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Original Paper

Novel plant transformation system by gene-coated gold particle introduction into specific cell using ArF excimer laser

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Abstract Novel methods for foreign-gene introduction into specific cells are required for the functional analysis of specific cells. We report a new transfection method for intact plant cells that uses ArF excimer laser-induced shock waves to introduce foreign gene-coated gold particles into plant cells through the cell wall. Foreign gene (*sGFP*)-coated gold particles were randomly scattered on the surface of *Torenia* tissue samples; the laser was irradiated toward the gold particles using a hollow optical fiber and a quartz light-condensing tip to introduce the particles into the target cells. Treated plant tissue samples were then cultured for several weeks on the selection medium, and transformation was assessed by fluorescence microscopy and PCR. Irradiation-induced damage to the cells and coated DNA was insignificant, and the transformation efficiency was similar to that of biolistic transformation based on the particle number. Although this method requires a suitable laser setup and is time-consuming, it could be applied in transformation experiments on specific target cells. Moreover, this method can possibly be used to introduce not only DNA but also biologically active substances such as protein regulators or inhibitors, whose introduction into cells is difficult through the cell wall.

Key words: ArF excimer laser, cell-specific transformation, gold particle, laser irradiation.

With the progress of biotechnology, novel plant-cell manipulation methods have been sought, particularly for foreign-gene introduction, because such novel methods are necessary for obtaining stable and/or transient transformants (Bock 2001). Several methods are currently available for introducing exogenous DNA into plant cells (Rakoczy-Trojanowska 2002). A wide range of dicotyledonous and monocotyledonous plant species have been transformed by PEG-mediated direct DNA uptake, electroporation, and fusion of protoplasts with liposomes (Prescott et al. 1998). However, these methods require protoplast formation and efficient cell-wall regeneration systems (Neuhaus and Spangenberg 1990). *Agrobacterium*-mediated (Hohn et al. 1989) and biolistic transformations (Daniell 1997) have routinely been used in many experiments for tissue explants and cells with intact cell walls. However, these methods also have their own drawbacks. In the former, the transformation efficiency largely depends on the susceptibility of the host plant; in the latter, the risk of tissue damage cannot be avoided because this method uses gunpowder or high-pressure gas for particle introduction. In addition, these 2 methods transform the cells in the tissue randomly and are not applicable to cell-specific transformation in principle (Neuhaus and Spangenberg 1990; Russell et al. 1993).

In the previous paper, we reported a novel plant cell manipulation technique using ArF excimer laser (Kajiyama et al. 2006). Although this technique enabled cell specific gene introduction into intact plant cell, the damage of the target cell was not avoidable because it required the insertion of glass capillary through the cell wall aperture made by the laser irradiation. Here we describe an alternative method for transfecting foreign materials into intact plant cells. This method uses ArF excimer laser-induced shock waves to introduce foreign DNA-coated gold particles into the cells. It has 2 major advantages: first, the robust delivery of exogenous DNA only into the target cell and second, minimal damage to the tissue samples.

Materials and methods

Plant material, tissue preparation, and regeneration conditions

The plant material was prepared from *Torenia hybrida* cv.

Abbreviations: CaMV, cauliflower mosaic virus; EtBr, ethidium bromide; FDA, fluorescein diacetate; HPT, hygromycin phosphotransferase; RT-PCR, reverse transcriptase polymerase chain reaction; sGFP, synthetic green fluorescent protein; TE, trishydroxymethylaminomethane-ethylenediaminetetraacetic acid

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Summerwave Blue (Suntory Flowers, Japan), an herbaceous plant that can regenerate from apical and stem epidermal cells (Tanimoto and Harada 1984). The *Torenia* plants (4–6 weeks old) were grown in a growth chamber (Model: MLR-350H; Sanyo Electric, Japan) at 25°C under a 16-h light (120 μE m^{-2} s^{-1})/8-h dark cycle. Shoot apices and stem segments were used for gene introduction. The shoot apices were excised under a stereoscopic microscope (Model: SZ60; Olympus, Japan) by using a scalpel and a stainless-steel needle. The stem segments (length, 2–5 mm) were prepared by cutting the second node of mature *Torenia* plants. They were then pretreated in regeneration medium (Murashige and Skoog medium containing 1 mg l^{-1} 6-benzylaminopurine, 3% (w/v) sucrose, and 3% (w/v) gellan gum; pH 5.8) for 2 days prior to exogenous gene introduction.

