(Figures 1 and 2) and UV spectrum analysis (Table 2, Figure 3). The experiments were repeated five times. The results mentioned above show that except from indirect lysis, all other DNA isolates, viz. by direct lysis, lysis by sonication as well as enzymatic lysis were pure and in good quantity. DNA by direct lysis was accepted as the method of choice for further studies (Figure 1) as it gave maximum amount of pure intact DNA (no DNA smear of lower molecular weight). DNA isolation was carried out from soils of different mechanical properties (Table 1) by the direct lysis method (Figure 3 *b*). Pure DNA was obtained in sufficient quantity (Table 1). The same method when applied to urine; enriched as well as pure cultures of both Gram-positive and Gram-negative bacteria (Figure 2), gave pure DNA which was subsequently used for PCR amplification of 16 SrDNA gene (Figures 2 and 4), TA-cloning (for community DNA and mixed culture) and sequencing. The novel sequences were submitted to GenBank. The applicability of DNA-based methods in the detection of pathogens would minimize the use of only culture-based detection. This becomes essential, as only 1 to 4% of the microbes can be cultivated under standard laboratory condition. Thus here we report one method of DNA isolation which works for a wide variety of samples ranging from soil of different kinds, water bodies with different organic and metal content (data not shown), and pure bacterial cultures of both Gram natures to pathological specimens.

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Agrobacterium-mediated genetic transformation of Nagpur mandarin (*Citrus reticulata* Blanco)

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Genetic transformation protocol was developed for Nagpur mandarin (*Citrus reticulata* Blanco), a choicest citrus variety grown in India and South East Asia. Cotyledon segments from mature seeds were co-cultivated with *Agrobacterium tumefaciens* for two days and cultured on an adventitive embryo induction

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medium containing MS basal medium supplemented with 8.87 μM BAP + 2.32 μM kinetin + 5.37 μM NAA with 60 μg/ml kanamycin and 300 μg/ml cefotaxime. The putatively transformed adventitive embryos that regenerated on the cotyledon segment were transferred for complete plantlet development on auxin-rich medium having MS basal salts fortified with 9.80 μM IBA and 100 mg l^{-1} activated charcoal. The transgenic plants were then hardened in glass jars filled with soilrite and perlite (1 : 1). GUS activity was detected in putatively transformed plants and maximum positive GUS assay frequency 34.5% was noted. Genetic transformation was confirmed by PCR analysis and Southern hybridization with α-^{32}P-labelled riboprobes of the *gus* gene.

Keywords: Adventitive embryony, *Citrus reticulata* Blanco, genetic transformation.

CITRUS is one of the most important fruit crops of the world due to its high nutritional value, huge fruit production and different processed products[1]. Citrus has its own merits and drawbacks with respect to a particular growing region of the world. Most of the cultivated *Citrus* species are diploid and have the basic chromosome number as 9 ($2n$ = 18), though triploid and tetraploid forms are also available[2]. Conventional genetic improvement of *Citrus* spp. faces a range of obstacles like long juvenility period, frequent inter- and intra-species incompatibility, polyembryony, etc. The common technique employed to reduce juvenility is grafting of scion on adult rootstock plants, which is not always effective. These characteristics make conventional breeding techniques difficult, expensive and also time-consuming[3]. The approach of using *Agrobacterium*-mediated gene transformation offers an attractive alternative to the conventional genetic improvement of *Citrus* sp. Genetic transformation of several *Citrus* genotypes has been achieved by co-cultivating different explants (mainly juvenile) with *Agrobacterium tumefaciens*, namely *Citrus sinensis* L. Osbeck[4], Washington Navel, calluses of Trovita sweet orange[5], epicotyl explant in Carrizo citrange[6–8], *Poncirus trifoliata*[9–11] and seedlings and inter-nodal stem segments of greenhouse-grown pineapple[12] and Ponkan[13] sweet orange, Swingle citumello[11]. However, only a few of these studies reported efficient production of transgenic plants mainly due to the difficulties in transforming citrus cells at sufficiently high frequency for producing shoots or embryos from the transgenic events and rooting the transgenic plantlets[7,9,10,12]. Strong influence of the genotype in citrus organogenesis[14–17] and genetic transformation suggested the need for specific studies on *in vitro* protocol adjustments[6,12,18]. Nagpur mandarin (*Citrus reticulata* Blanco) is a commercially important mandarin cultivar grown in India and adjoining countries and a few reports have described the *in vitro* regeneration for this cultivar[17,19]. We undertook the study for developing an efficient plant regeneration from cotyledon segment of matured seeds of Nagpur mandarin and establishment of transgenic plants via co-cultivation with *Agrobacterium tumefaciens* (strain EHA105 harbouring the binary vector pBI121 containing the *uidA* and *nptII* genes), with a view to develop virus-resistant genotypes in citrus.

