Research Article

Stable Transformation of the *Saintpaulia ionantha* by Particle Bombardment

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Background: A highly efficient genetic transformation system is essential for a successful genetic manipulation of the African violet (*Saintpaulia ionantha* Wendl.).
Objectives: Developing a particle bombardment-based genetic transformation system for the African violet.
Materials and Methods: A local cultivar of the African violet from Guilan province was used for transformation experiments. The pFF19G and pBin61-Ech42 vectors were used for transient and stable transformation experiments, respectively. The PCR and RT-PCR techniques were used to verify transgene presence and transcript levels in candidate transgenic lines, respectively.
Results: Using leaf explants as target tissues, we transferred an endochitinase gene cDNA into African violet. Transgenic plants were regenerated on selection medium at a reasonable frequency (in average, one stable transgenic line per shot). Molecular analysis of transgenic plants by PCR and RT-PCR techniques confirmed successful integration and expression of transgene in several independent transgenic lines.
Conclusions: Our results provide an efficient stable transformation system for genetic transformation of African violet.

Keywords: African violet; Biolistic; Genetic transformation; *Saintpaulia ionantha*

1. Background

Saintpaulia ionantha Wendl. (African violet) is one of the most important ornamental plant species of the genus *Saintpaulia* from *Gesneriaceae* family. To date many varieties of African violet have been produced with different colors and shapes largely using conventional breeding methods. Genetic engineering has proved to be a powerful complementary tool for conventional plant breeding. Efficient genetic transformation protocols are essential for successful genetic manipulation and for African violet, *Agrobacterium*-mediated transformation has prove to be successful (1, 2). However, *Agrobacterium* includes limited host range with relatively low success rate (3). In contrast, particle bombardment has proved to surpass such problems (4). Furthermore, biolistics is independent of using microorganisms as a mean of gene delivery mechanism. Moreover, the method is not based on special vector requirements; and foreign DNA of any conformation and size can be delivered into cells (5).

2. Objectives

Here, for the first time, we report a reliable biolistic-based genetic transformation system for African violet.

3. Materials and Methods

3.1. Plant Material and Sterilization Method

A local cultivar of African violet from Guilan province was used for transformation experiments. For surface sterilization, plant materials were washed under running tap water and disinfected with 70% (v/v) ethanol for 30 s followed by incubation in 5% (v/v) commercial bleach for 15 min. The plant segments were washed with sterile water under aseptic conditions and cultured on MS (Murashige and Skoog) medium (19).

3.2. Plant Transformation

The pFF19G vector (20) harboring the GUS gene under the control of CaMV 35S gene promoter and terminator, was used for transient integration of GUS gene into leaf tissues of African violet by biolistic method using a Bio-Rad Helium-driven PDS-1000/He particle gun (Bio-Rad Laboratories, Inc, USA).

Rupture disk membranes for the helium pressure of 1100 or 1350 PSI were used for proper experiments. Embryogenic calli were placed 5 or 12.5 cm away from the macrocarrier position. A vacuum of -0.8 bar was applied and the particles were discharged following the rupture membrane break by the helium. For stable transformation, leaves of *in vitro* grown sterile plants were placed from their adaxial side on regeneration medium (MS medium complemented with 1 $mg.L^{-1}$ BAP). The leaves were bombarded with plasmid-coated (pBin61-Ech42 (21)) gold particles. Bombarded leaves were cut into 3 mm^2 pieces, placed on selection medium (regeneration medium containing 50 $mg.L^{-1}$ kanamycin) followed by incubation in growth chamber (diurnal cycle of 16 h light of 25 $\mu E.m^2s^{-1}$ at 25°C followed by 8 h dark period at 22°C).

3.3. Histochemical GUS Staining

For histochemical GUS staining, bombarded leaves were incubated in GUS staining solution [1 mM EDTA (pH 8), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 200 mM potassium phosphate (pH 7.0), 1% Triton-X-100, 1 $mg.mL^{-1}$ X-Gluc] for 24 h at 28°C. To remove leaf pigments, stained leaves were incubated for 1 h in 50% (v/v) ethanol followed by incubation in absolute ethanol for several hours at 22°C.

