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In planta Transformation Method for T-DNA transfer in orchids

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Abstract. Transgenic plant technology is an efficient tool to study the function of gene(s) in plant. The most popular and widely used technique is *Agrobacterium*-mediated transformation in which cocultivation was done by immersing the plant tissues/organ in overnight bacterial cultured for about 30 minutes to one hour under *in vitro* condition. In this experiment, we developed more easier technique that omitted the *in vitro* step during cocultivation with Agrobacterium, namely *in planta* transformation method. Pollinaria (compact pollen mass of orchid) of *Phalaenopsis amabilis* and *Spathoglottis plicata* orchids were used as target explants that were immersed into bacterial culture for 30 minutes, then dried up the pollinaria, the transformed pollinaria was used to pollinate orchid flowers. The T-DNA used for this experiments were Ubipro::PaFT/*A. tumefaciens* GV3101 for *P. amabilis* and MeEF1α2 pro::GUS/ *A. tumefaciens* LBA 4404 for *S.plicata*. Seeds that were produced from pollinated flowers were grown onto 10 mg/l hygromicin containing NP (New *Phalaenopsis*) medium. The existance of transgene in putative transformant *protocorm* (developing orchid embryo) genome was confirmed using PCR with specific primers of either *PaFT* or *GUS* genes. Histochemical GUS assay was also performed to the putative transformants. The result showed that transformation frequencies were 2.1 % in *P. amabilis,* and 0,53% in *S. plicata*. These results indicates that *in planta* transformation method could be used for Agrobacterium-mediated genetic transformation, with advantage easier and more secure work from contaminants than that of the *in vitro* method.
Key words: Genetic transformation, *Agrobacterium, in planta*, pollinaria, orchids
PACS: 87.18.Cf

INTRODUCTION

Agrobacterium-mediated genetic transformation in orchids is now widely used. Successful methods are depending on the success of *Agrobacterium*/Ti plasmid system integrate the T-DNA into plant cell's genome, that has been facilitated by (1) the construction of modified *Agrobacterium* strains in which the genes responsible for pathogenicity have been deleted; (2) the design of intermediate vectors containing selectable markers for introducing foreign genes into the Ti plasmid and subsequently into plant cells; and (3) the development of efficient *in vitro* methods for transforming plant cells and tissues with engineered *Agrobacterium* strains [1]. The use of *in vitro* procedure in genetic transformation method often causes difficulties that resulted in failures of producing transformed plants. Therefore, modification of genetic transformation without *in vitro* inoculation procedure is needed. Chang *et al* [2] has published a stable genetic transformation method of *Arabidolpsis thallana* by simple *in plants* inoculation of *A. tumefaclens* strain LBA4404 harboring a binary vector pBI121 severing of apical shoots at their bases, that called *in planta* transformation. On average, 5.5% of the newly formed shoots produced transformed progenies, that confirmed by Southern hybridization method and *in situ* histochemical GUS assay. This simple, efficient, and reproducible transformation system has been developed in Dendrobium orchid using some modifications (our unpublished data), but the

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efficiency of transformation was still very low. In our previous works [3, 4], we established a method of Agrobacterium-mediated transformation using intact protocorms as target materials, but it still could not avoid *in vitro* innoculation procedure. In this work, we modified the *in planta* transformation procedure using pollen (pollinia and pollinaria) of two orchid species as target materials,
i.e a terrestrial sympodial orchid *Spathoglottis plicata* and epiphytic monopodial orchid *Phalaenopsis amabilis*. *In planta* transformation method was conducted by immersed the pollen in overnight cultured *Agrobacterium*, then the inoculated pollen were used to pollinate its flower (self pollination). The new generation from the silique were analyzed by both morphological and molecular approaches. The foreign genes are expected to be stably maintained and expressed in the resulting plants generated from the seeds and are inherited by progeny as typical Mendelian traits. The improvement technology for gene transfer in orchid plants will facilitate numerous studies on gene expression and regulation in orchids.

MATERIALS AND METHODS

Plant Materials and Bacterial Strains

Flowering plants of terestrial orchids *Spathoglottis plicata* and epyphitic orchids *Phalaenopsis amabilis* were used as plant materials.