Seeds were extracted from mature fruits (four-month-old) of Nagpur mandarin and washed in running tap water. The seeds were then agitated in 0.75% sodium hypochlorite (v/v) for 15 min, washed in tap water (30 min) followed by air-drying up to 12–15 h. Before storage, the seeds were treated with carbendazim (@ 1.0 g/kg) and packed in polythene bags (200 gauge) and stored at 4°C for further experimental use. For culture initiation the seeds were washed in running tap water for 30 min, and the outer and inner seed-coats were removed and surface-sterilized using 0.75% sodium hypochlorite solution (v/v) with 0.1% of Tween 20 for 10 min followed by rinsing five times with sterile distilled water. Zygotic and nucellar embryos were excised from seeds aseptically and cotyledon segments separated out with minimum damage. The cotyledon segments were then cultured onto the regeneration medium comprising MS basal medium[20] fortified with 8.87 μM 6-benzylaminopurine (BAP) + 2.32 μM 6-furfuryl-aminopurine (kinetin) + 5.37 μM α-naphthaleneacetic acid (NAA) and supplemented with 3% sucrose and solidified with 0.8% agar–agar. Before autoclaving (121°C for 15 min), the pH of the medium was adjusted to 5.7. All cultures were maintained in a culture room (26 ± 2°C) under complete darkness up to initiation of embryogenesis on the explant; thereafter they were transferred to 16/8 h light/dark regime (45 μmol m^{-2} s^{-1}). Enlarging adventitious embryos (3–4 cm long) formed on the cotyledon explant were excised and transferred for complete plantlet development on the MS medium fortified with 9.80 μM indole-3-butyric acid (IBA) and 100 mg l^{-1} activated charcoal (AC). *In vitro* developed plantlets were hardened and transferred to glasshouse[21,22].

To determine an appropriate concentration of kanamycin for the selection of transgenic shoots, cotyledon explants were cultured on regeneration medium with different concentrations of kanamycin (Sigma Chemical Co, St. Louis, USA) at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg/ml. Fifty cotyledons were used per treatment and adventitive embryo formation was recorded after eight weeks of culture. EHA105 strain of *A. tumefaciens* harbouring a binary plasmid pBI121 (Clontech, Palo Alto, CA, USA) was used as the vector system for transformation. The vector map is given in Figure 1. The bacterial culture was maintained on LB agar[23] plates (1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v sodium chloride; pH 7.0) containing 50 μg/ml kanamycin and 25 μg/ml rifampicin. A single colony was grown overnight on liquid LB at 28°C with appropriate antibiotics. Cotyledons from seeds were separated aseptically and were used as explant for the genetic transformation experiment. The cotyledons were gently shaken in the *Agrobacterium* suspension for about 10 min

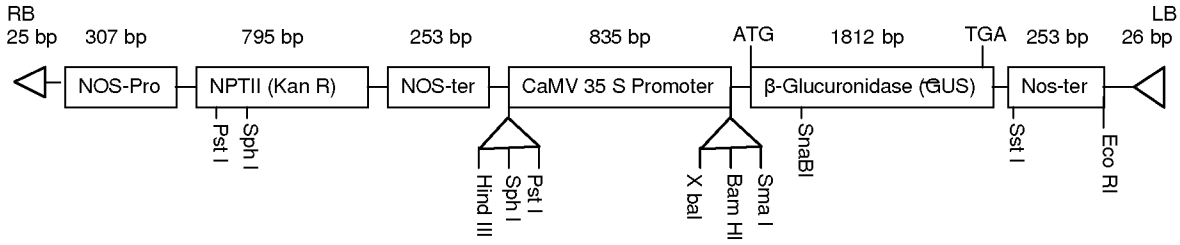

Figure 1. Diagrammatic representation of the pBI121 vector. The NOS-P, nopaline synthase gene promoter and NOS-T, nopaline synthase gene terminator signal control the expression of *nptII* gene.

