Development of an efficient Agrobacterium-mediated genetic transformation for an important ornamental plant, Phlox drummondii Hook

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Research Article

Keywords: Ornamental plants, Phlox drummondii, Genetic transformation, Agrobacterium, Floral variants

Posted Date: July 30th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7162932/v1

Abstract

In ornamental plants, continuous attempts are being made to introduce new quality traits that have market value. Of various modes of plant propagation that have been followed for production and propagation new traits, the genetic transformation technology has reportedly played an important role in creating novel varieties of ornamentals of rose, dahlia, carnation, lily, tulips, gerbera, etc. Many of transformed varieties of these ornamentals reportedly produce plants that have improved aesthetic traits like flower colour, fragrance, variant floral architecture, and vase life. *Phlox drummondii* Hook., also known as “Drummond” Phlox, is a prized ornamental among 67 species of the genus *Phlox*. In the present investigation, an efficient *Agrobacterium*-mediated genetic transformation protocol was developed to study its impact on floral morphology, an important commercial trait, of *P. drummondii*. About 3-4-week-old embryogenic calli derived from zygotic embryo on callus regeneration and multiplication medium (CRMM) comprising MS basal medium + 3% Sucrose + BAP (5 µM) + NAA (10 µM) were pre-cultured on shoot regeneration medium (SRM) comprising MS medium with 3% sucrose + BAP (15 µM) + IAA (2.5 mµ M) for 2–3 days. *A. tumefaciens* strain GV2260 carrying the *GUS* gene construct (PPZP 200) was grown overnight in YEM medium and calli were infected with bacteria (A_{600} 0.3–0.5) for 10 min. Infected calli were subsequently co-cultured on the medium SRM + acetosyringone (100 µM) for 2- days. Selection of transformed calli was achieved by transfer of these co-cultured calli on the SRM I (SRM supplemented with kanamycin (30 mg/l) and augmentin (300 mg/l), which allowed the production of 76% transformed shoots. For shoot elongation SRM II MS basal salts + 3%sucrose + BAP 10 µM + augmentin 300 mg + kanamycin (30 mg/l) was used. Rooting of transformed shoots occurred on MS + IAA (7.5 µM). Transformation status of putative transgenics was confirmed by PCR using primers(Supplementary Table No 5) specific for *GUS* and NPT-II genes, and by Southern hybridization using NPT-II gene as probe. Genetic transformation protocol has been standardized in this ornamental species for the first time and transformants were observed having unique changes in floral architecture hitherto unknown.

Key Messages

This study showed that a novel agrobacterium mediated genetic transformation protocol established in important ornamental plant *Phlox Drummondii* along with mutagenic changes reported in transgenics.

Introduction

Ornamentals constitute an important component of horticulture industry with direct impact on human life because of their aesthetic and economic importance. Flower crop cultivation is considered as a lucrative and income generating venture. More importantly, ornamentals play an important role in economic strengthening of many countries particularly in Kenya, Ethiopia, Costa Rica, Colombia and Ecuador (Sharma and Messar 2017). For economic purposes, floral industry mostly utilizes cut flowers,

loose flowers, potted- flowering and foliage plants. Further ornamental grasses, trees, shrubs and annuals are also grown, which fulfil the aesthetic needs and also form an integral part of ecosystem. Cut flowers in ornamentals like phlox have more market value, followed by flowering pot plants, nursery crops and trees (Shibata 2008). *Phlox drummondii* Hook. is an important ornamental species and a mark of eternal beauty in the genus *Phlox*, which belongs to family Polemoniaceae comprising Ca. 67 annual and perennial species. Plants of *P. drummondii* are grown all over the world because of its beautiful and attractive flowers, which occur in shades of purple, pink, Crimson, red, scarlet, violet and many other intermediate shades. Flowers are fragrant, trumpet shaped, 5- lobed with short narrow corolla tube appearing in clusters (broad flat-topped cymes) at the stem ends. Additionally, central eye of the flower emerging from corolla tube differs in colour from the rest of petals making it more attractive. Stigma and style with ovary emerge from the base of five epipetalous anthers growing up to the upper wall of corolla tube (Grant and Grant 1965). Leaves as well as stem are soft, hairy, and sticky. *P. drummondii* adores border clumps, central flower beds of lawns, window boxes, flower vases, hanging baskets, and sold as excellent cut flowers (Bailey 1950 ; Hay and Synge 1969; Herman 1973).

A winter annual *P. drummondii* is native to grasslands and open woods of Central and Eastern Texas. The genus name *Phlox* is derived from the Greek word “phlox”, meaning flame, in context of its intense flower colours. In 1835, Thomas Drummond originally collected seeds of this plant in Texas and subsequently sent them to England from where they were distributed to nurserymen in different European and other countries world over (Levin 1977). Considering the striking features of *P. drummondii*, its ornamental potential has not been exploited to full potential owing to sensitivity towards water stress, temperature, and fast maturing habit thus imparting limitations in its multiplication and floriculture trade. Even conventional practices like selection, sexual crossing, back crossing and mutation breeding could overcome these impediments as well as widen its genetic base only to a certain extent. Attempts needed to be initiated for achieving the horticultural excellence in *P. drummondii* using other non-conventional procedures (Razdan et al. 2008, Razdan-Tiku et al. 2014). Application of genetic transformation procedures is one of the biotechnological approaches that could be utilized to supplement the efforts of existing conventional methods. Through transformation procedures the potential to incorporate novel characters in plants is possible which may otherwise appear recalcitrant using conventional breeding procedures (Ali et al. 2017, Sharma et al. 2017). Molecular-based gene transformation technology in ornamentals has brought changes in plant morphology (Chandler and Lu 2005, Casanova et al.2005), flower shape (Casanova et al.2005), flower colour (Meyer et al.,1987, Tanaka et al. 2005, Debener and Winkelmann 2010, Chandler and Brugliera 2011, Umemoto and Toguri 2012), flower fragrance (Mori et al., 2004., Dudareva 2002., Underwood and Clarke 2011), pest as well as disease resistance (Milosevic et al., 2011a, 2011 b, Milosevic et al., 2015), stress tolerance (Lojic at al., 2015), and increased vase or post-harvest life necessary for cut flowers (Chandler and Sanchez 2012). Transgenic breeding in major cut flower crops like roses (Kim et al. 2004, Katsumoto et al. 2007), gladiolus (Graves and Goldman 1987, Kamo et al. 2009, 2010) carnations (Lu et al. 1991, Holton et al. 1993a, Meng et al. 2009) and chrysanthemums (Kazeroonian et al. 2018) was reported to have improved important traits in these ornamentals thus providing huge profits and avenues (Sharma et al. 2017).