3.4. Isolation of Nucleic Acids

Total genomic DNA was isolated from shoots of transgenic and wild-type plants using a cetyltrimethylammoniumbromide (CTAB)-based method (22). Total cellular RNA was extracted from leaves of transgenic lines and wild-type plants with TRIzol® reagent (Life Technologies, Carlsbad, USA) following the manufacturer's instructions. For removal of any contaminating genomic DNA, the samples were treated with RNase-free DNase I (Fermentas Inc, Burlington, USA).

3.5. Polymerase Chain Reactions (PCR)

PCR was used to verify transgene presence in candidate transgenic lines. The PCR program for *Ech42* gene (primer pair: *Ech42*-Forward (*Xba*I): 5′-A TTCTAGA ATG TTG GGC TTC CTC GGA AAA T-3′, *Ech42*-Reverse*(Bam*HI): 5′-A TGGATCC CTA GTT GAG ACC GCT TCG GAT-3′) was: an initial denaturation step at 94°C for 4 min followed by 35 cycles of 94°C for 40 s, 64°C for 30 s, and 72°C for 30 s, and a final 10-min extension at 72°C.

3.6. Reverse-Transcription Polymerase Chain Reactions (RT-PCR)

Reverse-Transcription PCR (RT-PCR) was used to evaluate the *Ech42* expression at transcript levels. Briefly, cDNA was synthesized using similar amounts of RNA (around 4 µg) from each sample using a Superscript III RT kit according to the manufacturer's instructions (Life Technologies, Carlsbad, USA). PCR was performed on similar amounts of cDNA using above mentioned program. The housekeeping gene, 16S rRNA (primer pair: 16S rRNA-For: 5′-GGA GCG GTG AAA TGC GTA GAG-3′, and 16S rRNA-Rev: 5′-TAC GGC TAC CTT GTT ACG AC-3′), was used as an internal control for normalization.

3.7. Statistical Analysis

The adopted experimental design was completely randomized with three replicas. The statistical comparison was performed by One-Way ANOVA using SPSS software version 19.0, and means were compared using the Duncan's Test ($P < 0.05$).

4. Results

4.1. Optimizing the Biolistic Transformation Parameters for African violet

Given that many parameters are involved in efficiency of biolistic method (23), we first aimed to optimize several main parameters for African violet transformation. In the first experiment, we set up rupture disk pressure and the distance between stopping screen and the target tissue. Two distances (5 and 12.5 cm) and two rupture disk pressures (1100 and 1300 PSI) were tested. Transient transformation of single sterile leaves of the same size and age using 0.6 µm Gold particles coated with pFF19G vector containing GUS expression cassette showed that the highest transformation efficiency (Evaluated through approximate number of blue spots on bombarded leaves following histochemical GUS staining procedure; Table 1) occurred at 5 cm distance from stopping screen at 1350 PSI (data not shown) that was used for the next experiment. In a second experiment, we included several other parameters including leaf orientation (abaxial/adaxial sides), particle type (tungsten/gold), and gold particle size (0.6, 1 and 1.6 µm). The best transient transformation and expression rates were resulted with leaf abaxial and 0.6 µm gold particles (Figure 1).

Data were analyzed using SPSS statistical software, and means were compared using the Duncan's Test. Values indicated by different letters in the superscript, show significant difference at $p<0.05$.

Next, we tried to use the optimized parameters to stably transform African violet with a functional gene. A construct (pBin61-Ech42) (21) containing *Ech42*

Table 1. The rough number of blue spots on single leaves of *S. ionantha* bombarded with pFF19G-coated particles following by histochemical GUS staining procedure. The values are means of three replicas derived from rough counting of blue spots on single bombarded leaves under a biological loupe (10× mag).

