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High Efficiency *in Planta Agrobacterium*-Mediated Transformation in Lilium cv. Pavia

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Authors' contributions

This work was carried out in collaboration among all authors. Author MJ conceptualized and supervised the study, author KBMS did tissue culture and molecular analysis experiment, authors MJ and PKM wrote and prepared the original draft, author MKS edited and finalized draft. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Absence of an efficient genetic transformation technique acts as a major barrier for development of new lily cultivars with desirable characters. Here we report a callus free*in planta Agrobacterium* mediated transformation protocol for the *Lilium cv.* Pavia. Tiny shoot buds obtained directly from the basal portion of the *Lililum* scales were used for *Agrobacterium* infection. *Agrobacterium tumefaciens* strain LB4404 harboring pCAMBIA 3301 vector was used for transformation. Putative transformants were selected on a selection media containing L-phosphinithricin. Further these putative transformants were confirmed by PCR analysis using primers specific for gus and bar

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gene. An average transformation efficiency of 31% was obtained using *in planta* transformation of *Lilium* shoot buds. The high efficiency and rapidity of this protocol make it suitable for commercial application and for future research on this valuable ornamental plant.

Keywords: Lilium; bulb scale; in planta; agrobacterium; transformation efficiency.

1. INTRODUCTION

Lilium‘ *Pavia*’ is a LA hybrid (cross between longiflorum lilies and Asiatic hybrid) which has enchanting and pleasantly scented yellow colored flowers. New lily cultivars with superior characters such as novel flower color, resistance to insects and diseases as well as longevity, are highly desirable for both producers and consumers [1]. Though there are reports of transformation in different lily cultivars, such as *Lilium oriental* [2], *Lilium formolongi* [3], and *Lilium tenuifolium* [4], *Lilium longiflorum* [5],still several factors like low transformation efficiency (up to 3%), lack of regeneration system, genotype dependent and poor genetic stability are major hurdles for the successful trait improvement by genetic engineering. The present study is aimed at establishing an *in planta Agrobacterium* mediated genetic transformation systems in *Lilium* cv. Pavia through the use of a simple *in planta* method.

2. MATERIALS AND METHODS

2.1 Explant Sterilisation and Culture

Lilium cv. Pavia bulb scales were used to obtain *in vitro Lilium* plants. The scales of bulbs were separated and thoroughly washed with running tap water for 20 min to remove the surface dirt, and treated with 0.1% Bavistin (fungicide) overnight. The scales were then surface sterilized using Tween 20 for 5 min followed by 0.1 % mercuric chloride (HiMedia) for 2 min. Finally the bulbs scales were rinsed five times with distilled water.The sterilized bulb scales of *Lilium* ‘Pavia’ were dorsally placed into MS [6] medium containing 30g/L sucrose and 8g/L Agar. The pH of the medium was adjusted to 5.8 using 1N NaOH and the medium was autoclaved at 121°C and 15psi for 20 min. The cultures were placed in dark at 25 ± 2°C for one month after which it was taken out for transformation. Three replicates of 50 bulb scales were used for the experiments.

2.2 Strain Used

Agrobacterium tumefaciens LB4404 and the binary vector pCAMBIA3301 were used for genetic transformation. The binary vector pCAMBIA3301 contains the bialophos/ phosphinothricin resistance (Bar) gene and GUS reporter gene (Fig. 1).