and then blotted dry on a sterile tissue paper cushion. After co-culture, the explants were washed in basal MS liquid medium, blotted dry on a sterile tissue paper cushion and transferred onto the medium for adventitive embryony with antibiotics (60 µg/ml kanamycin and 300 µg/ml cefotaxime). Co-cultivated explants were maintained at 26 ± 2°C under dark till embryogenesis. Two subcultures were made on the above medium. Later the concentration of kanamycin was reduced (40 µg/ml) and made completely devoid of cefotaxime. The adventitive embryos were allowed to grow on the original explant and then subjected to complete growth on auxin-rich medium. All the experiments were repeated at least three times keeping the different parameters unchanged. The data for each treatment were expressed as mean ± SE.

Isolated shoots from the regenerated explants after hardening were tested for histochemical GUS expression in a 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-Gluc) solution consisting of 2 mM X-gluc, 100 mM Tris-HCl (pH = 7.0), 50 mM NaCl, 2 mM potassium ferricyanide and 0.1% (v/v) Triton X-100. Shoots were stained overnight[24] at 37°C, fixed in 1% (v/v) glutaraldehyde for 3 h, cleared through ethanol series 30, 50, 70, 90 and 100 (5 min each; v/v) and rehydrated by inverting the ethanol series. Assayed tissues were observed under a stereomicroscope. For PCR analysis, DNA was isolated from 50 mg of plantlet leaves according to the standard method[25]. The *nptII*-specific primer sequences were obtained (Bangalore Genei, India): 5′-GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGG GGC GAT ACC GTA-3′ and used for PCR analysis. Each 25 µl PCR reaction mixture consisted of 10X reaction buffer, 15 ng DNA, 200 µM dNTPs, 15 mM $MgCl_2$, 100 ng of each primer DNA and 1 unit of *Taq* DNA polymerase (Bangalore Genei, India). PCR was carried out in a Biometra® thermocycler under the following conditions: 94°C for 4 min as preheating, then 35 cycles of 94°C denaturing for 1 min, 58°C annealing for 1 min and 72°C extension for 1 min. The final extension for 7 min at 72°C was carried out. Amplified DNA product was electrophoresed on 1.2% agarose gel, detected by ethidium bromide staining and photographed under ultraviolet light. Southern blot hybridization analysis was carried out using standard procedure[23]. DNA was extracted from fresh leaves of transformed and non-transformed (control) plants. Approximately 15 µg of total genomic DNA from transgenic plants and non-transformed control plant was digested with *Hin*dIII and *Eco*RI. DNA samples were separated on 0.8% (w/v) agarose gel, transferred to Hybond-N^+ membrane and hybridized with α-^{32}P-labelled riboprobes of the *gus* gene.

Regeneration of adventitive embryo from cotyledon was obtained as reported[19] earlier. The frequency of the adventitive embryo formation on the cotyledon segments in control recorded 72%, whereas average number of adventitive embryos per cotyledon segment was 18.3. The frequency of adventitive embryo formation was recorded 37.7, 42.0, and 31.6% when tested in triplicate on MS medium supplemented with 8.87 µM BAP + 2.32 µM kinetin + 5.37 µM NAA and also supplemented with 60 µg/ml kanamycin and 300 µg/ml cefotaxime (Table 1).

Kanamycin sensitivity of cotyledon explants was assessed prior to *Agrobacterium* transformation, to determine the concentration of kanamycin needed for effective growth of transgenic plants (Figure 2). Higher concentration, i.e. above 60 µg/ml kanamycin caused necrosis by the end of the fourth week, with gradual inhibition of adventitive embryo formation. However, adventitive embryo formation was at par in the lower concentration, i.e. 10, 20 and 30 µg/ml of kanamycin (Figure 3). Cotyledon segments without co-cultivation by *A. tumefaciens* EHA105 harbouring pBI121 vector when transferred on the medium containing 60 µg/ml kanamycin and 300 µg/ml cefotaxime (selection medium) became completely necrotic within four weeks of transfer. In contrast, cotyledon explants maintained on non-selective adventitive embryo induction medium (without kanamycin) exhibited adventitive embryogenesis (72%) by the end of the fifth week in culture. Adventitive embryos formed directly after co-cultivation without intervening callus and the maximum frequency of adventitive embryo formation was 42% (Table 1). All the co-cultivated cotyledon explants were maintained by regular subculturing on the selection medium for adventitive embryony at five-week intervals. Kanamycin concentration was then lowered to 40 µg/ml in the third subculture to reduce the antibiotic stress on the developing adventitive embryos. Average complete plantlet regeneration per explant was noted 8.8 in control whereas it was 1.8, 1.3 and 1.7 in co-cultivated explants in the three experiments (Table 1). Almost all the transgenic clones appeared morphologically normal in comparison to the non-transformed (con-

