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Evaluation of Parameters for High Efficiency Gene Transfer via *Agrobacterium tumefaciens* and Production of Transformants in Indian Mulberry, *Morus indica* cv. K2

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Abstract

Agrobacterium-mediated transformation of Indian mulberry, *Morus indica* cv. K2, employing the highly regenerative hypocotyl, cotyledon, leaf and leaf callus explants is reported. Preculture of explants on regeneration medium for 5 days and co-cultivation for 3 days was found optimal. With the GUS-histochemical assay up to 40% hypocotyl, 50% cotyledon , 50% leaf and 54% leaf callus explants tested positive. *Agrobacterium* strain LBA4404 was more infective than GV2260 and A281 and amongst the plasmids tested, pBI121 successfully transformed 100% of the explants followed by p35SGUSINT (90-100%). Approximately, 50% of the explants survived selection. Up to 35.7% hypocotyl, 38.5% cotyledon, 23% leaf and 40% leaf callus derived shoots tested positive for GUS gene activity by the spectrofluorometric analysis. Transgene integration in the regenerated transformants was confirmed by polymerase chain reaction (PCR) and Southern hybridization making this the first report of stable transformation of mulberry.

Key words: *gus, Morus, nptII,* regeneration, transformation.

Introduction

Recombinant DNA technology has opened a plethora of opportunities to genetically manipulate plants and supplement traditional plant breeding practices. The most successful technique used to transfer foreign DNA into plant cells employ the natural genetic engineer, *Agrobacterium*. The ability to transform tissues and to regenerate transgenics successfully using the natural gene transfer system of *Agrobacterium* depends on virulence of *Agrobacterium*, susceptibility of host plant, *Agrobacterium* cell density, period of co-cultivation, plant phenolics secreted in response to wounding, efficiency of selection that allows growth of the transformed cells and frequency of regeneration in the transformed cell population (Mahalakshmi and Khurana, 1997). Despite the established recalcitrance of woody plants (see Khurana and Khurana, 1999), Machii and co-workers reported *Agrobacterium tumefaciens* and particle bombardment mediated gene delivery in mulberry (Machii, 1990; Machii *et al.*, 1996). Oka and Tewary (2000) also reported injection of *A. rhizogenes* in the basal portion of the hypocotyls leading to the induction of hairy roots, which formed callus. Nozue *et al.* (2000) transformed the calli initiated from cotyledons, hypocotyls and roots of seedlings with *Agrobacterium tumefaciens*. However, all these reports failed to regenerate plantlets and hence, mulberry continues to remain recalcitrant with respect to genetic transformation efforts.

Our earlier work on mulberry successfully established highly efficient and reproducible regeneration protocols employing axillary buds (Tewari *et al.*, 1999; Bhatnagar *et al.*, 2001), hypocotyl, cotyledon, leaf (Bhatnagar *et al.*, 2000) and leaf callus (Kapur *et al.*, 2001) of Indian cultivars (Khurana *et al.*, 2002). The present study thus employs these protocols to transform the regenerative hypocotyl, cotyledon, leaf and leaf callus of *Morus indica* cv. K2 using *Agrobacterium tumefaciens* strain LBA4404 (p35SGUSINT). The present study reports the influence of diverse physical and chemical parameters like kanamycin sensitivity, bacterial density, duration of preculture, incubation and co-cultivation period, and infectivity of differ-

ent bacterial strains and plasmids towards achieving genetically transformed mulberry.