2.3 Transformation Protocol

Transformation procedure was essentially the standardized method reported earlier in tuberose [7]. The tiny shoot buds (receptor material) was obtained from bulbs within a month of culture were wounded by pricking with a sterile 30G syringeand immersed in *Agrobacterium* infection solution for 15 min, with occasional shaking to ensure complete contact of the tissue with *Agrobacterium* culture. The explants were removed from the medium, and blot dried on sterile filter paper for 20 min to remove the residual bacteria on the surface of the tissue. The explants were then transferred to the regeneration medium. The regeneration medium consisted of MS medium supplemented with 30g/L sucrose, 8g/L Agar, *2, 4-Dichlorophenoxyacetic acid (*2, 4-D) 1mgL^{-1}, α-naphthaleneacetic acid (NAA) 1mgL^{-1} and 6-Benzylaminopurine (BAP) 0.5mgL^{-1}, pH 5.8. Thecontinuous dark culture was carried out at 25±2°C for three days, followed by transfer to selection medium in light. Selection medium consisted of regeneration medium supplemented with 15 mgL^{-1} L- Phosphinothricin. This media

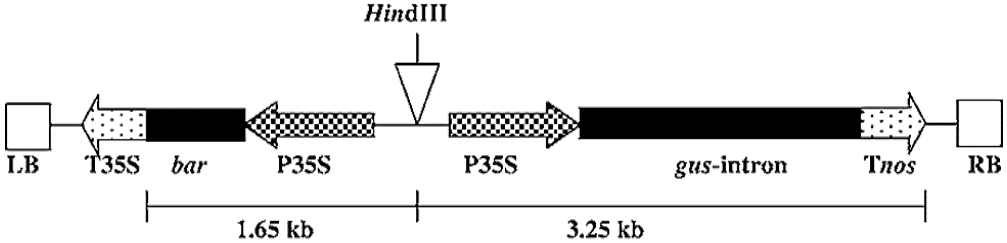

Fig. 1. Diagram of binary vector pCAMBIA3301

helped in selecting transgenic regenerates. The plants were subcultured to fresh selection media every two weeks. The number of plants obtained were counted and after four weeks the resistant shoot buds were transferred to MS medium for further culture under a 16/8-h light/dark cycle with a temperature of 25±2°C conditions to form complete plants. The rooting of the resistant shoots was done on ½ MS media with 0.5 mg L^{-1}Indole butyric acid (IBA) till sufficient roots were obtained. The well rooted plantlets were transferred to soil rite for primary hardening.

2.4 DNA Extraction and PCR Analysis

DNA was extracted from (100mg) leaves of putatively transformed shoots and from control material not inoculated, and processed according to the cetyltrimethyl ammonium bromide (CTAB) protocol of Doyle and Doyle (15). Putative transformed shoots were PCR-screened to detect the presence of gus reporter gene and bar selectable marker gene. Each reaction (25 µl) contained 20 mM Tris-HCl (pH 8.4), 50 mMKCl, 1.5 mM $MgCl_2$, 2 mM of dNTP, 1.0 U ofTaq polymerase, 50 ng DNA and 10 µM of each gus gene primers. Invitrogen's Taq DNA polymerase recombinant kit was used for DNA amplification. The following primer pair was used in the PCR assay: gus forward primer (Gus F1) was 5'-CAGCGCGAAGTCTTTATATACCG-3', gus reverse primer (Gus R1) was 5'- ATGCGTCA CCACGGTGATATCG-3' and bar forward primer (BarF1) was 5'-GGTCTGCACCATCGTCAACC-3' andbar reverse primer(BarR1)was5'-GTCATGCCAGT TCCCGTGCT-3'. The length of the amplified GUS and BAR gene fragment was 991 bp and 490bp, respectively. Plasmid pCAMBIA3301was used as positive control. Amplification conditions were performed as initial DNA denaturation at 95ºC for 4 min. followed by 35 cycles of 1 min denaturation at 95ºC, 1 min annealing at 37ºC and 2 min of extension at 72ºC with a final extension time at 72ºC for 10 min. After electrophoresis on 1% agarose (w/v) gel containing ethidium bromide (0.5 µg), the PCR products were visualized on a UV transilluminator and photographed by an Alphaimager Gel Documentation System (Alpha Innotech Corporation, USA).

