

ever pBR322::Tn5 (~10 kb) and pSUP2021 (~12 kb) were accepted easily by the culture. Therefore the preferential uptake of pBR322::Tn5 and pSUP2021 indicates that molecular weight of the plasmid alone does not determine the transformation frequency in *E. herbicola*. Similar observations have also been made in *Erwinia* sp. by other workers¹⁴. Attempts to mobilize pBR322 using *E. coli* S17-1 as the helper strain were also not successful. This is probably because of the fact that pBR322 lacks RP4 specific mob site.

Transformations of EhC1 with pBR322::Tn5 and pSUP2021 clearly show that pVQ1 (natural plasmid harboured in EhC1) has different origin of replication than that of pSUP2021 and pBR322. Both of these plasmids also co-existed stably with pVQ1 when transformed in the culture separately (Figure 3). This clearly shows that pVQ1 does not belong to Inc P group of plasmids and its origin is other than that of pMB1. During this study we did not come across any co-integrates formed as a result of homology between Tn5 present in pBR322::Tn5 and pSUP2021. This further confirms incompatibility between the two plasmids (pBR322::Tn5 and pSUP2021). Thus, it is evident that for the development of block mutants, the plasmid incompatibility strategy (using two different transposon vectors) could be exploited efficiently to facilitate transposition in *E. herbicola*.

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Agrobacterium-mediated genetic transformation in *Gerbera hybrida*

V. Nagaraju*, G. S. L. Srinivas[†] and G. Lakshmi Sita^{†,§}

*ICAR Research Complex for N.E.H. Region, Umiam 793 103, India

[†]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

Agrobacterium-mediated genetic transformation protocol was developed for the commercially important ornamental *Gerbera hybrida*. Petiole, leaf and shoot tip explants from 3 to 4 week old *in vitro* grown shoots of gerbera were co-cultured with *Agrobacterium tumefaciens* strain LBA4404. The strain harbours a binary vector containing neomycin phosphotransferase (*nptII*) and β -glucuronidase (*uidA*) genes. Callus formation and shoot regeneration were obtained on MS medium containing BAP, kinetin (1.0 mg/l each) and NAA (0.5 mg/l). Selection on the regeneration medium supplemented with 20 mg/l kanamycin allowed production of transgenic plants from 0.44% to 17.0% of the explants. GUS activity was detected by histochemical staining. Transfer of *uidA* and *nptII* genes was analysed by PCR and Southern hybridization. Transformed shoots were multiplied on MS medium containing 0.25 mg/l BAP, rooted in half strength MS medium supplemented with 40 mg/l kanamycin and successfully transferred to soil. This protocol could be used to introduce horticulturally important genes that govern pigment biosynthesis in flowers.

ORNAMENTAL plants are produced exclusively for their aesthetic values. Thus the improvement of quality attributes such as flower colour, longevity and form, plant shape and architecture and the creation of novel variation are important economic goals¹. *Gerbera* is one of the most popular ornamental plants in the world and is used as garden plants, potted plants, greenhouse flowers and cut flowers. Conventional breeding of gerbera by crossing and selection has generated several clonally propagated elite genotypes that have desirable traits, such as colour, shape, vase life and resistance against pests and diseases. However, one of the disadvantages of traditional breeding

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[§]For correspondence. (e-mail: sitagl@mcbl.iisc.ernet.in)

is the limited genepool of any single species. In addition, the heterozygous nature of the material makes the alteration of individual traits impracticable. Van Der Krol *et al.*² reported modification of flower pigmentation in petunia. Elomaa *et al.*³ developed a regeneration method and an *Agrobacterium*-mediated transformation using petiole explants of *Gerbera hybrida* variety Terra Regina. Mohamed *et al.*⁴ found that direct regeneration from vegetative tissue facilitates genetic transformation with minimal alteration of the target plant genome. Robinson and Firoozabady⁵ opined that success of *Agrobacterium*-mediated transformation depends on the cultivar, choice of the explants and the mode of regeneration⁶. The aim of our present study was to develop transgenic gerbera plants through *Agrobacterium*-mediated genetic transformation using appropriate explants of *Gerbera hybrida* line RCGH 164, *in vitro* shoot multiplication, rooting and *ex vitro* acclimatization.