In the present study, *Agrobacterium tumefaciens*-mediated genetic transformation procedure has been developed with an objective of producing *P. drummondii* plants with new improved floral traits since this technology acts as powerful tool for delivery of alien gene(s) of interest inside the host plant for integration with the host plant nucleus. Needless to state that a wide range of plant species have been transformed through application of this procedure for improvement of traits of agronomic, horticultural and ornamental value, including important traits in tree species. Moreover, an added advantage in this technology is that transfer of small copy number of T- DNA is good enough to result in stable integration of foreign DNA into host plant genome (Hwang et al., 2017). Considering these aspects an efficient *Agrobacterium*-mediated genetic transformation protocol was developed in present study for first time in *P. drummondii* or the genus *Phlox*.

Material and Methods

Plant material found suitable for genetic transformation

For genetic transformation of *P. drummondii* 3-week-old callus induced from zygotic embryo and multiplied by repeated subculturing on CRMM (callus regeneration and multiplication medium) was found suitable (Fig. 1) although calli derived from cotyledonary leaves were also tried. CRMM constituted of MS basal medium + 3% sucrose + BAP (5 µM) + NAA (10 µM). Prior to transformation the zygotic embryo induced callus was cut into 2-3 mm pieces and then they were pre-cultured on shoot regeneration medium (SRM), which comprised of MS basal salts +3% sucrose + BAP (15 µM) + IAA (2.5 µM) for zero days, 1-2 days, 2-3 days and 4-5 days.

Bacterial strain and binary vector

The *Agrobacterium tumefaciens* strain PGV2260 was used for transforming embryogenic calli of *P. drummondii*, which carried *GUS* binary plasmid vector PPZP20KWT with a marker gene and Nos promoter NPT-II: OcspA cassette (conferring kanamycin resistance) and a reporter gene *GUS* (intron)-35S promoter cassette (Fig. 2).

Agrobacterium-mediated genetic transformation

Agrobacterium strain PGV2260, carrying *GUS* binary vector, was cultured overnight in 100 ml conical flask containing 30 ml YEM (yeast extract mannitol medium) + antibiotics (carbenicithin, spectinomycin and refampicin) on a shaker, 250 rpm, at 28°C. OD (Bacterial density) of the culture was checked intermittently after the gap of few hours and cultures at different ranges of OD (0.1- 1.0) at A600 were tried for different sets of transformation experiments (Table 1). After adjusting the OD of the bacterial culture, it was transferred to autoclaved Oakridge tubes for centrifugation at 3000-4000 rpm for 10 min at 28°C. The supernatant was discarded and the pellet dissolved in MSO (MS liquid medium without hormones) + acetosyringone 100 µM. The callus pieces in this medium were then transferred to an autoclaved screw- capped wide – mouthed jam bottle and to each piece was added to *Agrobacterium* culture of different OD. The bottles were gently stirred manually so that the callus gets properly infected

with bacterial suspension. Along with the OD, infection time of bacteria was also standardized ranging from 5-30 min (Table 2). After pouring off the *Agrobacterium* suspension, the callus pieces were placed on autoclaved blotting paper. Blotted dry calli were then transferred to co-culture medium comprising SRM + acetosyringone (100 µM) for 2-3 days. The co-cultivated calli were finally transferred to selection medium containing SRM + augmentin (300 mg/l) + kanamycin (20- 50 mg/l) **(Table S1).**

Kanamycin assay to optimize the concentration for stringent selection for the recovery of the transformed callus in selection medium

Subculturing was done on the same selection medium after every 15 days up to 3 cycles of selection (Total 1½ months on selection medium). Untransformed callus did not survive on selection medium (Fig. 3A) whereas transformed callus could only regenerate on the selection medium (Fig.3 B). After 1½ months, callus was transferred to SRM II containing MS basal salts +3% sucrose +BAP 10 µM +augmentin 300 mg (Fig. 3C). After 1 month of subculturing on SRM II, 1-2 cm long shoots excised from regenerating callus were transferred to same medium but without kanamycin (Fig. 3 D).

Transformed shoot multiplication, rooting and transplantation

For multiplication of transformed shoots from the various experiments two approaches were followed. The small shoots (Ca 1-2 cm long) were individually transferred to fresh medium (SRM II without kanamycin) for further proliferation. Second, the longer shoots that had grown up to 4-5 cm, were cut into single node segments and each node segment transferred to fresh medium (SRM II without kanamycin) for further growth and multiplication (Fig. 3E). The number of shoots obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication. After 2- weeks shoots *in vitro* underwent flowering (Fig. 3F). However, for rooting, shoots measuring Ca. 4 cm, with 3-4 nodes, from transformed calli before *in vitro* flowering were excised and transferred to rooting medium (MS+IAA 7.5 µM). The rooted plants (Fig. 3G) were washed in tap water to remove agar and then transplanted to soil in small plastic pots containing autoclaved soil as per procedure followed by Razdan-Tiku et al. (2014). The soil composition was one part vermiculate and 3 parts of garden soil. The transformed plants in pots were then covered with polythene bags with small holes (2 mm diameter) to maintain high humidity at least for 20 days. These plants were kept in tissue culture room with temperature 25 to 27 °C, relative humidity 50 to 60%, and 16 h photo period. After one month of acclimatisation, these plants were transferred to bigger pots (Fig. 3H) and subsequently shifted to transgenic green-house.

GUS Assay

Analysis of GUS activity in transformed plant parts was performed by histochemical assay as described by Jefferson et. al. (1987). Transformed calli, leaves and roots were incubated in 500 µL of GUS assay buffer [10 mg of X- gluc (5 – bromo 4 – chloro – 3 indolyl – glucuronide) in 2 ml dimethyl formamide] , 2 ml 5 mM potassium ferrocyanide , and 20 ml 0.1 M sodium phosphate buffer , at 37°C overnight . The solution was removed next morning and incubated materials were rinsed in 70% ethanol and examined for detection of blue colour (Fig. 4 A-C) before being stored in 40 % glycerol.

