

A simple agroinfiltration method for transient gene expression in plant leaf discs

Kouki Matsuo,* Noriho Fukuzawa, and Takeshi Matsumura

Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan

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In the present study, we developed a simple transient gene expression system based on *Agrobacterium*-mediated transformation. Vacuum infiltration was applied to leaf discs from *Nicotiana benthamiana* plants with *Agrobacterium* suspension solution under conventional vacuum conditions in a needleless plastic syringe. Model proteins, green fluorescent protein, β -glucuronidase, mouse granulocyte-macrophage colony-stimulating factor, and human fibroblast growth factor 1 were successfully expressed in leaf discs within 4 days after infiltration. In addition, the functional evaluation of viral RNA silencing suppressors, *Artichoke mottled crinkle virus* p19 protein, was also performed. Using this method, the contamination and diffusion of genetically modified bacterium to the environment and important transgenic plants were prevented. This method can be conducted without specialized apparatuses or large amounts of *Agrobacterium* suspension solutions; thus, the simultaneous evaluation of multiple vectors will be easily possible.

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Transient gene expression systems have been widely used for the functional characterization of genes and recombinant protein production in plant cells. Both agroinfiltration (1) and plant viral vectors (2–4) have been frequently used for transient gene expression in plants. These methods have many advantages, including simple, rapid, and effective transformation compared with stable genetic transformations. The development of plant virus-based vectors has allowed a rapid and high-level transient expression of recombinant proteins in plants. However, the application of plant viral vectors is restricted by both the host range and size of the gene of interest without disturbing the virus particle assembly. The high cost of *in vitro* RNA transcription is also a marked problem with this system.

Particle bombardment has also been used for transient gene expression (5). However, this method requires special equipment for the transfer of genes into plant cells. Recently, *Agrobacterium*-mediated systems, agroinfiltration, have been aggressively used for the transient expression of genes, assessment of vector constructs, and production of recombinant proteins in plant cells. In agroinfiltration systems, a transgenic *Agrobacterium* with a binary expression vector is directly introduced into plant leaves. The gene of interest can be expressed for several days (depending on the recombinant protein and the host) after infiltration.

Agrobacterium-mediated systems are roughly divided into two methods, namely agroinfiltration using a needleless syringe (syringe infiltration) and vacuum infiltration (1,6). In syringe infiltration, an *Agrobacterium* suspension is infiltrated into the abaxial side of the leaves using a needleless syringe. Using this method, the ability of various constructs can be simultaneously assessed on the

same leaf. However, recombinant protein accumulation is markedly influenced by leaf age and leaf position effects (7); thus, the assessment of the constructs requires careful evaluation. For this reason, a method that enables simpler and more uniform evaluation of expression vector constructs is desired. The vacuum infiltration method is difficult to be conducted easily because vacuum equipment and a high amount of bacterial suspension are required for this operation. Thus, similar to plant viral vector systems, the high cost of a vacuum infiltration system is a marked problem (8). Furthermore, agroinfiltration applied plants were genetically polluted. Therefore, the application of agroinfiltration to important transgenic plants, such as T0 generation plants, should be avoided.

Zheng et al. (9) reported a conventional *Agrobacterium*-mediated transient gene expression system. In their system, seedlings and plantlets were co-cultivated with MS solution containing *Agrobacterium* for several days and transiently expressed the β -glucuronidase (GUS) gene. However, as the seedlings and plantlets were genetically polluted, further investigation of these plants was difficult. A high-throughput leaf disc-based infiltration assay that allows the assessment of expression constructs for production of recombinant proteins has also been reported (7). However, their method needed a custom-made leaf disc holder and an infiltration tank, making it difficult to be conducted in other laboratories. In contrast, a floating leaf disc assay using a needleless syringe and leaf discs has been used for photosynthesis (<http://www.grochbiology.org/FloatingLeafDiskAssay.htm>) and photoacoustic studies (10). Leaf discs in these studies were placed into a syringe body with appropriate infiltration solution. The leaf discs were infiltrated with the solution under conventional vacuum conditions made by the needleless syringe. In the present study, a simple and high-throughput transient gene expression system based on a combination of the floating leaf disc assay and the vacuum infiltration is described.

* Corresponding author. Tel.: +81 11 857 8994; fax: +81 11 857 8927.

E-mail address: matsuo-kouki@aist.go.jp (K. Matsuo).