Laser setup

The laser-irradiation setup used in this study is illustrated in Figure 1. It consisted of a laser oscillator (Model: ArF Excistar S-200; Tuilaser Inc., Germany), a microscope (Model: Ecrips ME600; Nikon, Japan) with a super-long working distance objective lens (Model: CFI Plan EPI SLWD 50X; Nikon, Japan), and a micromanipulator (Model: 5171; Eppendorf Inc., Germany). The laser beam (wavelength: 193 nm, pulse duration: 10 ns) was introduced into a hollow optical fiber (Matsuura and Miyagi 1999) (OD: 680 μm, ID: 500 μm; Soken, Japan) by using a converging lens, and it was condensed at the end of the fiber by using aluminum-coated quartz tips. The condensing quartz tips were prepared from quartz glass capillaries (Model: Q120-90-10; Sutter Inc., USA) by using a CO_2 laser puller (Model: P-2000; Sutter Inc., USA) with the following parameters: heat, 820; fil, 4; vel, 50; del, 130 and the outer surface of the tips were coated with aluminum by using vacuum deposition equipment (Model: VPC-260; Ulvac, Japan). The hollow fiber and laser-condensing tip were attached to the micromanipulator, and the laser could be focused onto any part of the sample surface. The laser energy from the tip was monitored using a power meter (Model: NOVA; Ophir Optronics Ltd., Israel) with a PD-10 or PE-10 counter head (Ophir Optronics Ltd., Israel). Before the laser irradiation toward the plant sample, the periphery of the sample stage was sterilized by 70% ethanol aq. and UV irradiation (10 W for 30 min) and gene introduction procedure was performed under the aseptic condition.

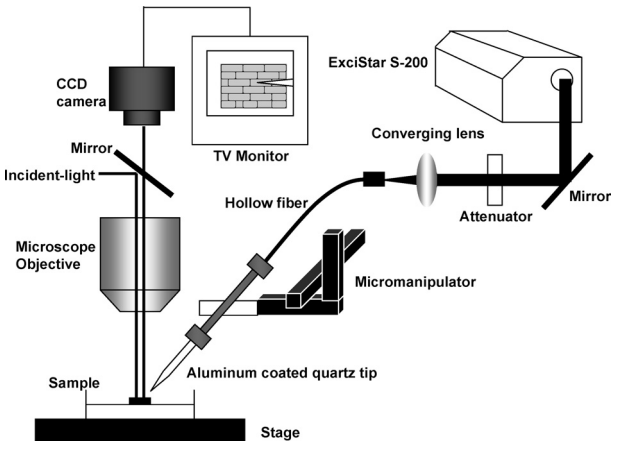

Figure 1. Excimer laser microirradiation setup.

Foreign-gene (plasmid) preparation

The DNA of the pBIsGFP (S65T) plasmid was used as the foreign DNA in this experiment, and it was supplied by Dr. Norihiro Mitsukawa (Toyota Central R&D Labs., Japan). This plasmid comprised CaMV 35S promoter-driven *sGFP* (*S65T*) (Niwa et al. 1999), *HPT*, and the *NOS* terminator. The plasmid was prepared using a plasmid maxiprep kit (Bio-Rad, USA) according to manufacturer's instructions. After assessing the purity by 1% agarose gel electrophoresis, the plasmid DNA was coated onto gold particles (2 μg DNA/mg gold particles) (1-μm gold particles for bombardment, Bio-Rad, USA) according to the Helios Gene Gun instruction manual (Bio-Rad, USA). The gene-coated particles were refrigerated at 4°C until use.