Molecular analysis of putative transformants of *P. drummondii*

The transgenic plants raised were analysed by PCR for the integration of the transgene. Using Techne PCR machine (U.K.), DNA was isolated from leaves in liquid nitrogen by a CTAB method as described by Doyle and Doyle (Doyle and Doyle 1987). About 100 ng of genomic DNA from untransformed control and the transgenic lines was taken separately, and mixed with 100 mM forward and reverse primers (Suppmentary Table No 2) in 7.5 µl of PCR buffer (10 mM of Tris HCl , 50 mM KCl , 2 mM $MgCl_2$, 100 µM dNTPS mix) and 0.1 Taq DNA polymerase (Biotools , Spain), pH 8.3. The volume of mixture was raised up to 25 µl with SDW. DNA amplification was carried out in the thermal cycler programmed for 40 cycles as follows: 1 cycle of 5 min at 94 °C, followed by 39 cycles, each of denaturation at 94°C for 1 min, annealing for 1 min at 53 °C, and synthesis at 72 °C for 2 min, and finally 1 cycle of 10 min at 72 °C. After completion of the PCR 2 µl of 10x loading buffer was added to each of the samples. The amplification product was separated by electrophoresis in 1.2 % agarose gel containing 0.05 µl/ml ethidium bromide (Roche Diagnostic Gmbh, Germany) in 0.5x TBE buffer (0.045 M Tris- borate and 1mM Na_2 EDTA). The *Hind*III digested λ DNA was loaded in one of the lanes to serve as molecular size markers. After agarose gel electrophoresis (AGE), the gel (Fig.5 A-B) was photographed in UV light (LKb Pharmalia, USA). PCR experiment was repeated with the same samples 2 -3 times for the confirmation of results. The details of the primers used for *npt*-II gene and *GUS* are given in Table S2.

Southern analysis with *NPT*-II gene as a probe

Southern blot hybridization was performed to detect the *NPT*-II gene integration. Genomic DNA (10 µg) from different PCR positive transgenic lines as well as the untransformed control was digested with *Eco*RI, which released the *NPT*-II gene from the T-DNA and blots were prepared (on Nylon membrane Sigma, USA) , hybridised and washed with high stringency as per the standard protocol (Sambrook et al. , 1989). The *NPT*-II gene probe was prepared by using the random priming kit (Takara, Japan) as per the manufacturer's guidelines. The probe was denatured before adding to the pre-hybridisation buffer. After hybridisation, membrane was washed and then exposed to X-ray film (Kodak); (Fig. 5C).

Statistical analysis

Each experiment was repeated thrice, data subjected to analysis of variance (ANOVA), and means were compared using SAS computer software according to Anderson – Cook 2004.

Results

For genetic transformation of *P. drummondii*, an efficient protocol has been devised based on the *Agrobacterium*-mediated transformation method. The details of procedures followed according to protocol are described in Section on material and methods. Based on this protocol, the following parameters were determined that facilitated efficient gene transformation in *P. drummondii*.

Optimum Density (OD) of *Agrobacterium* and infection period

Agrobacterium tumefaciens GV2260 strain carrying the *GUS* gene construct pPZp200 (Fig. 2) cultured in YEM medium at different ODs (0.1 – 1.0; Table 1) demonstrated the most effective transformation occurred using this bacterial strain at the optimum OD (0.3-0.5), A600. Explants infected with bacteria at same OD for different infection periods (5 min – 30 min) demonstrated gene transformation occurred at best at infection time of 10 min (Table 2). On higher OD at same infection time though the number of GUS positive calli were more but the number of transgenic plants regenerated happened to be very less. At lower OD the percentage of GUS transformed calli decreased with reduced potential for plant regeneration (Table 1). Similarly, with the increase in infection time (>10 min) plants regenerated from transformed calli had negligible or very low regeneration response (Table 2).

Preculture of untransformed callus

Prior to transformation as already mentioned, the untransformed callus was pre-cultured on shoot regeneration medium (SRM) for zero days, 1 -2 days, 2 -3 days and 4 -5 days to inculcate its better transforming efficiency. Most suitable callus for most effective genetic transformation was the one pre-cultured on SRM for 2-3 days. It contained high levels of cytokinin and low levels of auxin. Pre-cultured callus pieces (2-3 mm) were subsequently transformed by immersing in MSO medium containing *Argobacterium* (carrying gene construct) at standardised OD + Acetosyringone (100 µM) in a wide mouthed -autoclaved bottle and manually shaken for standardised period of 10 min. The infected calli were subsequently cultured on co-cultivation medium (SRM + acetosyringone (100 µM) for 2 – 3 days. These co-cultivated calli were finally transferred to a selection medium containing SRM + augmentin (300 mg/l) + kanamycin (30 mg/l). Although selection media with different concentrations of kanamycin were tested, proper selection of transformed callus (Fig. 3 B) occurred at antibiotic concentration 30 mg/l which regenerated more transformed shoot buds. At kanamycin concentrations below 30 mg/l although percentage callus survival was high but plants regenerated were mostly untransformed. At kanamycin concentrations above 30 mg/l, most calli turned pale -yellow or necrotic and died without showing any signs of regeneration.

Plant regeneration from transformed callus

Transformed callus were taken out from the selection medium and then transferred to SRM I containing MS salts + BAP (15 µM) + IAA (2.5 µM) + augmentin (300 mg/l) + kinetin (30 mg/l) and sub-cultured on same medium , every 15 days for at least one and a half month till small shoots appeared on the callus (Fig. 3 C) . Transformation frequency and regeneration frequency of *P. drummondii* transgenic plants were 76% and 62%, respectively (Table 3). GUS assay was done with 2-week-old calli growing on selection medium to test the incorporation of GUS gene (Fig.4 A) and percentage calli showing GUS activity calculated. Highest percentage of GUS activity was shown to be around 70% when infection time was kept at 10 min and bacterial density 0.3 – 0.5, A600 (Table 2). After shoot bud formation callus was transferred to shoot regeneration medium II (SRM II). This regeneration medium contained MS salts + 3% sucrose + BAP (10 µM) + augmentin (300 mg/l) + kinetin (30 mg/l). After one month of sub-culturing on SRM II, 2 cm long shoots were excised from the base of callus and transferred to the same medium

but without adding augmentin and kanamycin. This resulted in further elongation and multiplication of shoots (Fig. 3 D-E). *In vitro* flowering of transformed shoots also occurred on SRM II (Fig. 3F). Therefore, it became essential to induce rooting in transformed shoots on the medium MS + IAA (7.5 µM) before *in vitro* flowering (Fig 3G). The plantlets were finally transplanted to soil in pots (Fig. 3H) as per procedure described in material and methods.