The *Agrobacterium* carrying the plasmid pBI121 was used for transformation studies (Figure 1). The T-DNA of the binary vector contains the *nptII* gene, driven by the nopaline synthase (*nos*) promoter and terminator sequence. It provides resistance to kanamycin which was used as a selectable marker. The *uidA* gene, driven by the cauliflower mosaic virus (CaMV) 35S promoter and *nos* terminator was used as a phenotypic marker. Bacteria were cultured overnight at 28° in liquid LB medium (1% tryptone, 0.5% yeast extract and 1% sodium chloride, pH 7.0) containing 50 mg/l kanamycin and 25 mg/l rifampicin. The agro cells of 0.6 OD (50 ml) were pelleted at 4000 g for 5 min, resuspended and diluted in MS medium (50 ml).

Kanamycin, a widely used marker for plant transformation, can be phytotoxic and inhibit regeneration of transformed tissue. Experiments were conducted to determine the effect of kanamycin on morphogenesis. The effect of increasing kanamycin doses was evaluated using untransformed explants namely petiole, leaf and shoot tip explants cultured on regeneration medium containing 0, 10, 20, 30, 40, 60, 80, 100, 150, 200 and 300 mg/l kanamycin (10 explants/treatment). All the experiments were repeated thrice.

Petiole, leaf and shoot tip explants from one-month-old *in vitro* proliferated cultures of *G. hybrida* were used for this study. MS medium⁷ was used as the basal medium, with BAP, kinetin (1 mg/l each) and NAA

(0.5 mg/l) as growth supplements for the regenerating plantlets. The medium contained 3% sucrose and 0.8% agar. In all cases pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min at 121°C. Prior to transformation, the explants were pre-cultured on regeneration medium. After two days the explants were incubated for 5 min in an *Agrobacterium* suspension. They were blotted dry on Whatman No. 1 filter paper, placed on regeneration medium and incubated in dark. After 48 h of co-cultivation, the explants were washed 2–3 times in sterile MS, blotted dry, placed on regeneration medium and kept in dark. Cefotaxime (400 mg/l) was used to suppress the growth of *Agrobacterium* and transformants were selected using 20 mg/l kanamycin. The explants were subcultured onto fresh medium every 3–4 weeks. Regenerated shoots were transferred to MS medium containing kanamycin. Plantlets were considered kanamycin-sensitive if a newly developed leaf was mottled or bleached, or if there was no further growth. They were subsequently discarded. Elongated transgenic shoots were rooted on half strength MS medium supplemented with 40 mg/l kanamycin and acclimatized plants were transferred to soil.

Histochemical GUS assay was carried out according to the method of Jefferson *et al.*⁸. Explants were incubated overnight at 37°C in a reaction solution of 1 mM X-GLUC, 0.05 M potassium ferricyanide, 0.05 mM potassium ferrocyanide, buffered with 50 mM sodium phosphate pH 5.7. The materials were bleached, fixed in ethanol and observed under microscope.

DNA was extracted from transformed and untransformed plants by the CTAB method according to Murray and Thompson⁹. The final DNA pellet was treated with RNAase-A, dissolved in Tris-EDTA buffer and stored at –20°C until further use. DNA-PCR amplification was carried out in a thermal cycler using primers for *uidA* and *nptII*. PCR was performed with Taq DNA polymerase and 25 ng of template DNA. The upstream primer of 5'-TTC GCC TCG GCA TCC GCT CAG TGG CA-3' and 5'-GCG GAC GGG TAT CCG GTT CGT TGG CA-3', would yield a 500 base pair fragment of the *uidA* gene. PCR was performed initially for 5 min at 94°C, 35 cycles of melting (94°C 1 min), annealing (55°C 1 min) and synthesis (72°C 1 min). Two primers for *nptII* (5'-GAG GCT ATT CGGCTA TGA CTG-3' and 5'-ATC GGG AGG GGC GAT ACC GTA-