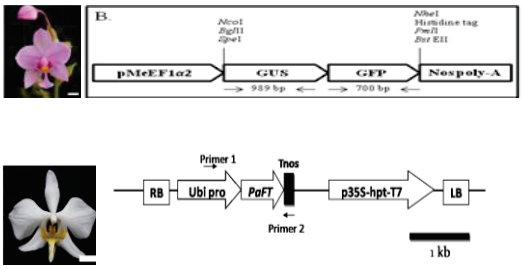


FIGURE 1. Structure of the T-DNA of plasmids MeEF1α2pro::GUS::GFP for *S. plicata* and pGAS102 for *P. amabilis*

Agrobacterium tumefaciens strain GV 3101 harboring the binary vector pCambia 1303 with MeEF1α2pro::GUS::GFP and 35Spro::GUS::GFP containing T-DNA (Fig. 1A) [5] were used for genetic transformation in *S. plicata*. A. *tumefaciens* strain LBA 4404 harboring pGA3426 (vector only) and pGAS102 that carry *Ubi*-pro*::PaFT* (P. *amabilis* Flowering Locus-T) for genetic transformation in *P. amabilis* (Fig. 1B).

In planta Transformation

Bacterial colonies of *Agrobacterium* that carry desired T-DNA were inoculated in 5 ml of liquid LB medium containing 100 $mg.L^{-1}$ Kanamycin, also Agrobacterium that carry pGA3426 and pGAS102 (*Ubi-pro::PaFT*) were inoculated in 5 ml of liquid LB medium with addition of 5 $mg.L^{-1}$ Tetracycline antibiotics, then incubated at 28°C for 2 days. One ml of this cultures were subcultured in 9 ml of new medium containing 50 $mg.L^{-1}$ Acetosyringone and incubated overnight. Ten ml of bacterial culture (OD600 = 0.8-1) were centrifuged at 5000 rpm for 10 minutes. Bacterial pellets then resuspensed with NP medium. Suspension of bacterial culture was then diluted with liquid NP medium at ratio 1: 4 (v / v) and used for innoculation.

For *S. plicata*, separated pollen that called pollinia were immersed in bacterial suspension for 30 minutes-1 hour, then it was dried up prior to be used for pollination. For *P. amabilis*, a pair of pollen that called pollinaria was immersed in bacterial suspension for 30 minutes up to one hour. The pollinaria was then used for self pollination. Seeds of 3 months old pod that produced from S. plicata pollination, and 4 months old pod that was produced from the *P. amabilis* pollination were sown on NP Medium as described in [4]. Regenerated protocorm is then cut into 2 pieces. One piece is regenerated, while another piece is used for transgene integration analysis by PCR. Protocorms are then selected on NP medium that contain 10 mg.L-1 hygromycin. Four weeks old green protocorms were counted to determine the efficiency of transformation in orchids.

GUS assay and Hygromycin resistancy analysis

To detect β-glucuronidase activity, the plant tissue were incubated for 6-12 h in the GUS staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide as a substrat as described by Jefferson *et al.* [6].

For hygromycin resistance test, 8 WAS developing protocorms were transfered onto NP medium + 10 $mg.L^{-1}$ hygromycin for selection of transformants. The survived green protocorms were counted. Regenerated protocorms were then cut into 2 pieces. One piece was continuing to be regenerated, while another piece was used for molecular analyses by PCRFor detection of GUS gene in transforman. the plant DNA(s) were amplified using GUS specific primers GUS A F (5'-CAACGAACTGAAA-CTGGCAGA-3'), GUS A R (5'-GGCACAGCAC-ATCAAAGAGA-3') and detection of GFP gene using GFP specific primers GFP B F1 (5'-CTT-TTC-ACTGGAGTGGTCCC-3'), and GFPB R1 (5-ATTTGAACTGGTGA-CACGAG-3').Detection of

Ubipro::PaFT insertion in *P. amabilis* transfor-mant genomes was performed using primer Ubi (forward: 5'-TTG-TCGATGCTCACCCTG-3') and TNos (reverse: 5'-GATCTAGTAACATAGAT-GACACCGCG-3'). Specific primers (trnL-F C (5'-CGA-AATCGG-TAGAC-GCTACG-3'), and trnL-F F (5'- GCCATGTGTAATCCTAGCAG-3') Chloro-plast DNA were used as internal control of PCR reaction.