Gene-introduction procedure

Each plant sample, i.e., the pretreated shoot apices and stem segments, was placed on a 0.8% (w/v) agar plate. The foreign DNA-coated gold particles were then randomly scattered on the surface of the tissue samples by using stainless-steel needles. Further, the laser was irradiated toward the gene coated-gold particles with an energy of 3 nJ/shot (energy density: 30 mJ/cm^2, irradiation area: 10 μm^2), using the laser setup (Figure 2). The irradiation area was set to approximately 10 μm^2 by controlling the distance between the sample and the end of the quartz tip with the micromanipulator. The number of particles introduced into a single cell was 1–5/shot. The transfected tissue samples were then transplanted onto the selection medium (Murashige and Skoog medium containing 1 mg l^{-1} 6-benzylaminopurine, 30 mg l^{-1} hygromycin, 3% (w/v) sucrose, and 3% (w/v) gellan gum; pH 5.8) and cultured in the growth chamber at 25°C under a 16-h light (120 μE m^{-2} s^{-1})/8-h dark cycle. Non-irradiated samples and samples introduced with gene-uncoated gold particles were separately prepared and used as controls.

Cell viability test

Twenty-four hours after laser irradiation (1, 3, 10, or 30 nJ/shot), 100 *Torenia* stem epidermal cells were vital-stained with FDA (Marfori et al. 2003), and the viable and nonviable cells were counted under a fluorescence microscope (Model: BX-50 with a U-MWB filter set; Olympus, Japan). The tests were repeated 5 times independently and the mean and standard deviation were calculated.

Estimation of DNA damage upon laser irradiation

The *sGFP* (*S65T*) plasmid-coated gold particles (100 μg) were placed on a 1×1 cm parafilm® square and irradiated with the ArF excimer laser at the energy density of 40 mJ/cm^2 which is *ca.* 30% higher than the energy density used for particle introduction experiments. DNA was extracted from both the laser irradiated and nonirradiated particles by using TE buffer. The extracts were quantified by the comparative Ct method, using the GeneAmp PCR system (Model: 9700; Applied Biosystems) with the forward primer 5′-GGAGCGCACCATC-TTCTT-3′, reverse primer 5′- ATGCCCTTCAGCTCGAT-3′, and the TaqMan probe 5′-AGACCCGCGCCGA-3′. A standard

curve was plotted using a known amount of the DNA sample (data not shown). The thermal cycling conditions were 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. The test was repeated 5 times independently, and the amount of DNA in each sample was calculated by comparing it with the standard curve. Data are presented as means and standard deviations.

PCR for transfected genes

The genomic DNA of the samples, including the adventitious shoots that were generated 6–8 weeks posttransfection, was extracted and purified using the DNeasy Plant Mini Kit (Qiagen, Japan). The DNA was checked for the presence of foreign genes by PCR with the primers 5′-CATCTGGTGGTG-TGAGCAAG-3′ and 5′-ATGCCGTTCTTCTGCTTGTC-3′ for *sGFP* and 5′-GAATTCAGCGAGAGCCTGAC-3′ and 5′-AC-ATTGTTGGAGCCGAAATC-3′ for *HPT*. The thermal cycling conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The PCR products were separated by 1% agarose gel electrophoresis and visualized by EtBr staining. The transformation efficiency was calculated as the percentage of trials with transgene-expressed tissues (from a total of 279 trials for stem epidermis and 277 trials for shoot apex samples).

Fluorescence microscope observation

The tissue samples were checked for *sGFP* expression at 24 h, 72 h, and 1 week after gene introduction by using a fluorescence microscope (Model: BX50 with a U-GFP-A mirror set; Olympus, Japan), and micrographs were obtained using a CCD digital camera system (Model: DP-70; Olympus, Japan). The samples that survived for a week after selection were monitored once a week using the same equipment.