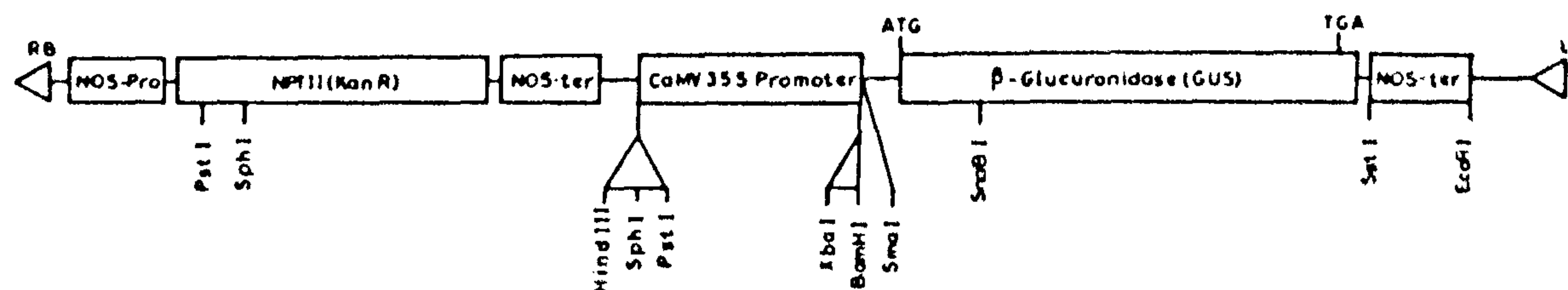


Figure 1. Schematic representation of pBI 121 plasmid. Abbreviation: *nptII*, coding region of the neomycin phosphotransferase gene; CaMV35S, cauliflower mosaic virus 35S promoter gene; *nos pro*, nopaline synthase gene promoter; *nos-ter*, nopaline synthase gene terminator

3') were used for amplifying a 700 bp product. Bangalore Genei Taq DNA polymerase (1 unit/50 µl reaction) and primers (0.25 µg/50 µl reaction each) were added. Amplification conditions were the same as above, but with annealing at 58°C, synthesis for 1.5 min/cycle and final soaking at 72°C for 6 min after all the cycles were completed. Amplified DNA was detected by ethidium bromide staining after electrophoresis on 0.8% (w/v) agarose gel. About 20 µg of plant DNA was digested with *Hind*III and *Eco*RI, fractionated by electrophoresis on 0.8% agarose gel, and transferred to Hybond-nylon membrane. Hybridizations were performed according to Sambrook *et al.*¹⁰. A 0.7 kb PCR fragment of the *npII* gene was used as a probe. Probe was labelled with [α -³²P]dCTP (Amersham) using a random-primed labelling kit (Amersham). After hybridization at 58°C, the filters were washed at high stringency conditions (65°C, 0.1 × SSC, 0.5% SDS).

In order to find out the appropriate selection conditions, kanamycin was tested in the regeneration medium. Though callus was produced from petiole, leaf and shoot tip explants on the medium supplemented with 40 or 60 mg/l kanamycin, the explants turned brown (data not shown). Hence kanamycin at a minimal concentration

of 20 mg/l was chosen as the selective factor in the transformation experiments and 40 mg/l for rooting of transgenic shoots.