MATERIALS AND METHODS

Agrobacterium for transient gene expression The plasmids pBE2113:GFP, pBE2113:aFGF, pBE2113:GM-CSF, and pBE2113:Hc-Pro were constructed based on the pBE2113 binary vector for the expression of green fluorescent protein (GFP), N-terminal polyhistidine-tagged (His-tag) human fibroblast growth factor 1 (aFGF), N-terminal His-tagged mature mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), and helper component-proteinase (Hc-Pro) genes, respectively. pBI121:p19, a pBI121-based expression vector for the production of *Artichoke mottled crinkle virus* p19 (11), a RNA silencing suppressor, was kindly provided by Dr. Benvenuto (ENEA, Italy). In pBI121:p19, Ω leader sequence was inserted into the 3'-end of Cauliflower mosaic virus 35S promoter sequence. For GUS expression, pBE2113 was used without any modifications. The binary vectors used in this study are shown in Fig. S1. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the respective expression vectors and used for the transient expression of these genes.

Leaf disc vacuum infiltration The *Agrobacterium* transformed to express the genes of interest were cultured in LB medium containing kanamycin (50 $\mu\text{g/mL}$), streptomycin (300 $\mu\text{g/mL}$), and rifampicin (100 $\mu\text{g/mL}$) overnight at 28°C with vigorous shaking. The *Agrobacterium* cells were then collected by centrifugation at 4800 $\times g$ for 10 min at room temperature. The pellet was resuspended in 10 mM MES-KOH (pH 5.7) containing 10 mM MgCl_2 and 150 μM acetosyringone. The *Agrobacterium* suspension solutions were placed at room temperature for at least 1 h before use. Bacterial concentrations were determined by measuring optical density (OD) at 600 nm. In a typical experiment, 10 mL of the bacterial suspension and a 20 mL plastic syringe (no needle) were used for each individual operation. Immediately before use, 10% Tween 20 solution was added to the bacterial suspension (final 0.01% (v/v)). For double-expression experiments, equal volumes of equal density bacterial suspension were combined and used for infiltration.

Leaf discs were cut from *Nicotiana benthamiana* leaves approximately 7–8 weeks after germination using a cork borer (ϕ 8.5 mm). After removal of the plunger from a 20-mL plastic syringe, leaf discs (10–50 discs per syringe) were placed into the body of the syringe followed by insertion of the plunger. *Agrobacterium* suspension solution was poured into a petri dish, and the tip of the syringe was inserted into the *Agrobacterium* suspension solution to draw all solution (10 mL) into the syringe. Air was removed from the syringe by holding the syringe tip upward and carefully depressing the plunger. The tip of the syringe was then sealed using quadruple-stacked parafilm held tightly, and the syringe was shaken vigorously to remove any discs from the wall of the syringe. Next, the plunger was pulled to create a small vacuum in the syringe (in a typical experiment, the plunger was pulled 1 mL). After vigorous shaking, the plunger was rapidly released. These infiltration steps were able to be repeated up to three times, but excessive operation was noted to cause leaf disc death. After infiltration, the parafilm was removed after pulling the plunger a little to prevent scattering of bacterial suspension. The bacterial suspension was then discarded, and the infiltrated leaf discs removed from the syringe. After removal of excess bacterial suspension, the well-infiltrated leaf discs were selected and incubated upper side down in Petri dishes with MS medium containing 3% sucrose, miconazole (10 mg/L) (12), and 0.8% agar under lighting programs (16 h of light: 8 h of dark) at 23°C.

GUS histochemical assay For histochemical staining, the *Agrobacterium*-infiltrated leaf discs were washed twice with 50 mM Tris-HCl buffer (pH 7.5) containing carbenicillin (500 $\mu\text{g/mL}$) and then twice with distilled water. The washed leaf discs were prefixed with chilled 90% (v/v) aqueous acetone and then washed with chilled water. They were then immersed in GUS staining solution consisting of 50 mM phosphate buffer (pH 7.2), 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 0.2% (v/v) Triton X-100, and 2 mM X-Gluc (5-bromo-4-chloro-3-indoxyl- β -D-glucuronide cyclohexylammonium salt). They were then incubated overnight at 37°C. Stained leaf discs were successively submerged in 25%, 50%, 70%, and 95% (v/v) ethanol to remove chlorophyll.

Real-time reverse transcriptase-polymerase chain reaction To evaluate the function of the p19 and Hc-Pro genes, these genes were co-expressed with the aFGF gene. *Agrobacterium* suspension solutions for expression of aFGF, GFP, GUS, p19, and Hc-Pro genes were mixed and used for transient gene expression experiments. Before total RNA and protein extraction, leaf discs were washed twice with 50 mM Tris-HCl buffer (pH 7.5) containing carbenicillin (500 $\mu\text{g/mL}$) and then twice with distilled water. Total RNA was extracted from the five leaf discs with an RNeasy plant mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. Contaminated genomic DNA in the total RNA was degraded with TURBO Dnase (ThermoFisher scientific, Waltham, MA, USA). One microgram of total RNA was used to generate first-strand cDNA using a PrimeScript II first-strand cDNA synthesis kit (Takara Bio, Otsu, Japan) with a random hexamer primer. For real-time reverse transcriptase-polymerase chain reaction (RT-PCR), 5 μL of each of the RT reaction mixtures (1:100 dilution) and 0.2 μM of the respective primers were used in 25 μL of PCR reaction solution using iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA). The primer pair aFGF-F (5'-TGCTCCAGGGAATTACAAG-3') and reverse primer aFGF-R (5'-GTATAAAGCCGTCGGTGTG-3') was used for aFGF-specific amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a housekeeping gene using the forward primer GDH-L-F (5'-