3. RESULTS AND DISCUSSION

In this study, the transformation of *Lilium* cv. Pavia was accomplished through the *in planta* pricking of adventitious shoot buds induced on the basal portion of bulb scales. After about a month direct regeneration of *Lilium* shoot buds was observed from the edges of scales (Fig. 2A.). The transformation of shoot buds induced on *Lilium* cv. Pavia bulb scale was accomplished through pricking of the shoot buds which formed on the basal portion of the bulb scale. When they were transferred to selection media the resistant buds developed and the untransformed shoot buds gradually died. The shoot buds were allowed to grow till sufficient length of the shoots have formed (Fig. 2B) and then separated and transferred to rooting media (Fig. 2C). A total of 150 scales inoculated (50 scales as three replicates), 98 (65%) shoots regenerated on selection media. The well developed plants with roots were transferred to soil rite in pots for primary hardening (Fig. 2D).

The genomic DNA was extracted from the transformed plants, and the plasmid pCAMBIA 3301 was used as positive control. PCR amplification was performed using the GUS and BAR gene specific forward and reverse primer and the band was detected using agarose gel electrophoresis. The PCR product of the GUS (991bp) and BAR (490bp) gene was amplified from the transformed shoot buds.A total of 47 (31%) plants were PCR positive. Fig. 3 gives the representative gel images of PCR confirmation. Molecular analysis with PCR on the transformants performed using the target genes confirmed the potential of this protocol to generate transgenic plants in lily. *Lilium* has always remained hard to transform with *Agrobacterium* due to low transformation efficiency. The transformation efficiency in *Lilium* is dependent on the genotype of the plant as well as the type of thereceptor tissue used for *Agrobacterium* transformation [8,9]. Different target tissues used for *Agrobacterium* infection has distinct merits and demerits associated to it [10]. *Agrobacterium* mediated transformation using embryogenic callus as target tissue has been largely reported due to its high proliferation rate and high transformation efficiency compared to any other receptor material. Although the major disadvantage being the development of genotype specific *in vitro* regeneration system and also the relatively long transformation cycles [9]. On the contrary, the use of vegetative tissue for *Agrobacterium* transformation is comparatively easier to perform and quicker, although lower transformation efficiency has been reported. The major advantage of *in planta*

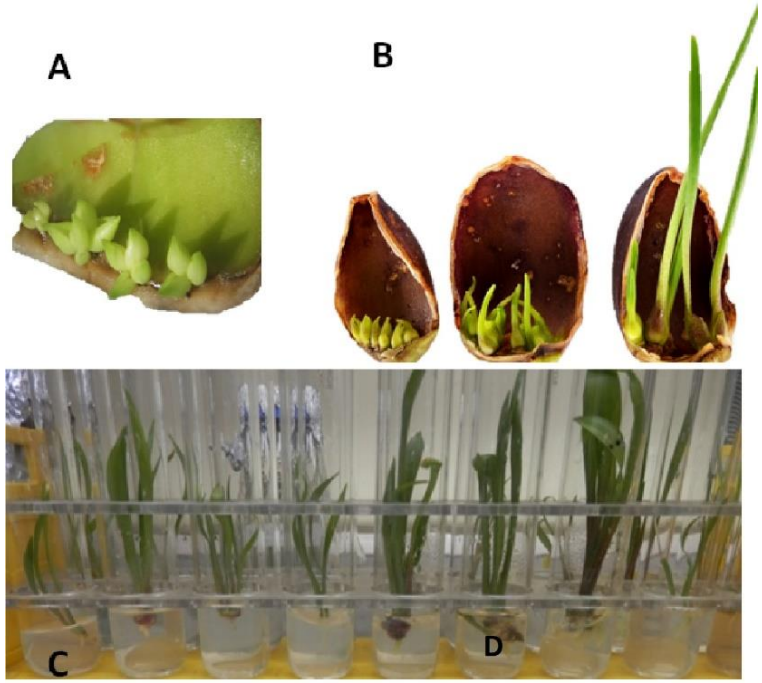

Fig. 2. A. Direct regeneration of shoot buds from scales which were pricked. B. Shoots growing in selection media along with the scales C. Rooting of individual shoots in rooting media D. Well-developed plants with roots