Material and Methods

Plant material and culture conditions

Seeds of *Morus indica* cv. K2 were collected from six- to seven-years old, healthy, field-grown plants and stored in a desiccator at room temperature. Seeds were surface sterilized with 0.1% mercuric chloride (Qualigens, India) for 8 min, rinsed 5-6 times and imbibed in sterile distilled water for 24 h prior to culture. Seeds were sown on MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg l^{-1} TDZ and kept in diffuse light. Hypocotyl and cotyledon explants were excised from 10-days old, *in vitro* raised seedlings. Leaf explants were taken from *in vitro* maintained axillary bud cultures (on MS medium + 0.1 mg l^{-1} TDZ). The callus explants were obtained from leaves taken from the field-grown plants and cultured *in vitro* on MS medium + 2 mg l^{-1} IAA + 1.1 mg l^{-1} TDZ + 2 mg l^{-1} $AgNO_3$ for over 2 months. All explants were precultured for five days on regeneration medium (RM: MS medium + 1.1 mg l^{-1} TDZ), unless specified otherwise, prior to co-cultivation. The pH of the medium was adjusted to 5.8 prior to autoclaving at 104 kPa at 121℃ for 15 min. Cultures were maintained at 25±1℃ under a daily photoperiodic regime of 16 h light and 8 h darkness. Four cool white fluorescent tubes (Philips, TL40 W/54) provided a light intensity of 65 μmol $m^{-2}s^{-1}$.

Bacterial strains and plasmids

Agrobacterium tumefaciens strain LBA4404 harboring the 13.2 kb p35SGUSINT (Vancanneyt *et al.*, 1990) construct was used for assessing the effect of bacterial density, duration of incubation and co-cultivation on the transformation frequency of various explants. The plasmid p35SGUSINT has the reporter gene *gus*, interrupted by a plant intron that restricts β-glucoronidase expression to plant cells and *nptII* as the selectable marker for kanamycin resistance. The *gus* gene is controlled by the CaMV35S promoter and polyadenylation signal, while the *nptII* gene is under nos promoter and termination signals. Plasmid pBI121 (Jefferson *et al.*, 1987) has the reporter gene *gus* driven by CaMV35S promoter and *nptII* gene by nos promoter and terminator. Infectivity of strain GV2260 and A281 was also compared.

Agrobacterium cultures were raised in Yeast Extract Broth (YEB: 5 g Beef extract, 5 g Bacto-peptone, 5 g sucrose, 1 g yeast extract, 0.5 g $MgSO_4$ per liter of medium, ph 7) medium supplemented with the antibiotics kanamycin (50 mg l^{-1}) and rifampicin (50 mg l^{-1}) and acetosyringone (200 μM) for 18 to 20 h at 26℃ and 240 rpm in dark.

Kanamycin sensitivity test

In order to check the sensitivity of the wild type (untransformed control) explants to kanamycin, explants were cultured on different concentrations of kanamycin sulfate ranging from 25 mg l^{-1} to 75 mg l^{-1}. The percentage survival and adventitious bud formation was calculated after a period of 30 days.

Cocultivation of explants

The explants pre-cultured on regeneration medium were treated with bacterial suspension at a density of $5x10^8$-$15x10^8$ cells ml^{-1} for varying periods (30-90 min). The explants were co-cultivated on RM for 2-5 days in dark. Samples were taken from each treatment and transient activity of the GUS gene was observed histochemically.

GUS histochemical assay

Explants were incubated overnight at 37℃ in the histochemical buffer [50 mM $NaHPO_4$, pH 7; 10 mM EDTA, pH 7; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 0.1% Triton X-100; 1 mM X-Gluc]. Transient expression of the β-glucuronidase was detected with the synthetic substrate (X-gluc), which upon cleavage formed a blue precipitate visual within transformed cells. Explants showing blue colour were scored as GUS positive.

GUS fluorometric assay

GUS Fluorometric assay was performed according to Jefferson, (1987). Leaf tissues from the shoots regenerated on the selection medium were used for fluorometric analysis. Tissue was homogenized in micro centrifuge tubes using liq. N2 and extraction buffer [50 mM $NaHPO_4$, pH 7; 10 mM EDTA, pH 8; 10 mM β-mercaptoethanol, 0.1% Triton X-100]. Crude protein extract (quantified according to Bradford, 1976) in extraction buffer was reacted with GUS assay buffer (1.0 mM MUG in GUS extraction buffer). The reaction was carried out at 37℃ for 15 h and 0.2 M Na_2CO_3 was added to stop the reaction. GUS activity was calculated as nanomoles of 4-MU formed (mg protein)$^{-1}$ h^{-1}. Explants showing more than 2 nanomoles of 4-MU (mg protein)$^{-1}$ h^{-1} were scored as *gus* positive.