Particle type	Particle size (μm)	Leaf orientation	
		Abaxial	Adaxial
Tungesten	0.7	165 ± 48^{b}	112 ± 22^{c}
Gold	0.6	278 ± 32^{a}	176 ± 28^{b}
Gold	1	153 ± 8^{b}	113 ± 15^{c}
Gold	1.6	160 ± 35^{b}	123 ± 51^{bc}

expression cassette as well as *nptII* selectable marker gene was used in bombardment of African violet leaves. Bombarded leaves were placed on 50 $mg.L^{-1}$ kanamycin-containing medium in about 3 mm^2 pieces. After 4-6 weeks, several green shoots were regenerated (Figure 2A). To reduce the possibility of non-transgenic cell line escapes on the selection medium, surviving cell lines were transferred to second round of selection on higher (100 $mg.L^{-1}$) kanamycin concentrations. In average, 5 independent antibiotic-resistant plants were recovered from each bombarded plates after two selection rounds. Seven well-grown green plants were selected for further molecular analysis (e.g., Figure 2B, C).

4.2. Molecular Analysis of Several Putative Transgenic Candidates

To confirm the physical presence of the *Ech42* transgene in the selected lines, PCR analysis was performed on seven independent plants survived on the second selection round. By PCR analysis using *Ech42* gene specific primer pair, a 1275 bp fragment was amplified from 4 out of 7 lines (~57%) (Figure 3), demonstrating successful integration of transgene into the genome of these plants. PCR-negative kanamycin-resistant plants possibly were resulted from mutations or escapes.

To demonstrate *Ech42* expression in putative PCR-positive plants, RT-PCR was carried out on the PCR-positive transgenic lines. Using above-mentioned *Ech42*-specific primer pair, in 3 out of 4 independent PCR-positive transgenic lines, a 1275 bp fragment was amplified from cDNA, confirming successful expression of *Ech42* transgene in these lines at transcription level (Figure 4). Note that the complete removal of any

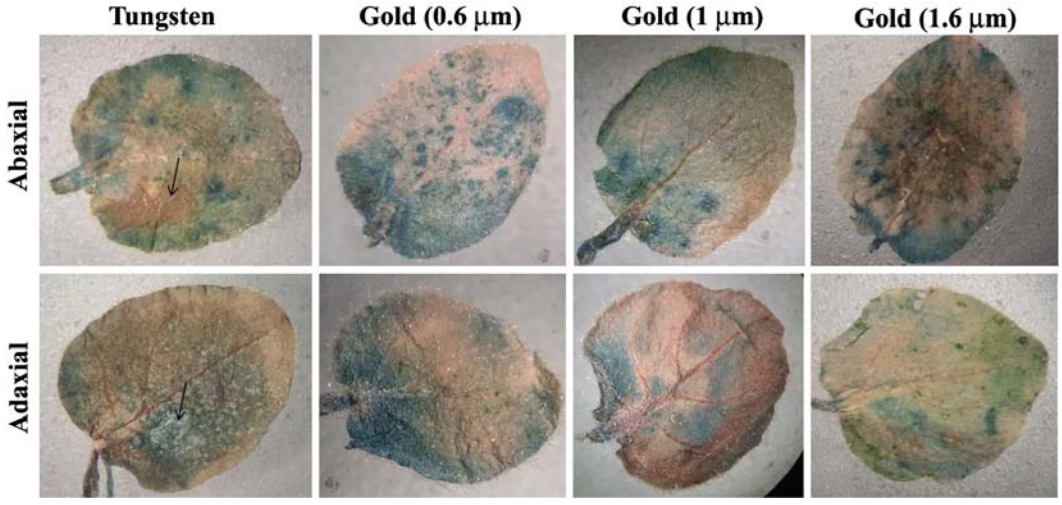

Figure 1. Histochemical GUS staining of single *in vitro* grown leaves of *S. ionantha* bombarded with pFF19G-coated tungsten/gold particles in different sizes. The distances of stopping screen to target tissue and the rupture disk pressures were adjusted to 5 cm and 1300 PSI, respectively. Photos are the best results of each treatment from three shots using mono-adaptor. Examples of leaf damage in tungsten-based experiments are indicated by arrows

Figure 2. Shoot regeneration from bombarded leaves of *S. ionantha* on selection media and adaptation of PCR-positive transgenic candidates. A: Green shoot regeneration (arrows) from African violet leaf pieces after 5 weeks incubation of bombarded leaves with pBin61-Ech42 vector, on 50 $mg.L^{-1}$ kanamycin-containing selection medium. B: Shoot multiplication, elongation and root formation in putative transgenic lines on hormone-free MS medium. C: Adaptation of several plants from putative transgenic lines in soil