Results and discussion

Effect of the laser irradiation on Torenia epidermal cells

The viability of the laser-treated cells was determined to estimate the extent of irradiation-induced damage. Laser-induced shock waves can be produced when a pulse laser is focused on a solid target (Yoshida 2004). In our experiment, using an aluminum-coated light condensing tip, an ArF excimer laser (wavelength: 193 nm, pulse duration: 10 ns) was focused on gene-coated gold particles placed on the surface of plant cells, and the particles were thus successfully introduced into the plant cells with intact cell walls (Figure 2). ArF excimer lasers have very low permeability, and the laser pulse should not damage important biological molecules within the cells. At an energy of 3 nJ/shot, which was sufficient for particle delivery into the cell, approximately 90% cell viability was retained (Figure 3). However, when the laser energy was increased to 30 nJ/shot, the cell wall

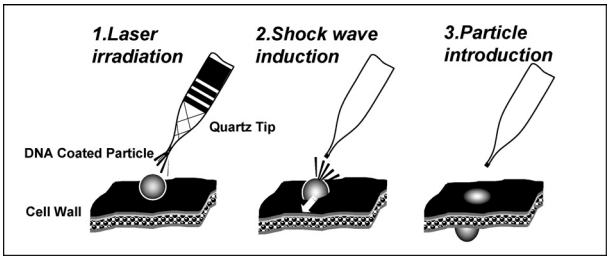

Figure 2. Schematic diagram of particle introduction by laser irradiation. The laser was irradiated through laser condensing quartz tip toward the DNA coated gold particle (1). Upon laser irradiation, the shock wave was induced on the surface of the particle and this generated the impelling force of the particle to smash into the cell inside (2, 3).

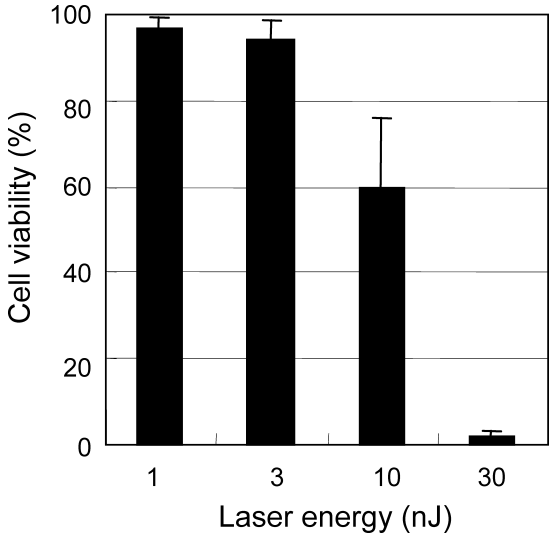

Figure 3. Cell viability of laser-treated *Torenia* epidermal cells 24 h after irradiation. One hundred cells were irradiated at various energies and vital stained with FDA. The values are the percentages of viable cells±SD.