After establishing the selection conditions, transformation was performed to obtain transgenic plants. Petiole, leaf and shoot tip explants from 3 to 4-week-old, *in vitro*-proliferated, etiolated shoots were pre-incubated for 48 h, infected with bacterial suspension, co-cultivated for 48 h and transferred to regeneration medium containing appropriate concentration of kanamycin and cefotaxime. Co-cultivation period beyond 48 h resulted in bacterial overgrowth and inhibition of callus formation and shoot regeneration. The explants were also bleached. The putative transgenic callus appeared 5 to 8 weeks after culturing the *Agrobacterium*-treated explants on the kanamycin medium. The transformation frequency was from 50 to 58% (data not shown). Transformed explants were transferred to fresh medium containing antibiotics. Shoots were regenerated from petiole (2.96%), leaf (0.44%) and shoot tip (17.0%) explants on MS medium (Figure 2a) containing BAP, kinetin (1.0 mg/l each) and 0.5 mg/l NAA. Six transgenic plants were selected for further analysis after being micropropagated on MS medium supplemented with 0.25 mg/l BAP. The putative

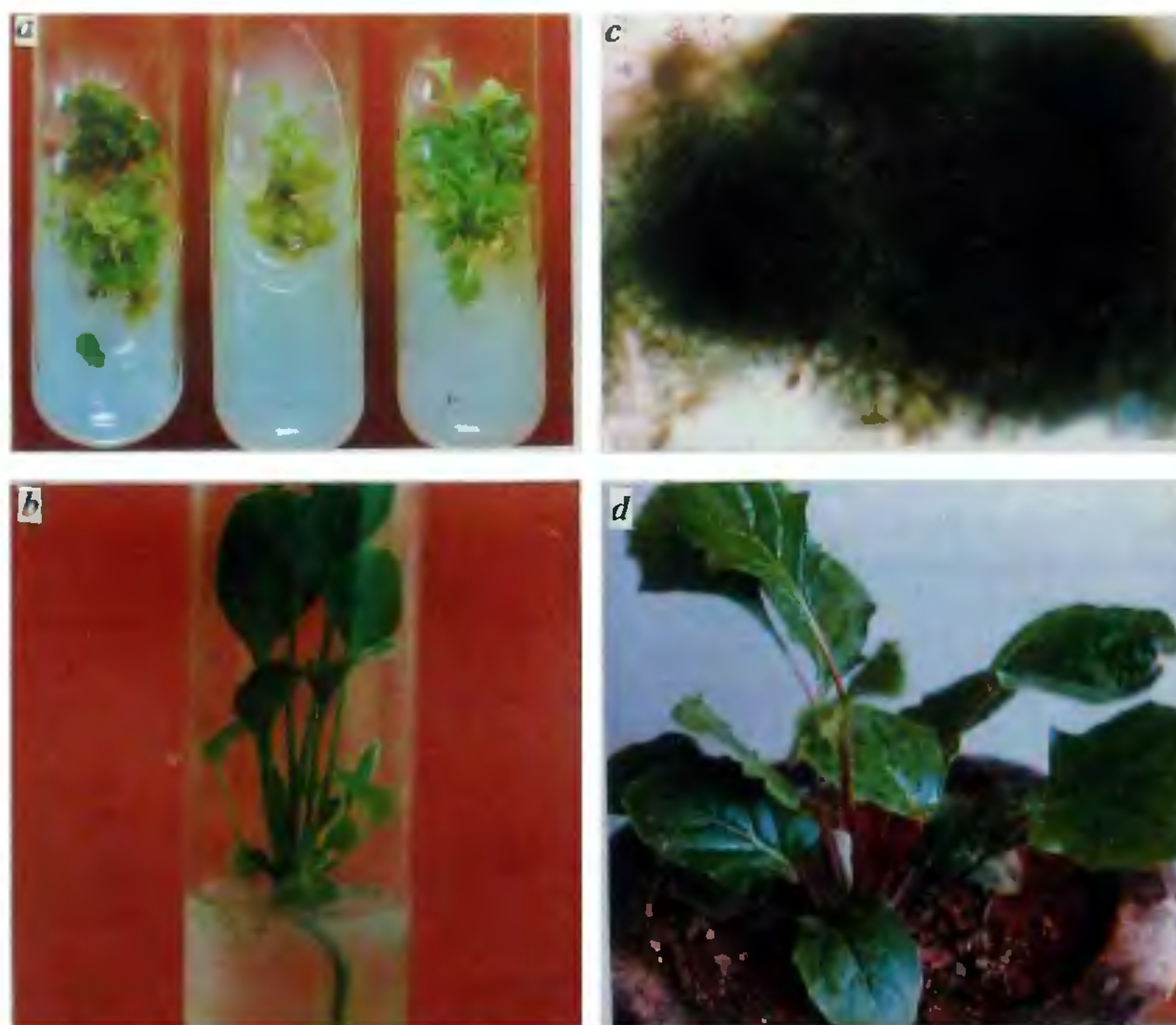


Figure 2. Regeneration and micropropagation of transgenic plants of *Gerbera hybrida*. a, Regeneration and multiplication of transgenic plants on kanamycin medium; b, Root development in selection medium containing 40 mg/l kanamycin; c, Transgenic plants showing characteristic blue colour of GUS; d, established plant in soil.