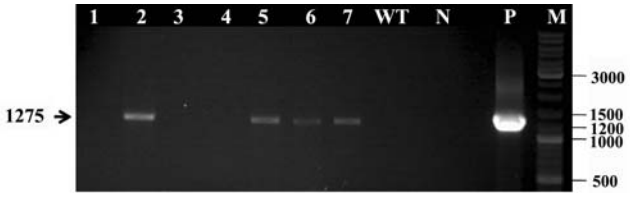

Figure 3. PCR analysis to study the presence of *Ech42* gene in putative transgenic candidates regenerated on selection medium from *S. ionantha* leaves bombarded with pBin61-Ech42 vector. Observation of a 1275 bp fragment in four independent lines (2, 5, 6 and 7), demonstrates successful integration of *Ech42* gene into the genome of these lines. N: negative control (without DNA), P: positive control (pBin61-Ech42 vector)

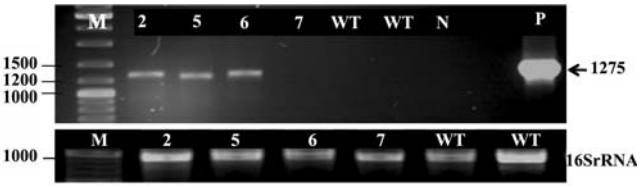

Figure 4. RT-PCR analysis to study the expression of *Ech42* gene at transcript levels from PCR-positive transgenic lines obtained from *S. ionantha* leaves bombarded with pBin61-Ech42 vector. Observation of a 1275 bp fragment in three independent transgenic lines (2, 5 and 6), verifies successful transcription of *Ech42* gene in these lines. The 16S rRNA was used as an internal control to normalize gene expression. N: negative control (without DNA), P: positive control (pBin61-Ech42 vector)

contaminating genomic DNA was proved by PCR analysis on the purified RNA samples, where no band was observed (data not shown). The lack of *Ech42* cDNA band in lane 7 (Figure 4) most likely is due to its integration in an untranscribed region of the genome. However, the gene could also have lost its transcription signals by mutation (24).

5. Discussion

S. ionantha is an important ornamental pot plant grown on a large scale both commercially and by amateurs. Several tissue culture and regeneration protocols have been established for African violet using different explant sources including leaves (6-12), anther (13), sub-epidermal tissue (14), protoplast (15, 16), petioles (8, 12) and floral buds (17, 18). In this study, we developed a biolistic transformation system for *S. ionantha*. In principle, *Agrobacterium*-mediated transformation and particle bombardment are two transformation methods for most plants. Because of limitations and disadvantages of each method (4, 25-27), that vary even for different genotypes of one species, the existing of alternative methods can be helpful for improving molecular breeding programs. *Agrobacterium*-mediated transformation has been previously described for African violet (1, 2); however, to the best of our knowledge, biolistic transformation method has not been reported yet. High genotype dependency is

the major obstacle of *Agrobacterium*-mediated transformation method, while, due to its simple physical principle, biolistics represents a universal genetic transformation technology (4). Although the target tissue damage is a crucial disadvantage of this procedure, however the method has successfully been used in nearly all groups of organisms (5, 28, 29). Moreover, it also provides the unique opportunity for stable transformation of mitochondria and plastids (28, 30-32). Here and for the first time, we describe a novel biolistic-based transformation system for African violet as an alternative method. Several important parameters involved in the efficiency of the particle bombardment system were adjusted. The parameters were rupture disk pressure, distance between stopping screen and the target tissue, leaf orientation, particle type, and particle size. Accordingly a stable transformation system using adjusted parameters was developed using mature leaves. Selection and regeneration of transgenic plants were also performed by direct organogenesis using routine tissue culture systems (6). Although the efficiency of the transformation and regeneration procedures can likely be improved by optimizing other parameters, the system described here is very time-saving that may reduce the possibility of somaclonal variation. This transformation system will provide a powerful alternative method for future improvement of African violet for important traits.

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