Selection and recovery of plantlets

After 3 days of co-cultivation, the explants were washed with MS liquid medium containing 0.1 mg

l^{-1} TDZ + 250 mg l^{-1} cefotaxime and selected on RM + 50 mg l^{-1} kanamycin + 250 mg l^{-1} cefotaxime for a period of 10 days. Thereafter, they were transferred on to the Shoot Elongation Medium (SEM: MS + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 2 mg l^{-1} $AgNO_3$) + kanamycin 50 mg l^{-1} for 15-20 days. The shoots regenerated under this selection pressure were then tested for GUS gene expression (spectrofluorometric) and were sub-cultured on SEM, without selection, for 15 days. These shoots with 6-7 leaves were then transferred on the Root Induction Medium (RIM: 1 mg l^{-1} NAA + 0.1% activated charcoal). Plantlets with well-developed roots were transferred to earthen pots containing autoclaved soil: Soilrite (1:1), and maintained in the growth room.

Polymerase Chain Reaction

For the rapid detection of transgenes in the putative transformants PCR analysis (Mullis and Faloona, 1987) was performed. Equal amount of DNA (200 ng) of the transformed and untransformed (negative control) and 20 ng of the plasmid DNA (positive control) were amplified with the nptII primer [nptF: 5′-TCG GCT ATG ACT GGG CAC AAC AGA-3′ and nptR: 5′-AAG AAG GCG ATA GAA GGC GAT GCG-3′] in a reaction volume of 25 μl. The PCR amplification was performed by initial denaturation at 94℃ (5 min hold), followed by 25 cycles at 94℃ (30 s), annealing 54.5℃ (30 s) and 72℃ (30 s) and finally holding at 72℃ (7 min) for extension employing a Perkin-Elmer Gene Amp PCR system 2400. The PCR products were run on 1.2% agarose gel in 1x TAE alongwith size markers (1kb ladder GeneRuler^{TM} MBI Fermentas, USA).

Southern analysis

Genomic DNA was isolated according to Dellaporta *et al.* (1983). 10 μg of transformant DNA and 100 picogram of p35SGUSINT DNA (positive control) were digested with *Eco*RI, which has a unique site within T-DNA region. 10 μg DNA of untransformed plantlet (negative control) was also run on a 0.8% agarose gel, along with 2 μg of undigested DNA samples from each of the transformants. These fractionated DNA segments were transferred to positively charged Hybond-N nylon membrane and hybridized with *nptII* probe. The 1750 bp product of *gus* gene and 721 bp PCR product of the *nptII* gene from p35SGUSINT were extracted from 1% low melting point agarose gel and used as the probe.

Results and Discussion

Agrobacterium is a natural genetic engineer, whose potential remains under-exploited for transformation of woody plant species (Khurana and Khurana, 1999) and thus continues to be the subject of active research. Various physical and chemical factors appear to affect the efficiency of transformation and optimization of conditions for culture and co-cultivation appear to be of paramount importance. The present investigation thus explores various parameters crucial for high efficiency gene transfer in mulberry, *Morus indica* cv. K2, by the *Agrobacterium tumefaciens* co-cultivation approach.

Kanamycin sensitivity

Various regenerative explants of *M. indica* cv. K2 were evaluated for their sensitivity towards kanamycin sulfate. Up to 50% of the explants placed on RM medium produced adventitious buds and addition of kanamycin had an inhibitory effect on the regeneration response. On the basis of survival and adventitious bud formation after 30 days on selection, a selection pressure of 50 mg l^{-1} kanamycin sulfate was selected for hypocotyl, cotyledon and leaf explants. Machii (1990) has also used this concentration to select the kanamycin-resistant adventitious buds in mulberry. However, the leaf calli were able to produce adventitious buds on even 75 mg l^{-1} kanamycin after 30 days of culture. Thus, in all the experiments with leaf callus, a selection pressure of 75 mg l^{-1} kanamycin for a period of 45 days was provided.