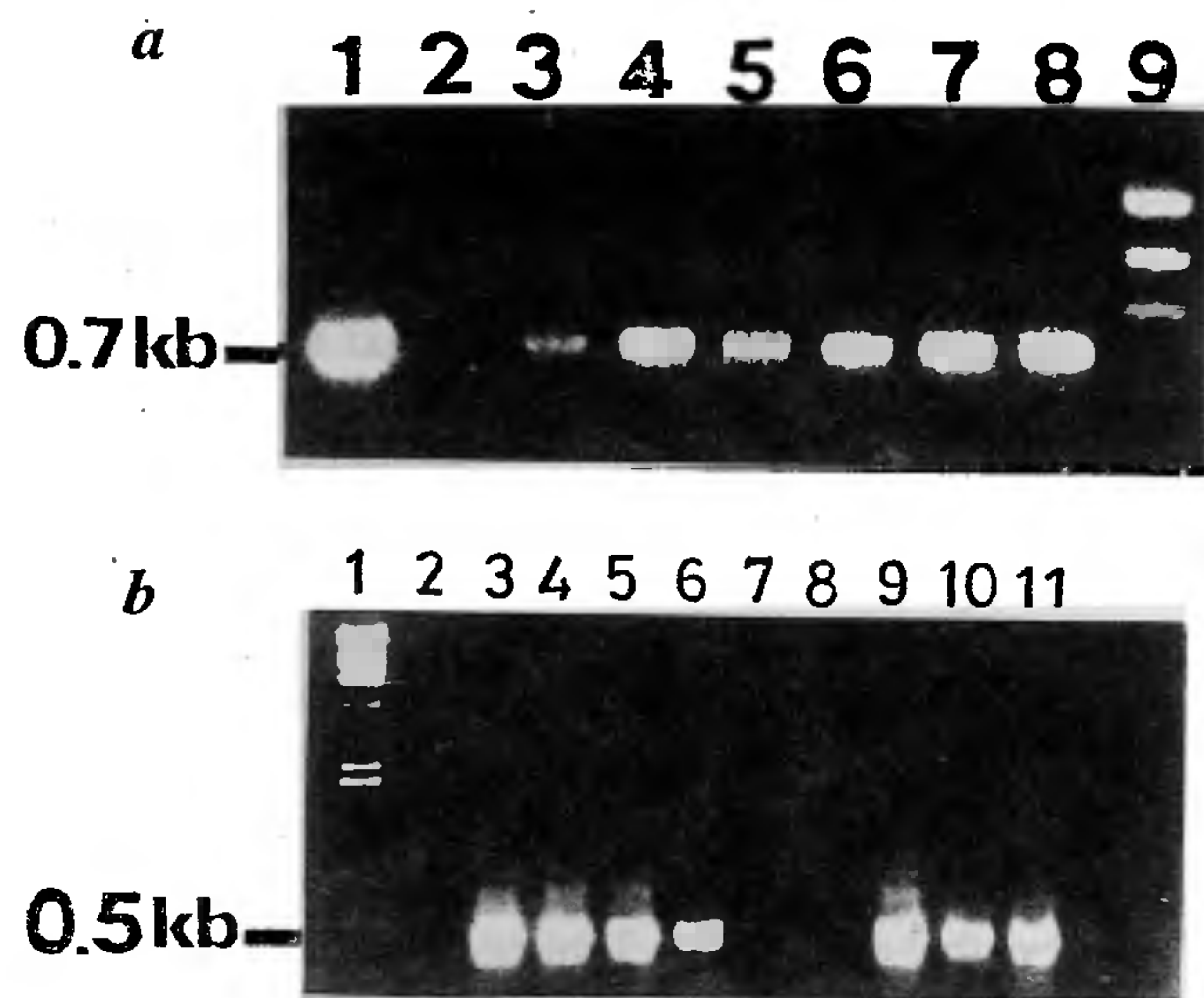


Figure 3. PCR analysis of kanamycin-resistant transgenic plants showing the presence of an expected fragment of (a) *nptII* (700 bp) gene. Lane 1, Positive control; Lane 2, Negative control (non-transformed); Lanes 3–8, Transgenic plants; Lane 9, Molecular marker. (b) *uidA* gene (500 bp). Lane 1, Molecular marker; Lane 2, Negative control; Lane 3, Positive control, and Lanes 4–11, Transgenic plants.

transgenic shoots and calli were transferred to antibiotic-free medium before the analysis of GUS activity. *Agrobacterium* also did not appear on this medium after 4–5 weeks of culture, indicating that no bacteria remained, which might interfere with the analysis. The individual shoots (>3–4 cm in length) excised from putatively transgenic plants, developed roots (Figure 2 b) in the presence of 40 mg/l kanamycin. Rooted plantlets were transferred to half strength MS solution for about 10 days and then planted in Majenta box filled up to one third with autoclaved soilrite and successfully transferred to soil after acclimatization (Figure 2 d).

Histochemical GUS assay was employed to determine the expression of *uidA* gene. Seven out of 59 kanamycin-resistant putative transformants gave positive results for GUS assay. Explants from control plants did not show the GUS activity. Some cultures showed a differentially stained pattern with some tissues exhibiting no detectable GUS activity and others showing a wide range of enzyme activity (Figure 2 c). The putatively transformed plantlets were subjected to PCR analysis to detect the presence of *uidA* and *nptII* genes. In the transformants with GUS activity, an expected fragment of 500 nucleotide *uidA* (Figure 3 b) gene was detected. An additional confirmation with PCR analysis of *nptII* was also done using appropriate primers. The *nptII* gene was observed from kanamycin-resistant plants and an expected fragment (700 base pair) of *nptII* gene (Figure 3 a) was amplified in all transformed DNA samples. No amplification was found in non-transgenic plants. Southern hybridization was also carried out in order to further confirm the T-DNA integration. A 0.7 kb PCR