Morphology of Transgenics

It was observed that floral and other characteristics of transgenic *P. drummondii* plants were quite different from control (normal /untransformed) plants. Height of transgenic plants was shorter than the normal plants. Stem and leaves of transgenics were very thick and dark green in contrast to the thin and light colour of stem and leaves of normal plants. Morphology of flowers in particular showed huge variations in different transgenic lines in comparison to normal untransformed plants (Fig. 6. A-E). In the genus *Phlox* (including *P.drummondii*) as well as in family Polemoniaceae to which it belongs, flowers are radially symmetrical and pentamerous (each floral whorl consists of 5 sepals and 5petals fused into a cup, or tube-shaped structure (Fig. 6A). Stamens are 5, epipetalous, the length of all anthers and their filaments being almost same they are attached at the base of petals and reach up to the mouth of the corolla tube (Fig. 6C). However, in our investigations on GUS transformed *P. drummondii* plants the flower structure was found paradoxically different as it showed marked variations due to the occurrence of partitions in the corolla tube resulting in petals becoming free except at the base (more like a polypetalous condition; Fig. 6 B). Corolla tube length in the control or untransformed plants being Ca. 1cm, the length of corolla tube in transgenic plants on contrary was Ca. 2 cm. While in control plants, epipetalous anthers reach the same height (Fig. 6 C), the transgenic plants exhibited variation in their height. Two stamens being long are slightly exerted towards upper mouth of the corolla tube, another two stamens are of medium size and reach till the middle of the corolla tube, whereas the fifth stamen is short and appears at the base of the corolla tube (Fig 6 D). Different transgenic lines also showed variations in flower colour ranging from light pink to reddish pink (Fig. 6 E2 and E5). Further, shape of the petals varied as they are broader in control plants whereas transgenic flowers had narrow petals pointed at the top (club shaped; Fig. 6 E2, E4 and E6). Another striking feature being that few transgenic lines showed flowers with an increased number of petals (6-8; Fig. 6 E3, E5 and E6) thus defying symmetrical pentamerous condition of 5 petals in control plants (Fig. 6 E1). GUS assay was also performed in shoots and roots of both transgenics and control. Appearance of blue colour confirmed the transgenic nature of plants (Fig. 4 B, C).

Molecular characterization of putative transgenics

As mentioned earlier 70 % of the calli infected by *Agrobacterium* carrying vector T-DNA *GUS* and *NPT*-II genes construct at 0.3- 0.5 OD, A600, and infection time of 10 mins showed maximum GUS activity (Table. 2).

The putative transgenics were characterised for the integration of vector T-DNA *GUS* and *NPT*-II genes into the host plant genome by doing PCR. The PCR was performed with the DNA isolated from

untransformed (control) and transformed lines using *NPT*-II (marker) and *GUS* (reporter) gene specific primers. The amplified product of both the genes was 500 bp gene fragment which was found in 70% putative transformants (Fig.5 A, B), thus confirming their transgenic nature. PCR positive lines were further checked by Southern hybridization using *NPT*-II gene specific probe (Fig 5C). For Southern hybridization, genomic DNA isolated from transformed and untransformed (control) plants were digested with the restriction enzyme *Eco*RI, which cuts the T-DNA fragment of 2.3 kb containing *GUS*-*NPT*-II gene (Fig. 5C). The results obtained from molecular analysis correlated with morphology-based identification of putative transgenic lines regenerated from transformed embryogenic calli of *P. drummondii*.

Discussion

Development of ornamental varieties that have much improved floral characters is

one of the important aspects of floriculture. Biotechnological approaches along with the classical breeding methods are being used to improve floral or other important traits in ornamentals to supplement their commercial value. Improvements desired in flowers generally relate to fragrance, morphological appearance of sepals and petals, their colour, diseases and stress resistance, enhanced vase life, etc. Transgenic strategies seem to have immense potential to produce novel flower phenotypes which otherwise are not found in nature or produced through traditional breeding procedures (Kim 2020). In this context the improvement of flower traits through gene transformation has been attempted in ornamental flowering plants like *Gerbera* (Chung et al., 2016), *Petunia* (Bashandy and Teeri 2017), rose (EL-Tarras et al., 2017), carnation (Yantcheva et al., 1997) and chrysanthemum (Shinoyama et al., 2012) with some success. Insertion of alien desired genes into a plant requires development of a reproducible protocol which was prime objective of this study for *P. drummondii*. Till date, no such studies have been carried out on any species of the genus *Phlox*. It required many efforts to devise and standardize each and every step of genetic transformation of *P. drummondii.* Certain very important parameters had to be standardized, such as choice of explant, selection of *Agrobacterium* strain with binary vector gene construct, infection time of this bacterium, optical density (OD) of the bacterial suspension culture, concentration of selection marker (kanamycin) and acetosyringone in the medium during co-cultivation, time period as well as temperature requirement for co-cultivation, and type of bacteriostatic agent used.

Transformation protocol for *P. drummondii* required the development of an efficient tissue culture technique for effective regenerative response of explant and transformed tissues. Selection of explant was important as different explants (leaf discs, florets, petals, stem cuttings, roots, cotyledonary leaves, embryogenic calli etc.) showed difference in transformation and regeneration potential. Elomma and Holton (1994) also observed this phenomenon in other ornamental plants (*Gerbera*, chrysanthemum, etc.). In *P. drummondii* zygotic embryo- derived callus was most suitable for *Agrobacterium*- mediated transformation using the protocol described and standardized in this paper. Preculture of calli proved an important parameter because most of the cells undergo rapid divisions and thus remain in an exponential growth phase which makes them more amenable to transformation. Such inferences were