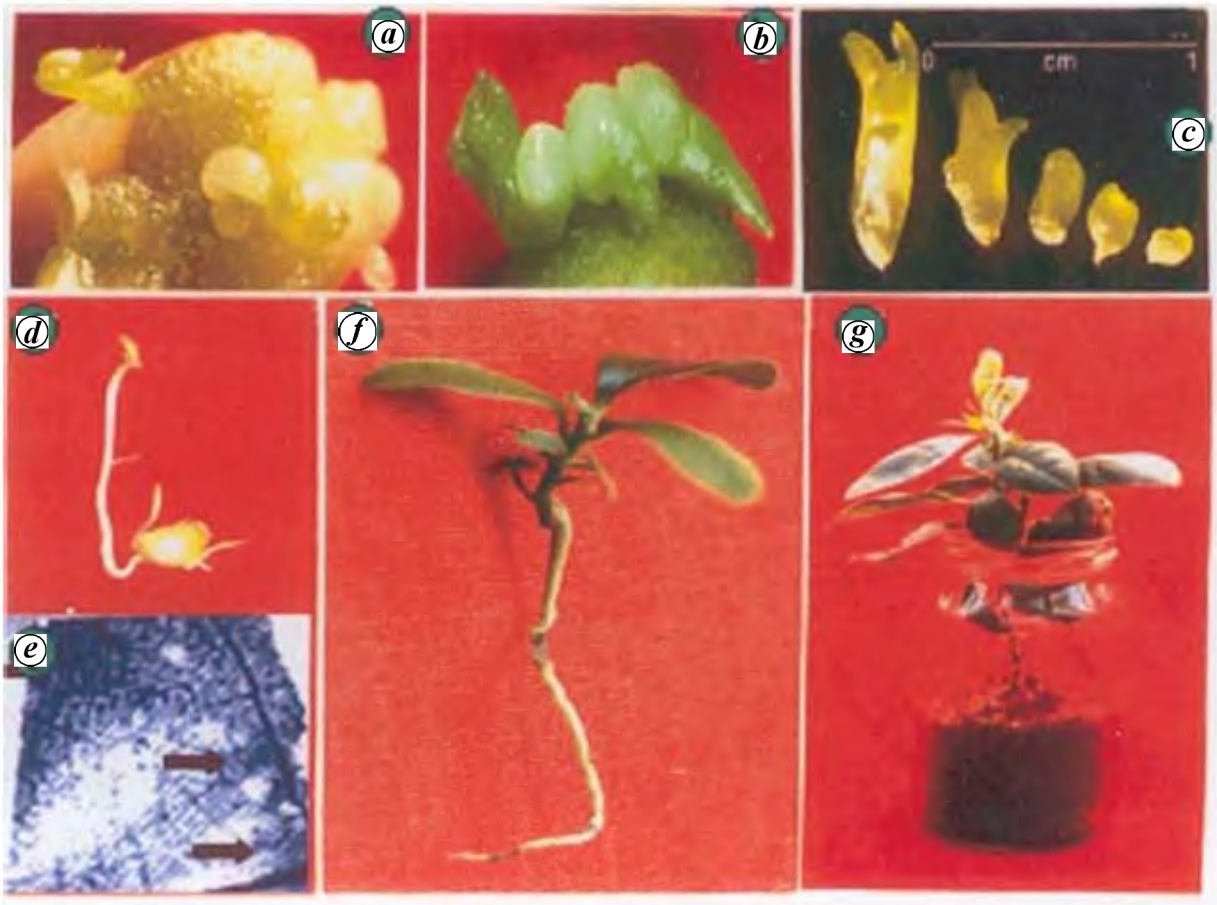

Figure 2. *Agrobacterium*-mediated genetic transformation and regeneration of transgenic plants from cotyledon segment of *C. reticulata* Blanco. ***a***, Cotyledon explants showing adventitive embryony; ***b***, Enlarged view of adventitive embryos on cotyledon explants; ***c***, Different developmental stages of *in vitro* adventitive embryos in citrus; ***d***, Transformed cotyledon explants showing adventitive embryony; ***e***, Arrow indicates GUS activity in young leaves; ***f***, Completely grown adventitive embryo with root; ***g***, Well-developed transformed plantlet after *in vitro* hardening.