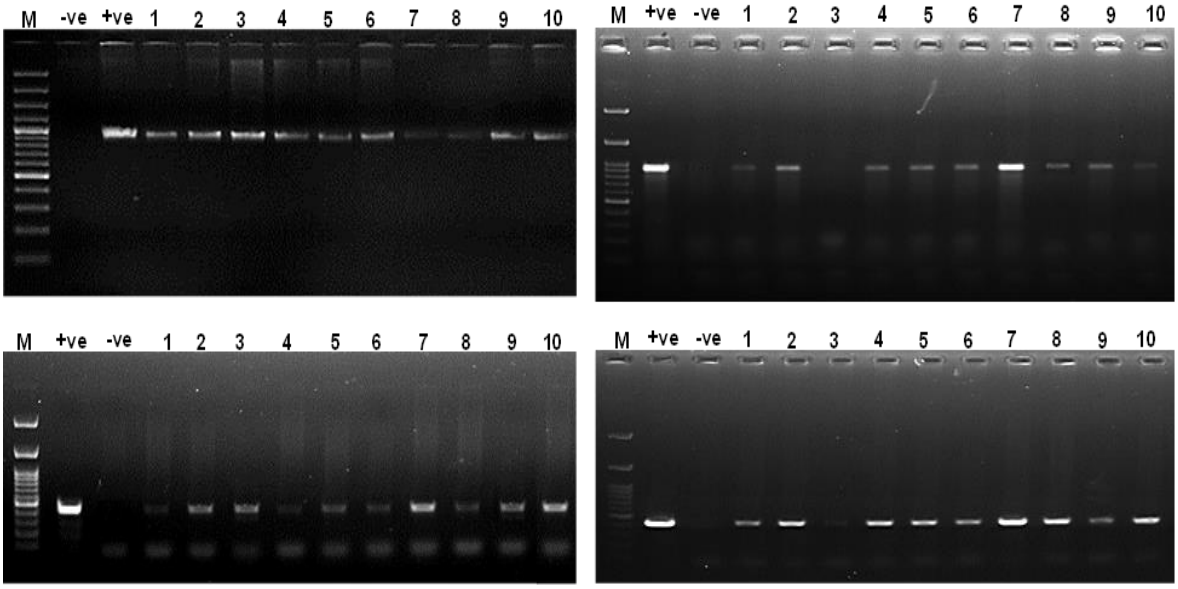

Fig. 3. PCR screening for positive transformants of *Lilium.* Gel image of PCR confirmed plants (Lane 1,2,5,6,7,9,11-18,22,23) (A) GUS gene showing a band of 991bp. (B) BAR gene showing a band of 490bp

transformation is production of a large number of plants in a short span of time. This type of callus free gene transfer called as "*in planta"* method is reported in several crops like tuberose, soybean, pigeon pea, strawberry, radish, rice, wheat and maize [7,11-16] but not so far reported in *Lilium*. Very recently this method of transformation has been reported for vegetatively propagated crop like sweet potato and they have named the method as RAPID which means regenerative activity-dependent *in planta* injection delivery and they report that this protocol has advantages of a

high transformation rate, short duration and user friendly operational protocol and does not require tissue culture [17].

4. CONCLUSION

In planta Agrobacterium mediated transformation method is a quick and efficient method that circumvents the need for an *in vitro* regeneration system. The pricking and wounding of the shoot buds at the initial stage of growth significantly enhanced the transformation efficiency (Average of 31%). This transformation efficiency is higher compared to transformation efficiencies reported earlier in *lilium* and also the transformed plants could be obtained within a short time of 2-3 months. The present study demonstrated that *in planta* transformation of *Lilium* cv. Pavia was more efficient and quick. Further the protocol reported here could be used to extend the genetic transformation of other important Lily cutlivars.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

We hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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