also reached based on the evidences of researches on other plant systems (Hiei et al., 1994; Vijayachandra et. al., 1995). According to Kumria et al. (2001) preculture of callus on high cytokinin (BAP 15 µM) or low auxin (IAA 2.5 µM) medium enhances the cell division as well as promotes *Agrobacterium* infection. OD of the bacterium suspension carrying gene construct was found equally critical. At lower OD (0.1–0.3) GUS activity was less pronounced depicting lower or negligible transformation frequency, while at higher OD (0.5-1.0) the extensive blackening of transformed calli proved detrimental for regeneration. Therefore, the agrobacterium strain PGV2260 suspension used in present study showed best GUS activity at O.D (0.3–0.5). The infection period of bacterium also varied and 10 min was found optimum for infection and transformation. Importantly, keeping the dry infected calli on SRM for at least 2 days was one more step required for successful transformation before shifting them to selection medium (SRM + kanamycin). Another interesting observation in our studies has been that the transformed callus showed no GUS activity in absence of acetosyringone in the medium. Therefore, acetosyringone (100 µM) in the medium was optimum and essential to promote both, GUS activity, and shoot regeneration. Acetosyringone, a phenolic compound, is emitted in wounded plant tissues which seems important for *Agrobacterium* to enter the host plant tissue-cell cytoplasm and transmit T-DNA along with associated proteins (T-complex) into the host pant genome for stable integration (Lacroix and Citovsky 2013). Cefotaxime and augmentin were tried as bacteriostatic in selection medium. Augmentin (300 mg/l) proved better as it restricted overgrowth of *Agrobacterium* and supported regeneration of transformed calli as well as shoot bud differentiation on selection medium SRMI (Fig. 3B). Differentiation of shoot buds on the transformed callus also occurred on the same selection medium but for further shoot development (elongation and multiplication of shoots) a different shoot regeneration medium (SRMII) was standardized and used. While developing the genetic transformation protocol for *P. drummondii* some very interesting and remarkable results were achieved. Transformed plants showed different floristic characteristics from the normal plants. A major change was the trend towards polypetalous condition of the flower (Fig. 6B) as against the floral architecture of gamopetalous tubular corolla in *Phlox drummondii*, and other members of the family Polemoniaceae. Another variation in floral architecture observed was that in some GUS transformed *P. drummondii* plants the number of petals increased (6–8) as against normal 5 petals found in normal plants (Fig. 6E3, E5, E6). Petal shape also changed along with its colour. The colour was more intensified and there was a paradigm shift in the position of anther filaments (Fig. 6E2 and E5). Reason behind these variations could be the impact of “insertional mutagenesis” in GUS transformed plants of *P. drummondii* leading to formation of new variant lines of this ornamental. Insertional mutagenesis is an alternative means of altering gene function caused by insertion of foreign gene into the genome of host plant. The foreign T-DNA carrying GUS : NPT-II by insertion changes the host plant gene expression with GUS acting as a marker for identification of variations in morphological characteristics of transgenic plants. Similar observations were reported in *Arabidopsis* due to the involvement of either transposable elements or T-DNA (Krysan et al., 2000; Bundock et al., 2012). Researchers have substantiated that T-DNA acts as an insertional mutagen and such mutations are chemically and physically stable through multiple generations (Wisman et al., 1998). T-DNA inserted mutants could be cloned easily as reported by Sangwan and Sangwan-

Norreel (1989). Several large- scale T-DNA insertional mutagenesis projects are being undertaken for cloning mutant genes (Gelvin 2017; Jupe et al., 2019 and Pucker et al., 2021).

In *P. drummondii*, the plausible explanation for noticeable variation in floral architecture of transgenic plants regenerated in present investigation could be that foreign T- DNA by insertion into host plant genome must have altered the functional genes responsible for expression of floral characteristics, thus almost warranting a rechristening of such transgenic taxa that show nearly polypetalous trend as against gamopetalous nature of normal plants in *Phlox*. Therefore, in present study a protocol devised successfully transformed *P. drumondii* resulting in transgenic plants that have striking variations in floral features hitherto not reported.

Conclusion

Genetic improvement of ornamentals is an ongoing process. Over the years new varieties of ornamental plants have been produced by conventional procedures, such as selection, cross hybridization, or mutation breeding. Success achieved in ornamentals following these traditional procedures has remained restricted. As a result, attempts are being made to apply non-conventional *in vitro* methods. GM technology could be one such tool useful for improvement of traits in original ornamental cultivars. Gene transformation techniques are known to modify target traits due to direct integration of bacterial T-DNA in to host genome. These techniques thus are used as a supplement to traditional breeding methods. In the present study, an efficient *Agrobacterium*- mediated genetic transformation protocol using reporter (GUS) and marker genes (NPTII) was developed for *P. drummondii*, an important ornamental, which impacted its floral structure. This transformation procedure is first attempt for any species in the genus *Phlox* and has potential for transfer of genes of interest in other *Phlox* species, e.g., polyamine biosynthesis genes for delaying senescence and increasing the vase life. The new transformed variant varieties could be propagated vegetatively or sexually for perpetuation of new traits.

Abbreviations

CRMM – Callus Regeneration and Multiplication medium

SRM – Shoot regeneration medium

MS – Murashige and Skoog medium

YEM – Yeast Extract Mannitol medium

SRM I – Shoot Regeneration medium I

SRM II - Shoot Regeneration medium II

MSO - Murashige and Skoog liquid medium

GUS: – Glucuronidase

NPT-II: – Neomycin Phosphotransferase II

Declarations

Conflict of interests

Authors declare that they have no conflict of interest.

Author’s contributions

SNR and MKR conceived the idea and MVR designed the experiments. ART carried out the experiments, generated the data. ART and MVR analysed the data. ART wrote the manuscript. MVR, SNR and MKR edited, and finalized the manuscript. All the authors read and approved the final version of the manuscript.

Acknowledgements

I thank Prof. M.V. Rajam, Department of Genetics & Prof. S.M. Raina, Department of Botany, University of Delhi for providing facilities for providing facilities to conduct this study.

Data availability

Data will be available on request.

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Tables

Table 1. Influence of bacterial density (OD_{600}) genetic transformation in 6-weeks-old callus of *Phlox drummondii* on selection medium and subsequent shoot regeneration from transformed callus

Bacterial density (OD_{600})	Percentage callus showing GUS activity	Average number of shoots obtained from tranformed explants
0.1 - 0.3	50 ± 0.10 c	30 ± 2.10 d
0.3 - 0.5	60 ± 0.25 bc	53 ± 0.10 c
0.5 - 0.7	80 ± 0.50 ab	20 ± 0.35 de
0.7 - 1.0	80 ± 0.28 ad	0 ± 0 e

Means ± SE, n=50; means in the same column followed by different letters are significantly different at P ≤ 0.05 and F = 96.56Anderson – Cook 2004).

Table 2. Effect of the infection time on genetic transformation during cocultivation, and subsequent shoot regeneration in 6-week-old transformed callus of *Phlox drummondii* on selection medium

Infection time (Min)	Percentage callus showing GUS activity	Average number of shoots obtained from responding explants
5	45 ± 0.50 c	30 ± 0.02 d
10	70 ± 0.35 b	53 ± 0.01 c
15	80 ± 0.00 ab	15 ± 0.30 e
20	80 ± 0.10 ab	10 ± 0.00 e
30	85 ± 0.15 a	0 ± 0 e

Means ± SE, n=50; means in the same column followed by different letters are significantly different at P ≤ 0.05 and F = 96.56 (Anderson – Cook 2004).

Table 3. Transformation of embryogenic callus of *Phlox drummondii* with binary vector (pPZP200) carrying *GUS* reporter gene and *NPT*-II marker gene

Number of calli co-cultivated	Number of calli surviving on selection medium (SRM I)	Average number of transformed shoots obtained from responding calli	Percentage transformation	Percentage regeneration
85	53 ± 0.1 c	40 ± 0.5 cd	62 ± 0.10 b	47 ± 0.25 c
85	50 ± 0 c	38 ± 0.3 d	63 ± 0.11 b	48 ± 0.05 c
85	65 ± 0.5 b	53 ± 0.25 c	76 ± 0.5 b	62 ± 0.10 b
85	49 ± 0.12 c	39 ± 0 d	58 ± 0.18 c	45 ± 0.0 c
85	47 ± 0.11 c	37 ± 0.10 d	63 ± 0 b	49 ± 0.01 c

Means ± SE, n=50; means in the same column followed by different letters are significantly different at P ≤ 0.05 and F = 96.56 (Anderson – Cook 2004).