Table 1. Transformation frequency of adventitive embryos derived on citrus cotyledon segment on MS medium containing 8.87 µM BAP, 2.32 µM kinetin, 5.37 µM NAA, kanamycin (60 µg/ml) and cefotaxime (300 µg/ml)

Treatment	No. of explants co-cultured	No. of explants showing embryo induction	Frequency of embryo formation (%)	Average no. of embryos/cotyledon	No. of plantlets regenerated/cotyledon	GUS positive assay (%)
Control*	50	36	72.0 ± 7.8	18.3 ± 2.1	8.8 ± 0.4	0.0
Control**	50	0	00.0	0.0	0.0	0.0
Exp. 1	45	17	37.7 ± 4.5	8.4 ± 0.4	1.8 ± 0.05	34.5 ± 4.7
Exp. 2	50	21	42.0 ± 4.8	6.7 ± 0.2	1.3 ± 0.06	22.7 ± 3.8
Exp. 3	38	12	31.6 ± 3.3	8.3 ± 0.5	1.7 ± 0.02	20.8 ± 3.7

*No co-cultivation, non-selective medium (without kanamycin).
**No co-cultivation, selection medium (with 50 µg/ml of kanamycin).

trol) plants after hardening. Thirty-four putatively transformed plants were selected and transferred to the soil after 30 days of glasshouse hardening. The survival rate was 93–95%. All the putatively transformed plantlets were subjected to *in situ* GUS assay. The expression of *uidA* gene was verified by histochemical staining of the young leaf. The positive regenerants showed typical indigo blue colouration of X-Gluc treatment, while the negative ones did not. Young leaves were more densely stained than other tissues of the plant. This seems to be the typical expression pattern of the CaMV35S promoter regulated *uidA* gene in young tissues. Leaf tissues from non-transformed plants did not show GUS activity. These results of extensive GUS expression in young tissues clearly demonstrated the stability of the inserted genes in the transformed plants (Figure 2 *e*). On the basis of GUS expression (i.e. number of putatively transformed plants showing positive GUS expression divided by total number of regenerated plants per cotyledon explant multiplied by 100), the maximum GUS positive assay was registered as 34.5% and the minimum was 20.8%. Presence of the *nptII* gene in the plants genome was confirmed by PCR analysis

which revealed that 0.8 kb *nptII* DNA fragments amplified from genomic DNA of putative transgenic plants. Figure 4 shows that all the samples from putative transgenic plants (lanes 3–9) gave 0.8 kb size of DNA fragment of *nptII* gene. No band was detected in the DNA sample from an untransformed control plant (lane 2). Based on resistance to kanamycin, GUS assay and PCR detection, presence of both *uidA* and *nptII* genes was confirmed in the transgenic mandarin plants. Integration of the GUS reporter gene into transformed plant genomic DNA was further confirmed by Southern blot hybridization analysis (Figure 5). Genomic DNA from randomly selected GUS-positive transgenic and one non-transformed control plant DNA was digested with *Hin*dIII and *Eco*RI and the *gus* gene was used as a probe. The *gus* gene probe hybridized to DNA from transgenic plants showed integration at different positions ranging from 2.4 to 20 kb. DNA from the untransformed control plant did not show any signal.

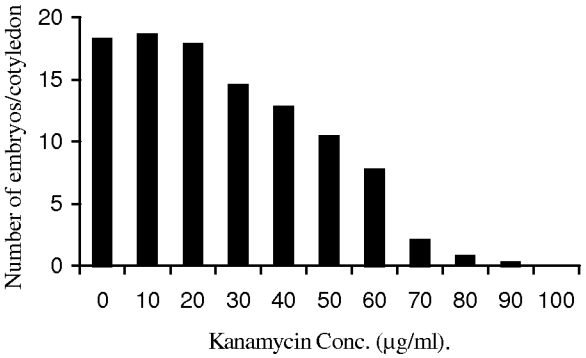

Figure 3. Effect of kanamycin on the number of adventitive embryos per cotyledon in *Citrus reticulata* Blanco after co-cultivation with *Agrobacterium tumefaciens* EHA105 harbouring PBI121 binary vector.