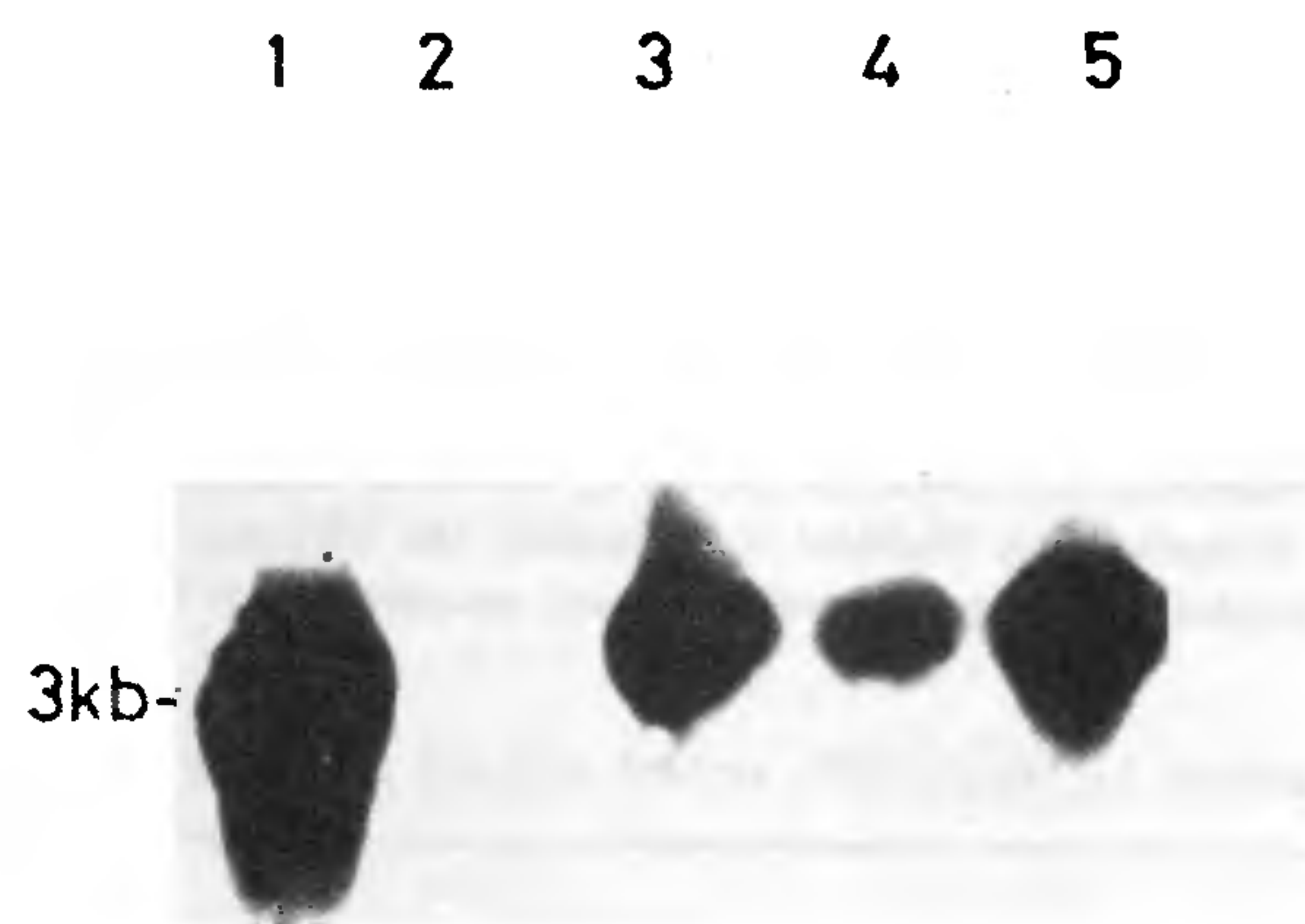


Figure 4. Southern blot analysis of DNA isolated from transformed and non-transformed gerbera plants. Lane 1, Positive control (pBI 121); Lane 2, non-transformed negative control; Lanes 3–5, Transformed plants. Filter was hybridized against 700 bp of PCR fragment containing the *nptII* gene.

fragment of the *nptII* gene was used as probe. The *nptII* fragment hybridized with DNA isolated from transgenic shoots and showed the signal at the expected size of 3.0 kb, indicating that the T-DNA of pBI121 was present (Figure 4). There was no hybridization to the DNA isolated from nontransformed control plants. Digested total DNA isolated from *Agrobacterium* (pBI121) also showed hybridization signal at the expected size (Figure 4). Six transgenic and five untransformed plants that were induced at the same time were transferred to pots containing soil. The transgenic plants showed normal morphogenesis. The results of our experiments indicated that transgenic plants were produced successfully using marker genes. This protocol could be used for introducing into gerbera, commercially important and horticulturally interesting genes that govern flower colour, longevity, creation of novel variation, insect and disease resistance. Further studies are required to produce viable seeds from transgenic plants for studies on heritability of introduced genes.