Figures

Figure 1

Three-week-old callus regenerated *in vitro* from zygotic embryo.

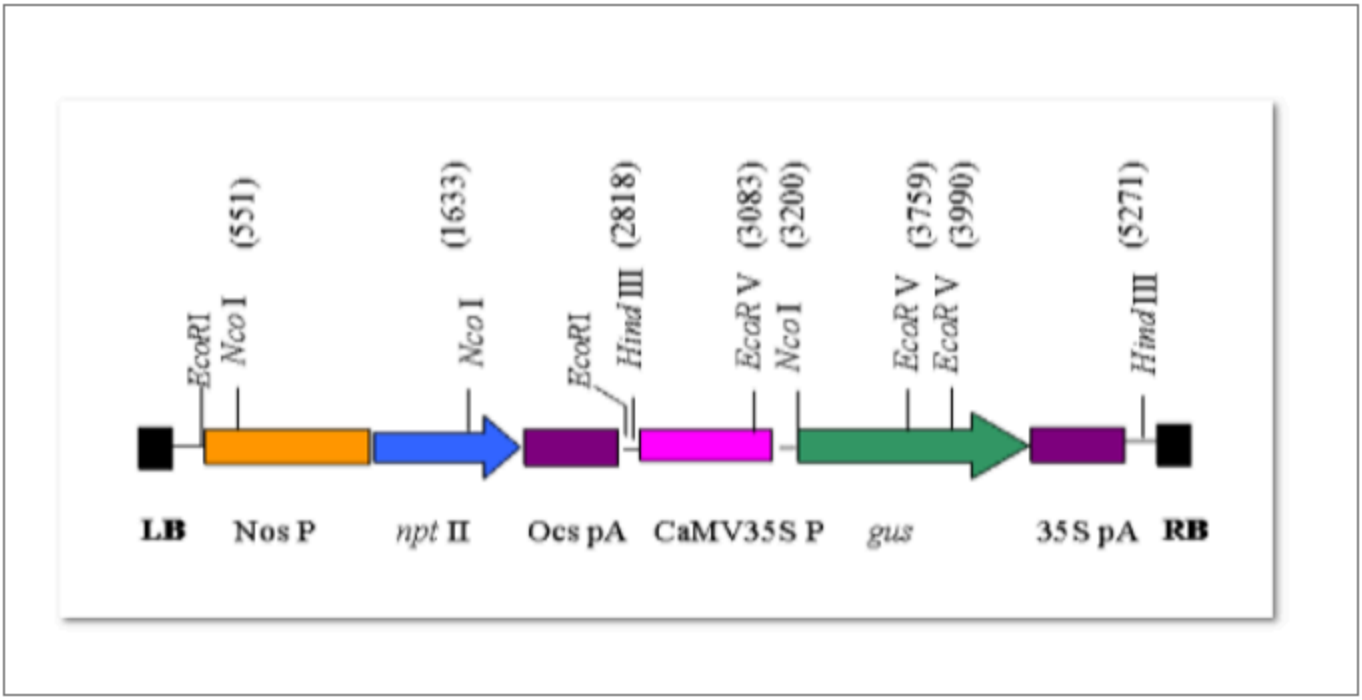

Figure 2

Showing construct of Binary plasmid vector PPZP200 KWT containing marker gene as Nos Pr nptii: OcspA cassette (conferring Kanamycin resistance) and a reporter gene as GUS (int)- 35S pA promoter cassette.