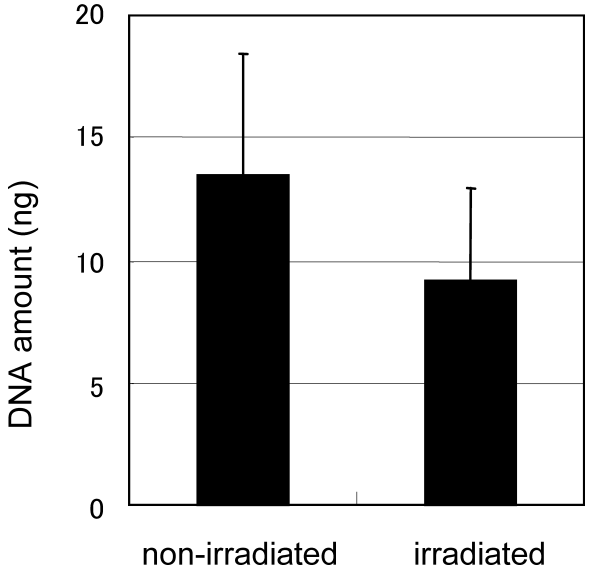

Figure 4. The effect of laser irradiation on DNA. The plasmid pBIsGFP (S65T) was independently extracted from both the laser-irradiated and nonirradiated particles by using TE buffer and quantified by quantitative real-time PCR. The test was repeated 5 times independently. Data are presented as means and standard deviations.

was partially perforated and part of the cytoplasm was observed to be extruded. At an energy of 1 nJ/shot, the particles did not penetrate the cells.

DNA damage upon laser irradiation

The diminution of the coated DNA was also evaluated by quantitative real-time PCR, in case the laser had caused serious DNA damage. Figure 4 shows the amount of DNA recovered from the laser-irradiated and nonirradiated particles. Although approximately 30% of the DNA was depleted by irradiation with the ArF excimer laser at the energy density of 40 mJ/cm^2 (*ca.* 30% higher laser density than that used in particle introduction experiment), over 10^2 copies of DNA per

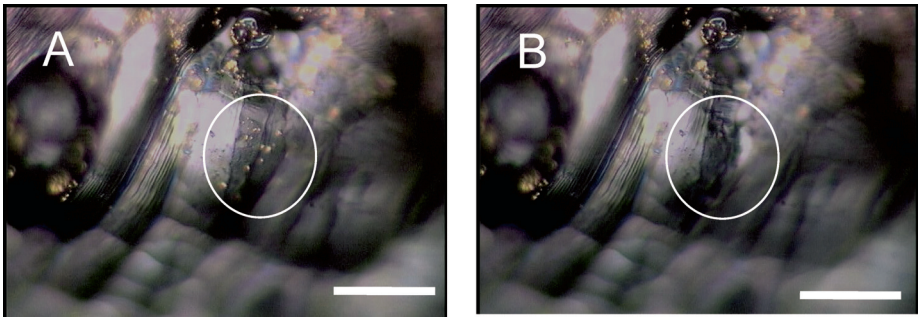

Figure 5. Micrographs of laser-treated samples. *Torenia* epidermal cells prior to (A) and after laser irradiation (B). The circles indicate the targeted gold particles. (Scale bar: 50 μm)