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Influence of pressmud on the enzymatic variations in the different reproductive stages of *Eudrilus eugeniae* (Kinberg)

L. S. Ranganathan* and S. P. Vinotha

Department of Zoology, Annamalai University,
Annamalainagar 608 002, India

Posterior gut of reproductively active, clitellate stage of the compost worm *Eudrilus eugeniae* when reared in pressmud (a waste byproduct of sugar mill) exhibits enhanced amylase, protease, acid and alkaline phosphatase and cellulase activity compared to activity of these enzymes observed in immature, pre-clitellate stage worms reared in the same media as well as in worms raised in cow dung. These enzymes activities are correlated to the significant growth exhibited by the clitellate stage.

EARTHWORMS, the most important soil invertebrates considering the amount of soil they move through their body and in their effects in improving soil properties, rely on microorganisms, particularly fungi, as their primary source of food¹⁻³. It has been recently shown that the clitellate stage of *Eudrilus eugeniae* reared in pressmud prey on and harbour more microorganisms³. Further, pressmud rich in organic matter (OM), nitrogen (N), phosphorus (P) and iron (Fe) has been shown to aid growth in terms of increased size with high fecundity in the clitellate stage⁴.

Our present knowledge of enzyme physiology related to reproduction and biomass formation is not adequate in the present context of sustained organic farming using vermitechnology. The present paper analyses the enzymatic activities of the economically important compost

worm *E. eugeniae* during its different phases of reproduction when the worms are reared in pressmud.

30-day-old immature pre-clitellate worms and 45-day-old mature clitellate worms were reared in separate cement tanks 50 × 35 × 30 cm, each containing 8 kg of feed with approx. 70% moisture, 29°C ± 1°C. Two-month-old, weathered, dry pressmud and dried powdered cow dung was used as feed. Required water was sprinkled to maintain moisture. The worms were pre-adapted to these feeds for three days and then were allowed to grow for 10 days. Homogenate of anterior gut (5-100 segments) cleared of contents and posterior gut (101-205 segments) was made in buffer solution. After centrifuging the homogenate at 2000 rpm for 15 min, the supernatant was used as an enzyme extract. The protein content of the gut homogenate was determined by the method of Lowry *et al.*⁵ using bovine serum albumin as standard. Amylase activity was measured following the method of Raghuvamulu *et al.*⁶, cellulase activity by the method of Malik and Singh⁷, protease activity by the method of Gowenlock⁸ and acid phosphatase and alkaline phosphatase activities by the methods of Gutman and Gutman⁹ and King and Armstrong¹⁰ respectively. Statistical significance between anterior and posterior gut and preclitellate and clitellate stage of worms was tested at 1% level.

The activities of enzymes in the different regions of the gut of pre-clitellate and clitellate stages of *E. eugeniae* when fed on pressmud and cow dung are given in Table 1. Amylase, protease, acid and alkaline phosphatase activities were higher in the posterior gut of reproducing, mature, clitellate stages of worms reared in pressmud compared to preclitellate stage reared in pressmud as well as compared to worms (both stages) reared in cow dung.

Pressmud (OM 50%; N 1.2% and P, 2.9%) was earlier found⁴ to support better growth measured as length and weight than when raised on cow dung (OM, 47%, N 1.1% and P 0.5%) in *E. eugeniae*. Significant growth and biomass was attained by this worm during sexual maturity (from 1500 mg to 3000 mg from day 30 to day 45) and probably this was supported by phenomenal increased activities of the amylase and protease in the clitellate, reproducing worm than pre-clitellate worms raised on pressmud as well as worms raised on cow dung (Table 1).

Enzyme activity in worms is regionally specialized as in other animals and influenced by physiological state, age and microorganisms¹¹. The observed highest enzymatic activity in amylase followed by protease in the posterior gut of clitellate *E. eugeniae* raised on pressmud was likely due to (i) substrate availability, (ii) regional specialization, and (iii) enhanced presence of microorganisms²⁻³. Higher protease activity in the posterior region of other worms was reported by Mishra and

*For correspondence.