Competence of explants

The age and physiological status of explants at the time of co-cultivation plays an important role. The plant material should contain cells competent for both regeneration and transformation. It was found that pre-culturing of explants before co-cultivation enhanced the susceptibility of cells to infection and T-DNA transfer. Preculture on a variety of hormones ranging from 2,4-D, NAA to TDZ is known to enhance regeneration in other tree species such as *Malus* (Schaart *et al.*, 1995), *Malus* x *domestica* Berkh. (De Bondt *et al.*, 1994), *Populus nigra* (Confalonieri *et al.*, 1994), *Populus tremuloides* (Tsai *et al.*, 1994), *Prunus americana* (Machado *et al.*, 1992) and *Prunus elulcis* (Miguel and Olivera, 1999). In the present investigation, best response (50%) was obtained when the explants were pre-cultured on regeneration medium for a period of 5 days (**Table 1a**).

Table 1 Effect of preculture, bacterial density, duration of incubation and co-cultivation, on frequency of transformation of different explants of *Morus indica* cv. K2 with *Agrobacterium tumefaciens* strain LBA4404 (p35SGUSINT), five days after co-cultivation.

Explants → Treatment	Hypocotyl (%GUS+ve)	Cotyledon (%GUS+ve)	Leaf (%GUS+ve)	Leaf Callus (%GUS+ve)
a. Pre-culture duration (days)				
2	20 ± 0.58	40 ± 1.00	25.0 ± 2.90	NP
5	35 ± 0.58	50 ± 0.00	50.0 ± 0.00	NP
7	16 ± 0.67	30 ± 1.00	36.6 ± 3.30	NP
10	15 ± 0.58	30 ± 0.58	23.3 ± 3.30	NP
b. Bacterial density (x 108 cells/ml)				
5	20 ± 0.58	30 ± 0.58	26.6 ± 1.67	44.4 ± 2.33
8	35 ± 0.58	50 ± 0.00	43.3 ± 1.67	48.0 ± 2.75
10	20 ± 0.00	30 ± 0.58	50.0 ± 5.80	53.5 ± 05.0
15	05 ± 0.00	05 ± 0.58	43.3 ± 4.40	53.1 ± 03.1
c. Incubation duration (hours)				
0:30	40 ± 0.00	46 ± 0.33	50.0 ± 5.80	45.4 ± 5.58
1:00	36 ± 0.33	50 ± 0.58	43.3 ± 6.70	51.6 ± 6.78
1:30	30 ± 0.58	50 ± 0.00	40.0 ± 0.00	50.0 ± 4.73
d. Co-cultivation duration (days)				
2	20 ± 0.00	33 ± 0.33	26.6 ± 3.30	44.4 ± 2.33
3	35 ± 0.58	46 ± 0.33	46.6 ± 6.60	46.4 ± 5.06
4	16 ± 0.33	13 ± 0.33	40.0 ± 0.00	51.2 ± 5.89
5	00 ± 0.00	00 ± 0.00	06.6 ± 3.30	50.0 ± 4.74

NP: Not performed

Bacterial density and duration of co-cultivation

The early log-phase was found ideal for the preparation of bacterial suspension. Hypocotyl and cotyledon explants gave maximum GUS-expression (**Fig. 1A, B**) when co-cultivated with a bacterial density of $8x10^8$ cells ml^{-1} while for leaf and leaf callus explants (**Fig. 1C, D**) the ideal density was $10x10^8$ cells ml^{-1} (**Table 1b**). *Agrobacterium* cell density varying from 10^7 to 10^9 have been used routinely for different tree species. The optimal time of incubation of explants in bacterial solution varied for different explants (**Table 1c**). If left in bacterial solution for more than 90 min it was very difficult to recover the explants due to bacterial overgrowth after co-cultivation. Duration of co-cultivation is thus an important factor as during this time the transfer and integration of bacterial plasmid-DNA with the plant genomic-DNA takes place. In our study, the optimum time at which maximum GUS positive expression is obtained has been found to be 3 days (**Table 1d**). Due to overgrowth of bacteria around the explants, it was difficult to remove the bacteria during washing, if co-cultivated for 5 days or more. Generally, the period of co-cultivation in tree species is varied from 2 days in papaya (Fitch *et al.*, 1993), plum and apricot (Machado *et al.*, 1994) and poplar (Howe *et al.*, 1995) to 3 days in Liquidamber (Sullivan and Lagrimini, 1993) and 4 days in pear (Mourages *et al.*, 1996) and apple (Sriskandarajah *et al.*, 1993).