RESULTS AND DISCUSSION

Analysis of Hygromycin resistancy

In *planta* transformation in orchids is an improvement from the common Agrobacterium-mediated transformation method that omit *in vitro* procedure when innoculation of bacterial culture for T-DNA transfer into plant genome. *In planta* technique is very simple, in which pollinia /pollinaria (orchid pollens) just simply deepen in Agrobacterial culture, dried out the pollen, then used it for self pollination of the orchid flower. (Figure 2). Prior to in planta transformation, it is important to check and decide the concentration of antibiotic used for selection of transformants. In this work, we used Hygromycin resistant gene as selection marker. We analyzed wild type plants of both *S. plicata* and *P. amabilis* by sowing seeds on various concentration (0-15) mg.L^{-1} of Hyg selection medium. Both orchids shows that 10 mg.L^{-1} Hyg is the best concentration for selection, based on the data of LC 50 (Table 1) First we observed that after innoculation, the pollen became swollen, then used it for pollination. Twenty four infected pollinia were successfully pollinated with its original flower, some fruits produced. The next generation (seeds) were sown on 10 mg.L^{-1} Hygromycin containing medium. Three of them were intensively analyzed for morphological and molecular analysis.

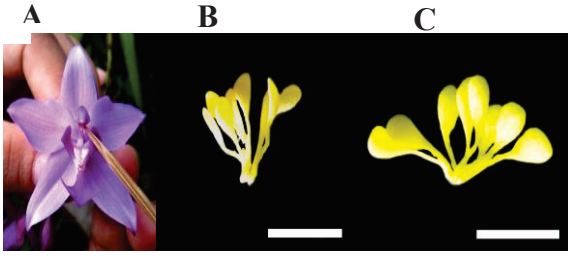
In this experiment, we used pollen/pollinia of two orchid species as target materials. Without any *in vitro* procedure, the technique become simpler, cheaper, and faster. Figure 2 shows the technique, that first pollen (pollinia/pollinaria) were taken from a flower by a clean toothpick, then immerse into a suspension of Agrobacterial culture in half strength of NP liquid medium (no LB medium at all) for 30 minutes-1 hour. After taken out and dried the pollen, it was put into gynoecium/column to pollinate the ovule in the original flower (self pollination). Three months after pollination, the fruit with matture seeds inside could be gathered. Seeds were sown on to NP medium for 3 weeks, then transferred the protocorms on to 10 mg.L-1 Hygromycin selection NP medium. The number of transferred protocorms was indicated as Total protocorm examined, the survival protocorms in this selection medium is termed as transformant candidates. The transfor-mant candidates were checked in DNA level by PCR using Hygromycin resistance gene's primers, GUS and GFP reporter genes primers. The result shows that half of transformant candidates do not contain the T-DNA. A pair of trnL-F primers of intergenic region between tRNA Leucine and tRNA Phenylalanine of cp DNA as internal control that could amplified 1.2 kb DNA fragmen. (Figure3).

GUS assay reported that T-DNA has integrated into orchid genome, inwhich blue color can be detected in the midvein of orchid leaves (Figure 3). pMeEF1a2 promoter originally isolated from seeds of *Manihot esculenta* [5], we did a transient experiment using a young leaves of *M. esculenta,* and got the same result as the same as in orchids.

TABLE 1. Hygromycin resistancy of *S. plicata* Protocorms

Concentration of Hygromicin (mg. L^{-1})	Number of protocorms	Survive protocorm	Death protocorm	Percentage of death protocorm (%)
0	163	163	0	0
2,5	396	352	44	11,11
5	288	248	40	13,89
7,5	372	236	136	36,56
10	252	124	128	50,79*
12,5	320	132	188	58,75
15	368	140	228	61,96

*LC50 of S.plicata to Hygromycin was reached at the concentration of 10 mg.L^{-1} Hyg



