagnostics, Mannheim, Germany), with a change of medium after 1 week. Clumps of tissue exhibiting bright green lobes were then transferred to fresh FN Lite medium containing hygromycin B at the concentration of 30 mg/l and were maintained for 3 weeks, with a change of medium weekly. The hygromycin-tolerant tissue was finally subjected to selection in FN Lite medium containing hygromycin B at the concentration of 45 mg/l for 1 week.

Plants were recovered according to a modified version of a previously described procedure (Finer and McMullen 1991). Hygromycin-tolerant embryogenic tissue was transferred to FNLO5535 liquid medium [FN Lite macro-salts, MS micro-salts, B5 vitamins, asparagine (1 g/l), 3% sucrose, and 3% sorbitol, adjusted to pH 5.8] and maintained on a rotary shaker at 110 rpm. After 4 to 5 weeks, excess liquid was removed from the somatic embryos with sterile filter paper and they were placed in dry petri dishes for 3 to 5 days before transfer to MS0 medium (MS salts, B5 vitamins, 3% sucrose, and 0.2% Gelrite, adjusted to pH 5.8). The germinating plantlets were subsequently grown on 0.5 × B5 medium (0.5 × B5 salts, 0.5 × B5 vitamins, 3% sucrose, 0.05% MES buffer, and 0.2% Gelrite, adjusted to pH 5.8). After root and shoot elongation, the presence of transgenes in the plants was confirmed by polymerase chain reaction (PCR) analysis. The transformed plants were then transferred to pots containing soil and maintained at a high humidity level until acclimation. The plants were gradually adapted to ambient humidity and placed in a glasshouse.

PCR analysis of regenerated plants

Total DNA was extracted from putative transgenic T0 plants using a Kurabo PI-50α machine (Kurabo Industries, Osaka, Japan) according to the plant DNA ver. 2 method. PCR was performed in a 20-µl reaction mixture containing 10 ng of genomic DNA, 200 µM of each deoxynucleoside triphosphate, 0.2 µM of each primer, and 2.5 U of Ampli-taq Gold polymerase (Applied Biosystems, Foster City, CA, USA) in polymerase buffer. The reaction protocol comprised an initial incubation for 9 min at 94°C; 30 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C; and a final incubation for 7 min at 72°C. The primer set for hpt (5'-ATCTCTTC GCAAGACCCCTCCT-3', 5'-GGTGTCGTCATCAGT TTG-3') resulted in the amplification of a 560-bp product. The primer set for sGFP(S65T) (5'-AAGGTACCGGATCC CCCCTCAGAA-3', 5'-AAGAGCTCCGATCTAGTAACA TAGATGACACC-3') yielded a 708-bp product.

Southern blot analysis of transformed plants

Total DNA was isolated from both nontransformed control plants and transformed plants as previously described.

Fig. 2. Scoring of the intensity of fluorescence attributable to transient expression of sGFP(S65T) in soybean embryogenic tissue grown in suspension culture. Embryogenic tissue was examined 24 h after particle bombardment with pUHG(SK). Panel W, embryogenic tissue observed under white light. Panels 0 to 4, embryogenic tissue showing the five different scoring levels of fluorescence associated with the transient sGFP(S65T) expression. A black bar in panel W represents a scale bar of 3 mm.
(Murray and Thompson 1980) and was subjected to Southern blot analysis to confirm the stable integration of the transgenes. Total DNA (10 µg) was digested with the restriction enzyme HindIII, and the resulting fragments were separated by electrophoresis through a 1% agarose gel and then transferred to a Hybond N+ membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Labeling of probes and detection of hybridization were performed with an ECL kit (Amersham Biosciences). DNA fragments corresponding to the hpt and sGFP(S65T) genes were amplified from pUHG(SK) by PCR with the primers described above for use as probes for Southern hybridization.

Results and Discussion

It is important for transient or stable transformation of any plant tissue by particle bombardment that optimal parameters be established (Schöpke et al. 1997). We previously developed a particle bombardment-based transformation procedure that we applied to generate stable transgenic soybean plants (El-Shemy et al. 2004). The transformation efficiency of this method was insufficient, however, for routine use on a small scale. To improve the transformation efficiency achieved with this approach, we therefore performed two independent experiments to optimize the conditions for DNA delivery. For these experiments, we tested the effects of different particle bombardment parameters on the transient expression of the sGFP(S65T) reporter gene in soybean embryogenic tissue.

Gold particle size, acceleration pressure and target distance

In the first optimization experiment, we assessed the effects of the gold particle size, acceleration pressure, and bombardment target distance by scoring for transient expression of sGFP(S65T) 24 h after bombardment. The number of bombardments was set at two and the DNA load per bombardment was set at 0.8 µg, as in our previous study (El-Shemy et al. 2004).

Both acceleration pressure and bombardment target distance significantly affected the level of transient sGFP(S65T) expression in embryogenic soybean tissue (Fig. 3). Increasing the target distance from 6 to 9 or 12 cm thus resulted in a significant decrease in the mean expression score. A similar negative effect of increasing target distance was described for the transient expression in wheat (Rasco-Gaunt et al. 1999). Although the level of transient sGFP(S65T) expression did not differ significantly among the three gold particle sizes tested (Fig. 3), a significant interaction between the gold particle size and bombardment target distance was observed (Fig. 4). The combination of a small particle size (0.6 µm) with a short target distance (6 cm) yielded the highest level of transient sGFP(S65T) expression. Particles with a diameter of 1.6 µm have a superficial dimension and weight that are ~7.1 and 19 times, respectively, those of particles with a diameter of 0.6 µm; they may therefore cause severe damage when they penetrate into cells. Similar analysis with Arabidopsis, tobacco and birch also revealed that particles with a diameter of 0.6 µm yielded the highest transformation efficiency (Helenius et al. 2000). Our results showed that the level of transient sGFP(S65T) expression was significantly higher in soybean tissue bombarded at an acceleration pressure of either 7.6 or 9.3 MPa than in that treated at 6.2 MPa (Fig. 3). Previous studies with cassava (Schöpke et al. 1997) and Chrysanthemum (Jaime et al. 2002) also showed an acceleration pressure of 7.6 MPa resulted in a higher level of transient expression than did a pressure of 6.2 MPa. No significant interaction between the
acceleration pressure and either gold particle size or target distance was apparent (data not shown).