Suitability of Agrobacterium strains and plasmids

Agrobacterium strains play an important role in the transformation process, as they are responsible for infectivity and efficiency of gene transfer. The suitability of different strains harboring various plasmids was observed on the leaf and leaf callus tissue incubated for 30 min at a bacterial density of 10^9 cells ml^{-1} and co-cultivated for 3 days. The explants were subjected to GUS histochemical assay after washing. *Agrobacterium tumefaciens* LBA4404 (p35SGUSINT) and LBA4404 (pBI121) were found more infective than GV2260 (p35SGUSINT) and A281 (pBI121) (**Fig. 2**), The GUS positive explants obtained were above 90% with LBA4404, 70-75% with GV2260 and 25-35% with A281 (**Fig. 2**). LBA4404 (p35SGUSINT) has been found to be effective for other tree species also by James *et al.* (1989) in apple, Kajita *et al.* (1994) in

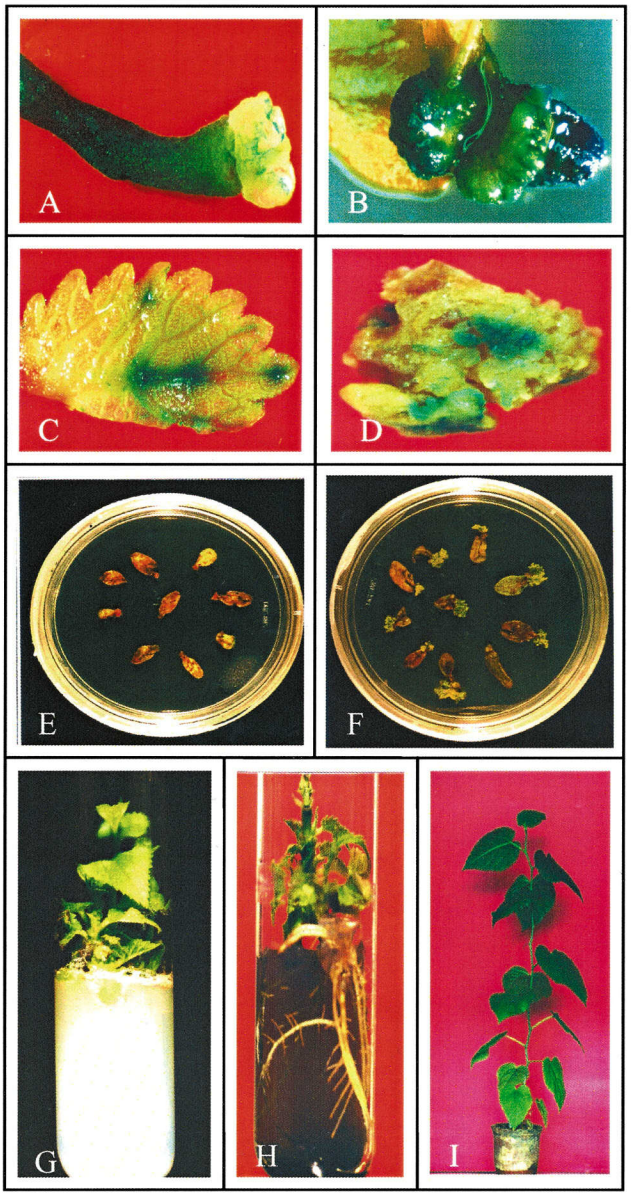

Fig. 1 Transformation of Indian mulberry via *Agrobacterium tumefaciens* LBA4404 harboring p35-SGUSINT. GUS positive hypocotyl (A), cotyledon (B), leaf (C) and leaf callus (D). Cotyledon explants on MS medium + 1.1 mg l^{-1} TDZ + 50 mg l^{-1} kanamycin, control (E) and transformed explants producing adventitious buds (F), Regenerated shoots on MS medium + 2 mg l^{-1} IAA + 1.1 mg l^{-1} TDZ + 2 mg l^{-1} $AgNO_3$ (G). Rooting on MS medium + 1 mg l^{-1} NAA + 0.1% activated charcoal (H), Putative transformant in pot (I).