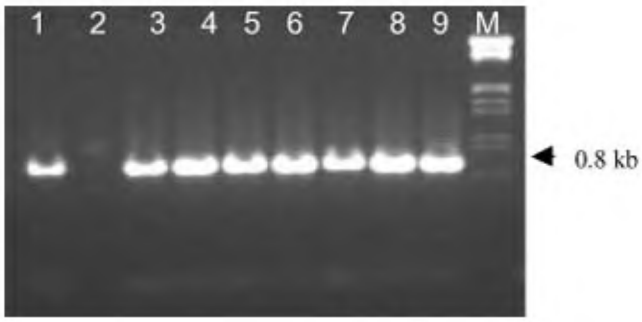

Figure 4. PCR analysis of transgenic plants by amplification of *nptII* gene from the DNA extracts. Lane 1, Plasmid pBI 121 (positive control); Lane 2, Untransformed plants (negative control); Lanes 3 to 9, Transformed plantlets; M, DNA size marker *Eco*RI/*Hin*dIII digested λ-DNA.

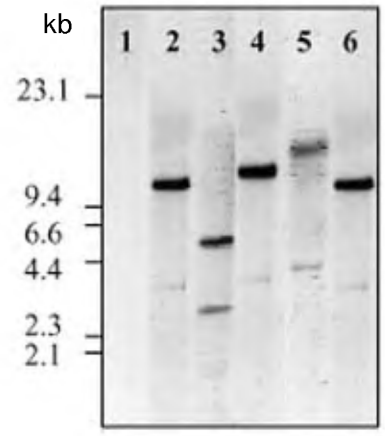

Figure 5. Southern blot analysis of transgenic *C. reticulata* Blanco plants. Total genomic DNA (15 µg) was digested with *Hin*dIII and *Eco*RI, subjected to electrophoresis, blotted and hybridized with α-^{32}P-labelled riboprobes of the *uidA* gene. Lane 1, Negative control consisting non-transformed Nagpur mandarin plant DNA; Lanes 2–6, GUS-positive plants.

In vitro regeneration using cotyledon as an explant was achieved in the present work. Results show that adventitive embryony was noted when explants were cultured and kept under dark in both the conditions, i.e. in control as well as in transformed tissues. Similar observations were reported in lime[7,15]. Regeneration of transgenic shoots at high frequency was reported when explants were maintained under darkness[7,12]. Kaneyoshi *et al.*[9] have obtained a high frequency of regenerated *P. trifoliata* transgenic plants without exposing the explants to darkness. These results suggest that different culture conditions may be needed for efficient regeneration of transgenic plants in different citrus species. Pena *et al.*[26] suggested that co-cultivation in auxin-rich medium caused conditions favourable for better integration of foreign DNA. The frequency of GUS positive that was comparatively[11] higher in the present study could be due to regeneration of adventitive embryos on auxin-rich medium and their subsequent transfer to auxin-rich medium for complete development.

In the present study, kanamycin concentration (60 µg/ml) was used for the initial selection of transformants and the positive GUS assay confirming transformation was noted as 34.5%, whereas this frequency was found to vary in different replications. A major problem in citrus genetic transformation is the occurrence of escapes[6]. Regeneration of escapes in *Citrus* could be explained by the high-efficiency shoot formation opposed to the low *A. tumefaciens* mediated transformation frequencies[5,6] or by ineffective kanamycin selection due to nonspecific *nptII* activity or by persisting *A. tumefaciens* contamination[27,28]. That the *gus* gene has become a reliable scorable marker and has allowed checking the putative transformants have been earlier reported[7]. In the present study, GUS assay has been used as a reliable source for characterization of putative transformants and confirmed by Southern blotting.

Biotechnological tools such as somatic hybridization[29,30] and genetic transformation[8,31–34] are already integrated in *Citrus* breeding programmes in several countries. The results presented here are an important contribution to the genetic transformation of an important *C. reticulata* Blanco cultivar, thus demonstrating the possibilities for

introduction of foreign gene(s) such as resistance to different pathogens (viruses) and horticultural interest.

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