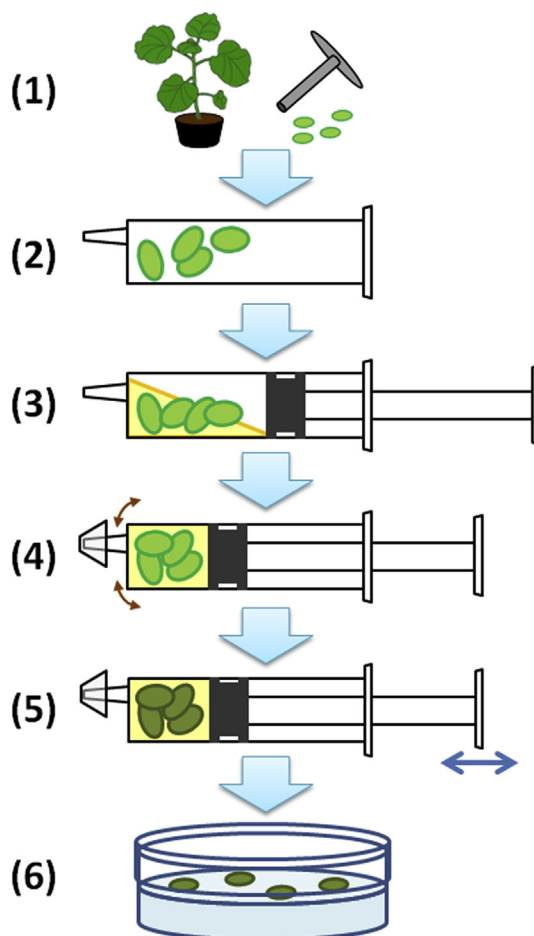


FIG. 1. Summary of the present infiltration method. 1, Cut leaf discs (ϕ 8.5 mm, 10–50 discs) from *Nicotiana benthamiana* plant. 2, Place the leaf discs into the body of the 20-mL plastic syringe, and then insert the plunger. 3, Draw up 10 mL of *Agrobacterium* solution suspended with 10 mM MES-KOH buffer. 4, Remove air from the syringe, and then seal the tip of the syringe using stacked parafilm or a plastic cap. Shake the syringe vigorously to release the discs from the inside wall of the syringe. 5, Pull the plunger approximately 1 mL to create a small vacuum in the syringe, shake vigorously then release the plunger rapidly. 6, Incubate leaf discs on MS agar plate (23°C, 16 h light/8 h dark).

GGAGGAGGGAACAACAAGAGG-3') and reverse primer GDH-L-R (5'-AGATGCCCTCAGTGCCGA-3') (13). To estimate contamination of DNA in the total RNA, real-time RT-PCR was also conducted using the total RNA as a template.

Estimation and detection of recombinant proteins Concentration of total soluble protein was estimated using a DC protein assay kit (Bio-Rad). Bovine serum albumin was used as a standard protein. The production of His-tagged human aFGF was confirmed by Western blot analysis using anti-FGF-1, a mouse-monoclonal (2E12) antibody (Abnova, Taipei, Taiwan) as a primary antibody. Recombinant Human FGF acidic (aa 2–155) (R&D Systems, Minneapolis, MN, USA) was used as a standard. The amount of recombinant aFGF was estimated by enzyme-linked immunosorbent assay (ELISA) using human FGF acidic DuoSet ELISA development system (R&D Systems) according to the manufacturer's instructions. The production of His-tagged GM-CSF was confirmed by Western blot analysis using GM-CSF antibody (Rockland Immunochemicals, Limerick, PA, USA) as the primary antibody. His-tagged GM-CSF was purified using His Mag Sepharose Ni (GE Healthcare, Uppsala, Sweden) and then subjected to Western blot analysis. Production of GFP was observed under blue LED light. GFP expression level in leaf discs (average fluorescent value of ten leaf discs) was estimated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

RESULTS

Transient expression of GFP and GUS genes A summary of the method presented in this study is shown in Fig. 1. Briefly, leaf discs from the *N. benthamiana* plant were placed into the body of

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