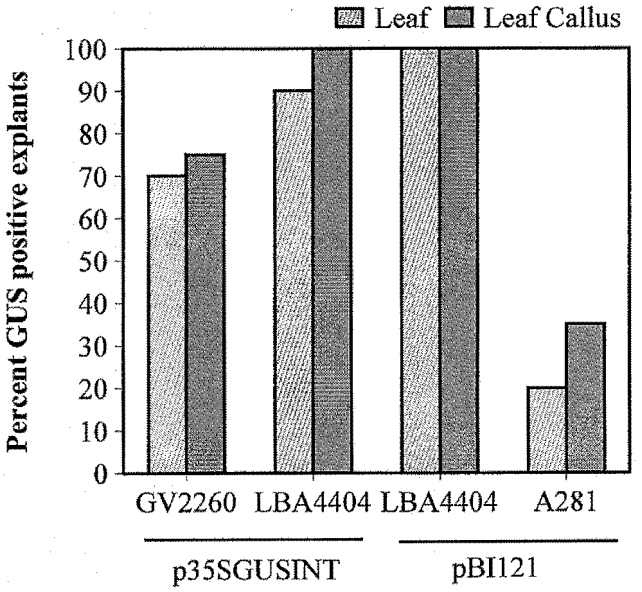

Fig. 2 Percentage of GUS positive leaf and leaf callus explants of *Morus indica* cv. K2 co-cultivated with different strains of *Agrobacterium tumefaciens* harboring two different plasmids, after three days of co-cultivation.

poplar, Machado *et al.* (1994) in apricot and plum and Archilletti *et al.* (1995) in almond. Amongst the various plasmids in LBA4404, pBI121 successfully transformed 100% of the various explants tested followed closely by p35SGUSINT (90-100%) **(Fig. 2)**.

Potential of various explants/tissues

Five days after co-cultivation, the various explants i.e. hypocotyl, cotyledon, leaf and leaf callus, were analyzed histochemically for expression of the GUS gene. Percentage GUS positive explants varied from 40% in hypocotyls, 50% in cotyledons and leaves to 54% of leaf calli, although, there was not much difference in terms of the number of blue spots and their intensity in the different explants **(Fig. 1A-D)**. Since the importance of the transformed cell lies in its subsequent division and differentiation into an adventitious bud, the leaf callus thus emerged as the explant of choice.