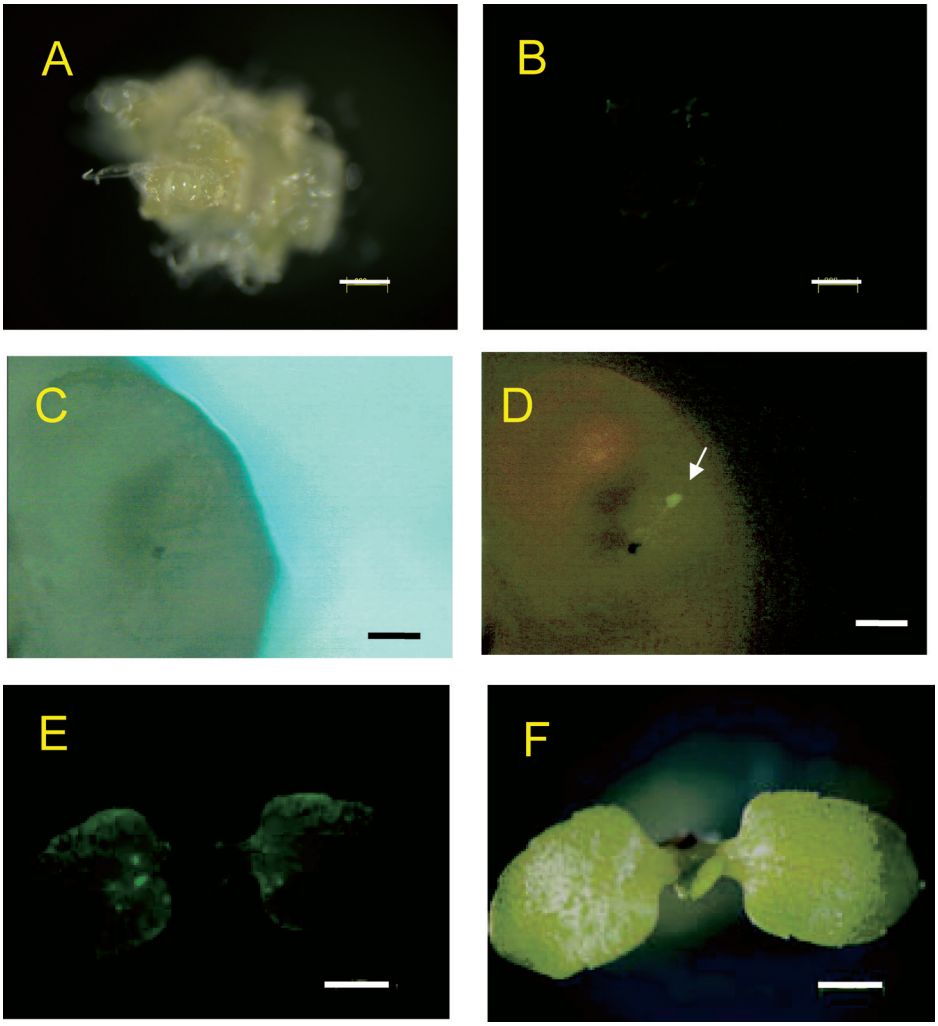

Figure 6. Micrographs of transformed *Torenia* apex samples. Expression of *sGFP* was observed under the fluorescence microscope just after (A), 24 h (C) and 55 days (E) after gene introduction. (B), (D) and (F) are the bright-field images corresponding to (A), (C) and (E) respectively. The arrow in (B) indicates the single cell expressing *sGFP*. (Scale bar: 200 μm in A and B, 500 μm in C and D and 1 mm in E and F)

particle were intact (calculated based on the molecular weight of the plasmid, i.e., 9.3 MDa); according to previous reports, this amount is considered to be sufficient for transformation (Rasco-Gaunt et al. 1999). This result indicates that the laser-irradiation conditions used in this experiment had practically no harmful effect on the foreign genes.

Foreign-gene introduction into Torenia by laser irradiation

Gene introduction into *Torenia* tissue was performed under the irradiation condition decided based on cell-viability and DNA-damage data (3 nJ/shot). Shoot apices (277 samples) and stem segments (297 samples) were tested; 42 of the former and 85 of the latter survived. These viable samples were analyzed for their uptake of the foreign genes. All the control samples died during the selection process.

Analysis of the introduced genes

Figure 5 shows micrographs of the epidermal cells before (A) and after (B) the introduction of the gene-coated gold particles by laser irradiation. The circles indicate the targeted gold particles. Although a slight trace was observed on the cell-wall surface, no major extrusion of the cell contents was observed. The treated tissue samples were observed under a fluorescence microscope and checked for *sGFP* expression (Figure 6). Approximately 10% of the treated samples possessed cell(s) that expressed *sGFP* 24 h after the gene introduction (Figure 6C), whereas the non-transformed samples did not exhibit fluorescence (data not shown). Furthermore, the green fluorescence was not observed around the treated cell in the immediate aftermath of gene introduction procedure (Figure 6A), indicating that the laser treatment did not cause autofluorescense of the treated region. Figure 6E indicates a shoot that developed on the selection medium from the gene-introduced apical cells. Fluorescent microscopic observation of the shoot clearly indicated that the transformant was chimeric. This means that target cells such as germ cells can be selected to produce a complete transgenic plant.