On the basis of these data, we selected the following optimal transformation parameters: a gold particle size of 0.6 µm, an acceleration pressure of 7.6 MPa, and a bombardment target distance of 6 cm. These conditions were used in subsequent experiments.

**DNA quantity per bombardment and number of bombardments**

The optimal parameters defined based on the results of the first experiment were used in the second experiment, which was designed to assess the effects of both the number of bombardments and the DNA load per bombardment. The DNA load significantly affected the level of transient sGFP(S65T) expression. Increasing the amount of plasmid DNA per bombardment from 0.4 to 0.8 µg thus increased the expression of the reporter gene, although further increases in DNA load to 1.6 or 3.2 µg did not further increase the expression (Fig. 5). A similar effect was previously observed with cassava somatic embryos, in which the highest level of transient gene expression was achieved with a single bombardment with 0.5 µg of plasmid DNA (Schöpke et al. 1997); an increase in the amount of plasmid DNA from 0.5 to 1.5 µg resulted in a significantly lower level of expression. The level of transient expression of sGFP(S65T) in the present study was not significantly affected by increasing the number of particle bombardments from one to two or three (Fig. 5). In addition, we did not detect any significant interaction between the number of bombardments and the amount of DNA per bombardment (data not shown). The highest level of transient expression of uidA induced by particle bombardment in cassava was achieved with two bombardments (Zhang et al. 2000). We adopted two bombardments with 0.8 µg of DNA per bombardment as optimal conditions for the transformation of soybean embryogenic tissue.

**Application of optimized bombardment conditions**

We next applied the optimized bombardment conditions determined for the transient expression of sGFP(S65T) to generate stable transgenic plants. We compared the transformation efficiency achieved with this optimized method with that achieved with our previous method (El-Shemy et al. 2004). Application of the optimized conditions resulted in >10-fold increase in the integrative transformation efficiency compared with that achieved with the previous method (Table 1). The smaller particle size and acceleration pressure in the optimized method compared with those in the previous method might result in a reduced amount of damage to the target tissue and hence improve the proliferation potential. Similarly, a previous study of wheat tissues revealed that bombardment with smaller gold particles at lower pressures resulted in improved shoot regeneration compared with bombardment with larger particles at higher pressures (Rasco-Gaunt et al. 1999).

We performed a Southern blot analysis to confirm the stable integration of the transgenes in the transformed plants. All the transformants analyzed yielded three to > 10 bands that hybridized with either the hpt or sGFP(S65T) probe (Fig. 6). Each plant exhibited a distinct band pattern. Hybridization signals corresponding to DNA fragments of hpt or sGFP(S65T) gene cassette were observed, as were differences in the banding patterns between the two probes. These results suggest that genetic rearrangement or fragmentation of the vector had occurred in some of the transgenic plants.

The optimization of the particle bombardment conditions in the present study has revealed that a gold particle

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**Table 1.** Comparison of the efficiency of generation of transgenic soybean plants by particle bombardment under the optimized conditions developed in the present study and the conditions in our previous study (El-Shemy et al. 2004)

<table>
<thead>
<tr>
<th>Particle bombardment parameters</th>
<th>Particle bombardment parameters</th>
<th>No. of embryogenic clones/plate</th>
<th>No. of transgenic plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPS(µm) AP(MPa) BTD(cm) NBT DLP(µg)</td>
<td>Hygromycin-tolerant</td>
<td>Transformed&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Optimized conditions 0.6 7.6 6 2 0.8</td>
<td>15.0±5.8</td>
<td>2.2±1.0&lt;sup&gt;2&lt;/sup&gt;</td>
<td>8.2±3.1&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Previous conditions 1.0 9.3 6 2 0.8</td>
<td>8.8±5.2</td>
<td>0.2±0.4</td>
<td>0.6±1.2</td>
</tr>
</tbody>
</table>

Particle bombardment was performed with six plates for each set of conditions. Data are means±SD for the six plates.

<sup>1</sup> Transformed embryogenic clones positive by PCR for transgene incorporation and expressing sGFP(S65T).

<sup>2</sup> P<0.01 versus corresponding value for previous conditions (Student’s t test).
size of 0.6 μm, an acceleration pressure of 7.6 MPa, a target distance of 6 cm, and bombardment twice with 0.8 μg of DNA per bombardment resulted in a superior transformation efficiency with soybean embryogenic tissue compared with that achieved in our previous study (El-Shemy et al. 2004). Our results further indicate that transformation protocols optimized for transient gene expression are applicable to the generation of stable transgenic plants.

Acknowledgments

We thank Yasuo Niwa (School of Food and Nutritional Science, University of Shizuoka) for providing the sGFP(S65T) gene and Yumi Naganuma for the technical assistance. M.M.K. and R.S.M. were supported by postdoctoral fellowships from the Japan Society for the Promotion of Science. H.A.E-S. was supported by a postdoctoral fellowship from the Japanese Science and Technology Corporation. This study was supported by funding from CREST (Core Research of Science and Technology) to K.W. and M.I.

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