Selection and regeneration of putative transformants

After co-cultivation the explants were washed

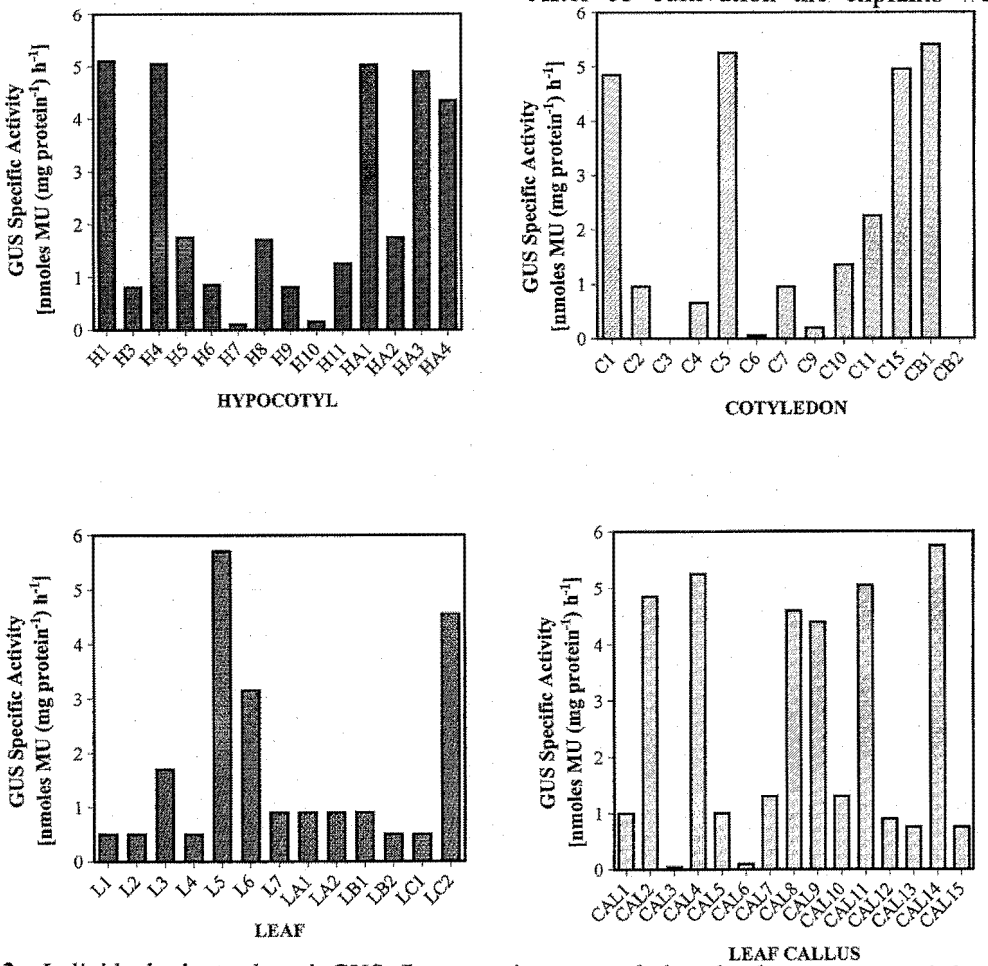

Fig. 3 Individual plant-based GUS fluorometric assay of the plantlets regenerated from hypocotyl, cotyledon, leaf and leaf callus explants of *Morus indica* cv. K2 transformed with *Agrobacterium tumefaciens* LBA4404 (p35SGUSINT), one month after co-cultivation.

thoroughly with liquid RM containing 250 mg l^{-1} cefotaxime to remove the adhering bacteria. Explants were then placed on RM + kanamycin supplemented with cefotaxime for 10 days, to restrict the bacterial growth while selecting the transformed explants. Some of the explants turned yellow and were discarded, while the kanamycin resistant green ones were transferred to fresh selection medium (SEM + kanamycin) for another 20 days. Approximately 50% of the explants survived the selection and formed adventitious buds (**Fig. 1F**). After 15-20 days on selection medium, the regenerated shoots were transferred to a selection-free medium for elongation (**Fig. 1G**), and *gus* expression levels estimated fluorometrically in individual regenerated shoots. Although most of the shoots showed GUS activity, only the shoots having very high activity [more than 2 nanomoles of 4MU formed (mg protein)$^{-1}$ h^{-1}] were scored as positive. Based on this criteria, the number of shoots testing positive for *gus* activity were 35.7% for the hypocotyls, 38.5% for the cotyledons, 23% for the leaf explants, and 40% for the leaf callus tissue (**Fig. 3A-D**). The *gus* positive shoots were then transferred to root induction medium for plantlet formation. Healthy roots developed in 80% of the shoots transplanted (**Fig. 1H**), and 90% of these plantlets were subsequently established in pots and maintained successfully in the growth room (**Fig. 1I**).

PCR analysis

The PCR analysis of the genomic DNA was performed using primers specific to *nptII*. In one of the representative experiments, three out of five samples tested displayed the presence of amplified product of 721 bp (**Fig. 4**). No band was observed in the untransformed control. Of the putative transformants analysed 50% tested positive for both *nptII* and *gus* by PCR analysis. PCR amplification of *gus* (Nozue *et al.*, 2000) and rol (Oka and Tewary, 2000) genes have been demonstrated in the *Agrobacterium* transformed calli of mulberry, however, these calli failed to regenerate plants.