The uptake of foreign genes (*sGFP* and *HPT*) was further confirmed by PCR. Figure 7 indicated the example of PCR results from randomly selected 7 regenerants after gene introduction (lane 1–3: regenerants from shoot apices, lane 4–7: regenerants from stem segments). In this example, the transgenes were observed in one plantlet both in the case of *sGFP* and *HPT*. Based on the trial number, the transformation efficiency was calculated to be approximately 0.4% in both the apical and epidermal samples. In biolistic transformation, the transformation efficiency is approximately 30% based on the trial number (Baum et al. 1997); however, over 10^6 particles are required per trial in this method (Helios

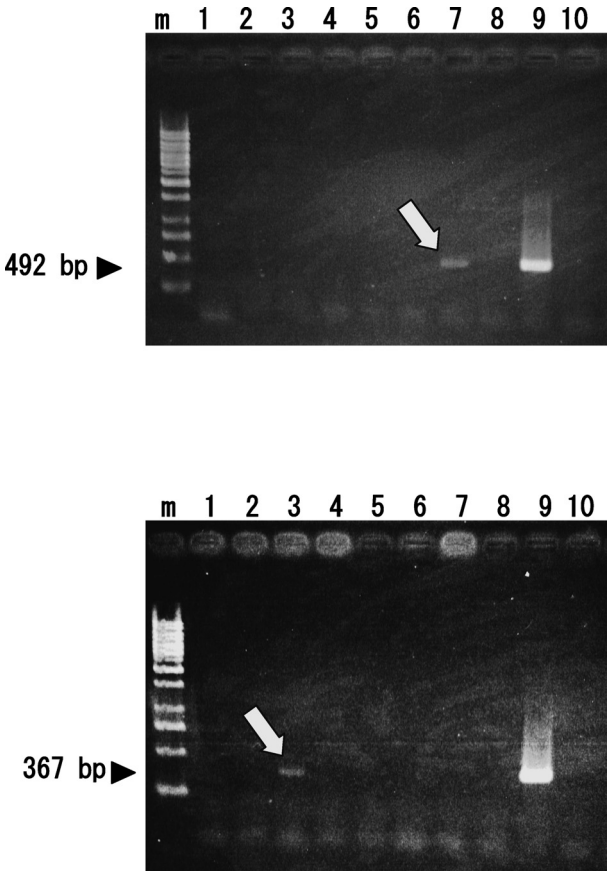

Figure 7. PCR detection of transgenes (*sGFP* (*S65T*) and *HPT*) in shoots after gene introduction. (A) 492-bp fragment of *sGFP* (*S65T*). (B) 367-bp fragment of *HPT*. m: marker; 1–3: regenerants arising from shoot apices; 4–7: regenerants arising from stem segments; 8: non-transformant (negative control); 9: pBIsGFP (S65T) (positive control); and 10: water. The white arrows indicated the positive bands.

Gene Gun manual, Bio-Rad). In contrast, our method requires only 1–10 particles per trial. Therefore, the transformation efficiency can be considered to be even higher than that of biolistic transformation when compared in terms of the number of particles introduced. Moreover, the biolistic and *Agrobacterium*-mediated methods cannot be used for cell-specific transformation, e.g., in transient gene-suppression experiments using RNAi constructs. In this regard, our new method is a beneficial transformation method.

In conclusion, we have developed a new approach for the transfection of exogenous materials, e.g., foreign genes, into intact plant cells. In this method, damage to the target tissue is minimal, and foreign genes are robustly introduced only into the targeted cell. This method differs from other transfection methods in this regard. Although this method requires a suitable laser setup and is time-consuming, these drawbacks are expected to be overcome by automated equipment. Moreover, this method can possibly be used for the introduction of not only DNA but also biologically active substances such as protein regulators or inhibitors, whose

introduction into cells is difficult through the cell wall. The use of this method for the introduction of protein compounds is currently under investigation.

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