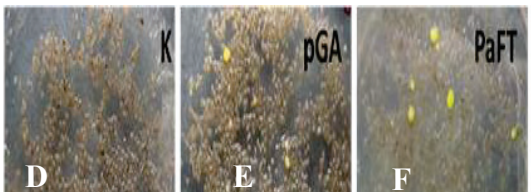
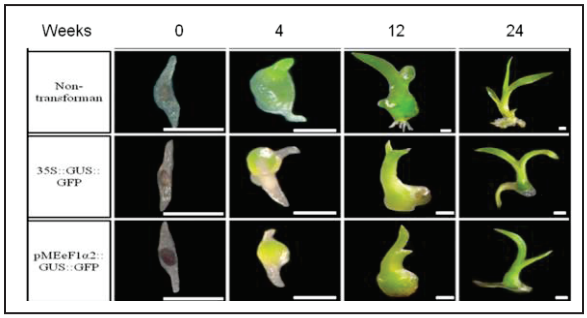


FIGURE 2. *In planta* Transformation in Orchids. (A-C) S. plicata. A. Pollinia was taken out from flower, (B) Pollinia before innoculation, (C) After innoculated with Agrobacterial culture. (D-F) P. amabilis. (D) Protocorm of non transformant (NT) as control, (E) Protocorms of pGA transfered, and (F) Protocorms transferred by pUbi::PaFT.



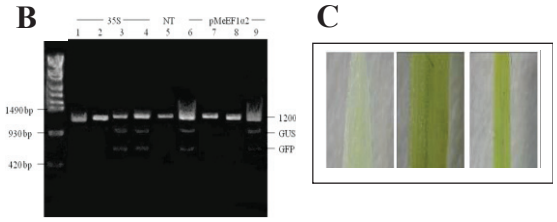


FIGURE 3. Transfoormants of *S. plicata* and detection of transgenes. (A) Growth of shoot from protocorms, (B) PCR products of transformant using 3 sets of primers: GUS, GFP, and trnL-F intergenic of chloroplast DNA as internal control. T-DNA were integrated into genome of half of survival transpormants. (C) GUS analysis of leaves of NT, and two transformants. The blue color is detected at the midvein in the leaves of transformants but not in NT leaf. Bar = 1 cm

Efficiency of transformation

Transformants of both *S. plicata* and *P. amabilis* orchids, that grew on Hygromycin selection medium were confirmed by PCR and GUS assay. It is likely that the efficiency of *in planta* transfor-mation in *P. amabilis* is higher than that in *S. plicata* (Tabel 2), although it is not extreemely different, i.e (0.53-0.72)% in S. plicata and (0.8-2.1)% in *P. amabilis.* It might be related to the structure of pollinia/pollinaria, inwhich polli-naria of *P. amabilis* consists of two compact pollinia that connected by a structure of *discus viscidium*, that make it easy to be handled. Intact protocorm as target plant materials were used for infection of Agrobacterium with efficiency of transformation in a range of (0.1-1.7) % [7].. Chang *et al* [2] got high efficiency of transforma-tion (5.5%) in Arabidopsis, by using inflorescence for *in vivo* transformation. It might be due to the natural biological relation of soil bacteria Agrobacterium and dicot plants, in which dicot plant cells produced chemostatic signal molecules acetosyringone as wounding response.In monocots, usually we added lycopene, vit C or fruit extract as triggers for wounding response to Agrobacterium [4, 7].

TABLE 2. Efficiency of *In Planta* Transformation in Orchids

Plant	Genotype	Total Protocorm examined	Hygr (+)	PCR (+)	Eff. Transform. (%)
S. plicata	NT	1011	186	0	0
	35S::GUS::GFP	1307	279	2	0.72
	pMeEF1α2::GUS::GFP	1508	379	2	0,53
P. amabilis	NT	2091	0	0	0
	pUbi:: HPT	2203	279	17	0,80
	pUbi::PaFT	2246	49	49	2.10

CONCLUSION

Agrobacterium-mediated transformation in orchid can be approached by *In planta* Transfor-mation using pollen/pollinia to omit tissue culture step during T-DNA transfer. Efficiency of *in planta* transformation in *S.plicata* orchid is (0.53-0.72)% and in *P. amabilis* orchid is (0.8-2.1)%.

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