Southern analysis

The genomic DNA of the putative transformants obtained by co-cultivating leaf callus with *Agrobacterium tumefaciens* LBA4404 (p35SGUSINT) was digested with *Eco*RI and blotted onto a nylon membrane and probed with *nptII*. Presence of an intron in the *gus* coding region ensures eukaryotic expression and eliminates chances of bacterial contamination. In a representative experiment, four transformants were probed with *nptII*, the autoradiograph demonstrated bands in the digested samples of all the four transformants tested while no signal was observed in the untransformed control (**Fig. 5**). In transformants C-2 and C-5 a single band at 7.5 kb was observed which indicates integration of single copy of *nptII* in plant genome. Multiple bands in the range of 4-9 kb were observed in transformants A-5 and A-1. This indicates integration of *nptII* gene in 5-6 copies. Presence of same band in C-2 and C-5 and similar pattern and copy number in A-1 and A-5, confirms these to be the clones obtained through different transformation events. This is thus the first report to show transgene integration in the host genome of mulberry. In the earlier reports on transformation of mulberry transgene integration has not been substantiated by Southern hybridization. Characterization and detailed analysis of other PCR positive

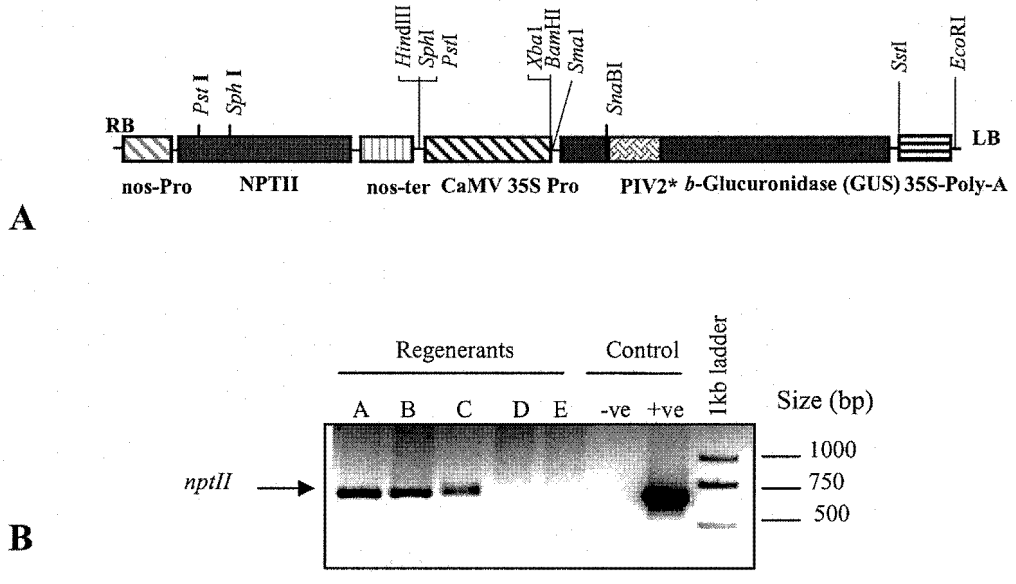

Fig. 4 (A) Partial map of p35SGUSINT. (B) PCR analysis of genomic DNA samples of five putative transformants of *Morus indica* cv. K2 using primers specific for *nptII* gene.

transformants is in progress.

In the present study, we have not only optimized parameters for high frequency transformation of mulberry via *Agrobacterium tumefaciens* strain LBA4404, but also shown that co-cultivation of regenerative explants of mulberry with *Agrobacterium* at a density of $8-10x10^8$ cells ml^{-1} for 3 days induces GUS gene expression and confers kanamycin resistance. The regenerants were checked for stable integration of the transgene by both PCR and Southern hybridization. This is thus the first report of stable transformation of mulberry (*Morus indica* cv K2). The transgenics are growing successfully for over six months and detailed characterization is in progress. The protocol developed hereby, can thus be used for stable transformation of mulberry with genes of desirable agronomical traits.

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