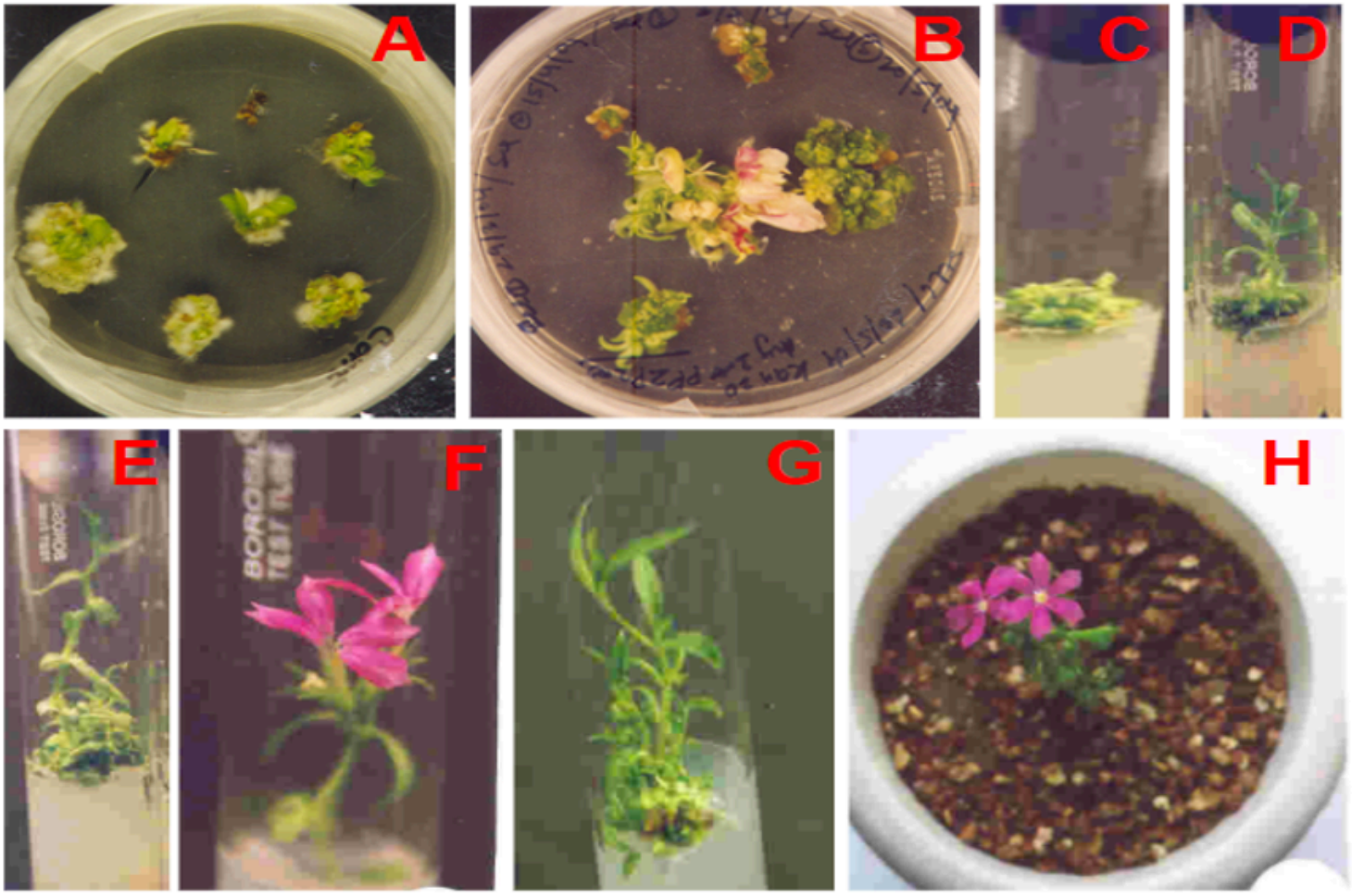

Figure 3

(A-H). Showing different stages of regeneration in 3-week-old transformed embryogenic callus (obtained from zygotic embryo culture) of *P. drummondii*

A. Shoot bud formation on the callus (untransformed) on SRM (shoot regeneration medium) with no antibiotics.

B. Shoot bud formation on the transformed callus on selection medium (SRM + 30 mg/l Kanamycin).

C. Transfer of shoot bud containing transformed callus to shoot proliferation medium (SRM II without kanamycin).

D – E. Elongation and multiplication of transformed shoots on shoot proliferation medium (SRM II)

F. Well-developed shoot obtained from transformed callus showing in vitro flowering on µG. rooting in vitro of transformed shoots of *P. drummondii* on the medium MS + IAA (7.5 M).

H. Transplantation of in vitro rooted transformed shoots to soil and vermiculite mixture.

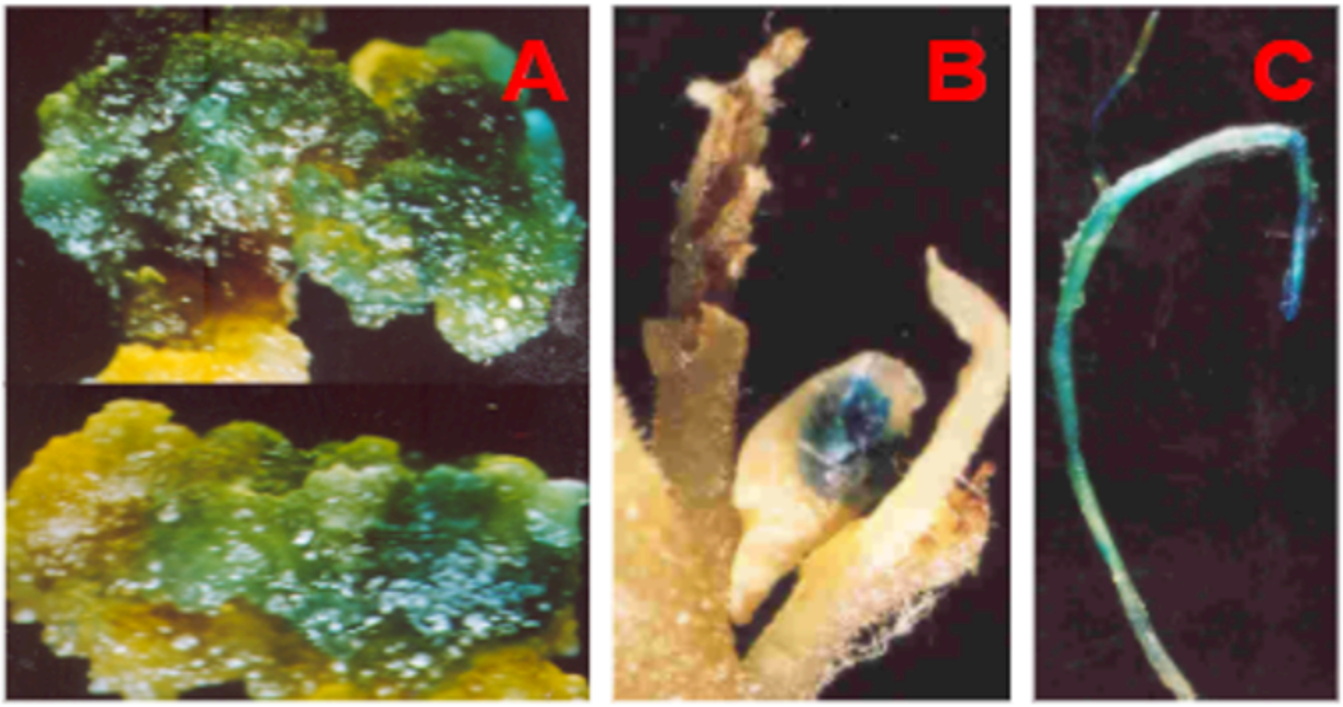

Figure 4

A-C). GUS Assay in different parts of transformed plant

A. Conformation of GUS gene (blue colour) in transformed callus.

B. Conformation of GUS gene (blue colour) in shoot of transformed plant.

C. Conformation of GUS gene (blue colour) in roots of transformed plant.

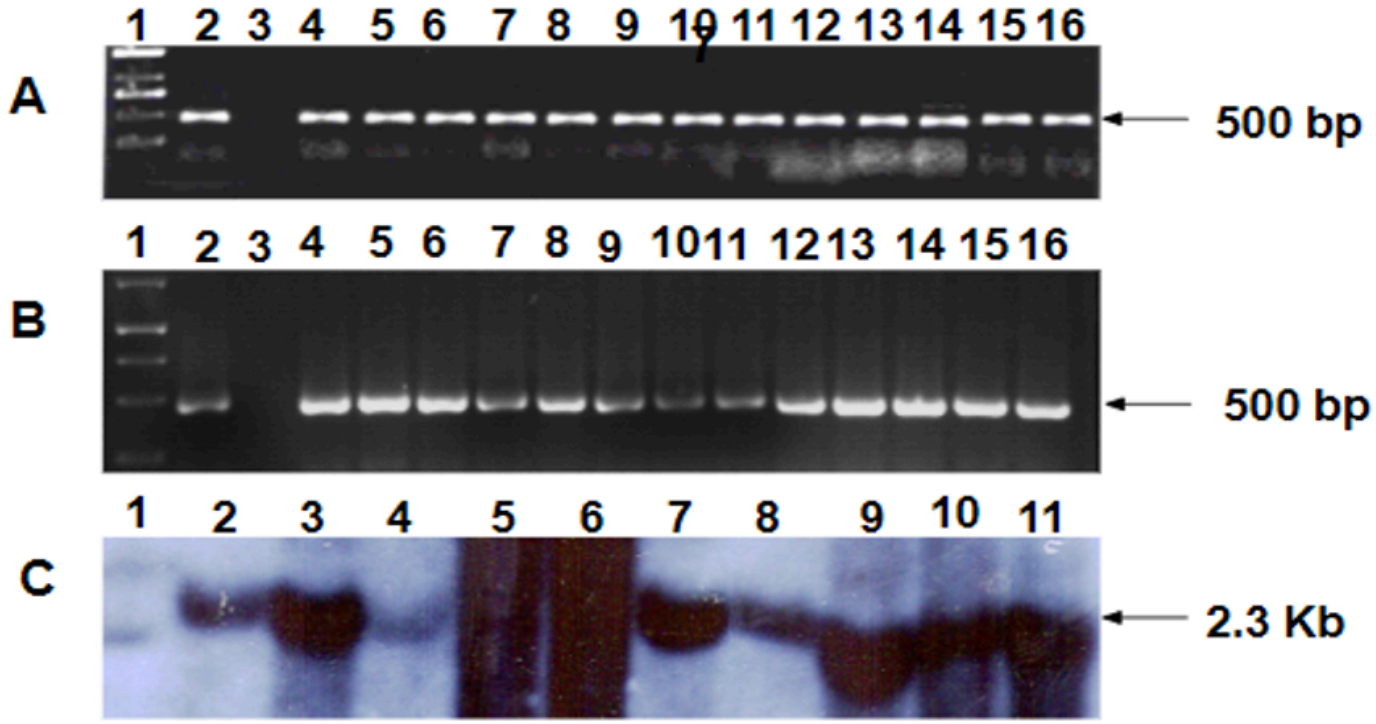

Figure 5

(A-C). Molecular characterization of putative transgenic plants

(A) PCR with *NPT*-II gene-specific primers: Lane 1- 1 kb ladder; Lane 2 – plasmid DNA: Lane 3- untransformed control; Lanes 4-16 putative transgenic lines.

(B) PCR with *GUS* gene- specific primers: Lane1- 1 kb ladder; Lane 2 - plasmid DNA; Lane 3- untransformed control; Lane 4 -16 putative transgenic plants.

(C) Southern analysis with *NPT*-II gene as probe: Lane 1 - 1 kb ladder; Lanes 2 -11PCR- positive transgenic lines.

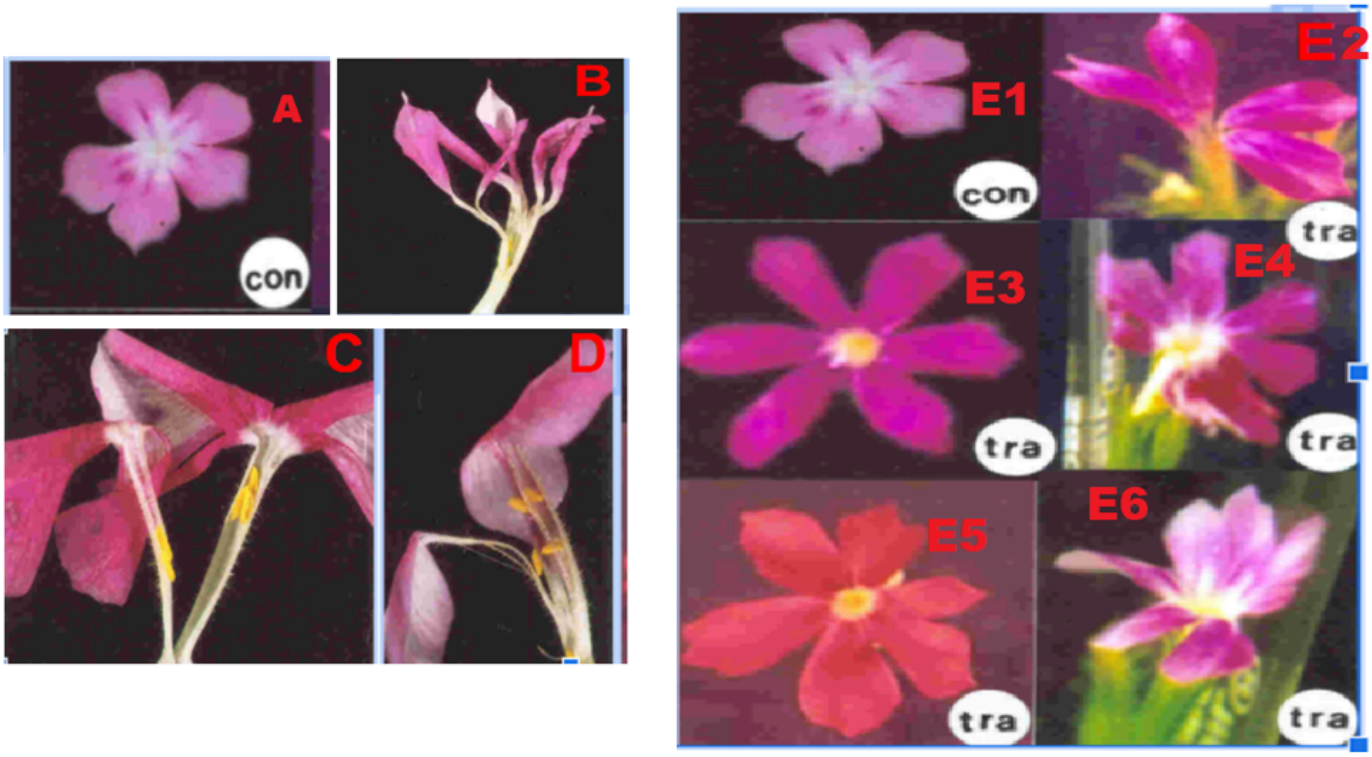

Figure 6

(A-E)

Variations in colour, shape, position of anthers and number of petals in the flowers of transformed (tra) plants in comparison to the normal (con).

A. Flower from control untransformed plant.

B. Flower from transformed plant showing nearly polypetalous condition.

C. L.S Flower from control plant showing epipetalous position of anthers (all the anthers have same position and same size of filament).

D. L.S Flower from transformed plant showing change in position of anthers (2+2+1) and variation in size of filaments of different anthers.

E. Flowers from different transgenic lines showing variation in colour: dark pink (E2-E4, E6), Red (E5); and shape: club shaped and nearly polypetalous (E2, E4 and E6) with increase in number of petals :6-8 (E3, E5 and E6) in comparison to flower of control (untransformed) plant, i.e. pink, gamopetalous with five petals (E1).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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