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Efficient Regeneration System for Genetic Transformation of Mulberry (*Morus indica* L. Cultivar S-36) Using *in Vitro* Derived Shoot Meristems

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ABSTRACT

Shoot meristems used for the study were exercised from the *in vitro* regenerated shoots cultured on MS medium supplemented with 0.5 mg/L of BAP for multiplication. The sensitivity of the *in vitro* regenerated was studied using shoot meristems of 0.5 cm. Shoot meristems were cultured on medium containing 10 - 100 mg/l kanamycin to determine the concentration that was lethal for multiple shoot induction and root induction. The response of shoot multiplication decreased (66.2% - 6.2%) as the concentration of kanamycin increased (10.0 - 70.0 mg/L) with complete inhibition of shoot proliferation at 100 mg/L kanamycin. The rooting phase was very sensitive to kanamycin compared to shoot multiplication. The percentage of shoots that rooted decreased (53.8% - 4.8%) with increase in the concentration of kanamycin (10.0 - 70.0 mg/l) on IBA and 2,4-D supplemented medium. For transformation studies, the shoot tips that were infected with *Agrobacterium* strain were placed on selection medium containing MS medium with 0.5 mg/L BAP and 100 mg/L kanamycin and scored for the putative transformed shoots. An average of 62.2% of shoot tips developed shoot buds from the base and the shoots reached a length of 0.5 - 1.0 cm at the end of 30 days of culture on the selective medium in comparison to control which showed no response. An average of 66.7% of the regenerated plants showed GUS expression on selection medium where 43.2% and 65% of GUS expression was recorded in the leaves and callus. Leaves and callus induced from the controls did not show GUS activity. Stable integration of *npt*II gene with the genomic DNA from these transformed plants was confirmed through PCR analysis. Our result presents an efficient regeneration system using *in vitro* derived shoot meristems for *Agrobacterium* mediated gene transfer.

KEYWORDS

***Morus indica* L. Cultivar S-36; *In Vitro* Regeneration; Shoot Meristems; Kanamycin; Genetic Transformation**

1. Introduction

Mulberry (Morus spp.), a woody perennial tree plant plays a significant role in sericulture, as its foliage constitutes the main diet for silkworm (*Bombyx mori* L.). The most important factor in the management of sericulture is the improvement of mulberry cultivation for achieving higher leaf yields. Mulberry leaves are essential for the survival of silkworms, since silkworms are monophagus insects, which grow only by feeding on mulberry leaves. Thus the cultivation of mulberry is the most important factor in the production of silkworm eggs, rearing of silkworm eggs, rearing of silkworm cocoons and on the whole in the entire operation of sericulture. Mulberry cultivation is fraught with many problems in the form of biotic and abiotic stresses. Among the various biotic stresses, fungal diseases cause a major damage to this crop [1]. Mulberry is improved qualitatively and quantitatively by conventional genetic approaches. How-

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ever, the perennial and highly heterozygous nature of the plant coupled with prolonged juvenile period limits the speed of improvement using conventional methods [2]. Further, the dioecious nature of the taxon and the genetic linkage of desirable and weak traits limit the success of genetic improvement.

Genetic engineering offers a suitable alternative as it facilitates the introduction of desirable genes from different sources for developing resistance to various abiotic and biotic stresses. However, this approach demands the development of genetic transformation technology for integration of desirable genes. Among the various genetic transformation techniques, *Agrobacterium* mediated transformation is preferred because of its simplicity, efficiency and relatively neat packaging, and stable integration of transferred DNA into the plant genome [3].

Although several reports on efficient transformation using diverse explants of mulberry have been published [4-6] but still success with genetic transformation of mulberry species is not satisfactory. This is due to the lack of efficient protocols to regenerate the whole plants. We describe here an improved system for routinely developing transgenic mulberry plants (*Morus indica* L. cultivar S-36) through the use of *Agrobacterium tumefaciens*. To the best of our knowledge, there have been no reports of *Agrobacterium*-mediated transformation of mulberry using *in vitro* multiplied shoot meristems as explants. This transformation system has the advantages of efficient, simple and rapid regeneration and transformation (with no need for sterilization or a greenhouse to grow stock plants), flexibility (available all the time) for *in vitro* manipulation, uniform and desirable green tissue explants for both nuclear and plastid transformation using *Agrobacterium* mediated and biolistics methods, no somaclonal variation and resolution of necrosis of *Agrobacterium* inoculated tissues.

2. Materials and Methods

2.1. Plant Material

Mulberry cultivar S-36 (*Morus indica L.*) was procured from Department of Sericulture, P2, L. R. Seed Farm, Kammadanam, Mahboobnagar, Andhra Pradesh, India. Initially nodal explants bearing axillary shoots were cultured on MS medium with 0.3 mg/L of 2,4-D where the axillary buds sprouted with high frequency [7]. When the shoots attained 1.0 - 1.5 cm of length shoot meristems of 0.5 cm were excised and cultured on 0.5 mg/L of BAP. For transformation experiments shoot meristems of 0.5 cm from healthy shoots at the end of 3rd stage of shoot proliferation was used. The work dealing with *Agrobacterium* mediated transformation was done in strict observance of national safety regulations.

2.2. Agrobacterium Construct

A. tumefaciens strain GV2260 harbouring binary plasmid p'GUSINT'' used for the transformation experiments of mulberry. It contains a scoreable reporter gene GUS (*β*-glucuronidase) driven by a CaMV35S promoter and NOS terminator and a selectable maker gene nptII fused between NOS promoter and NOS terminator encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance [8]. The *Agrobacterium* strain was maintained on LB agar medium containing 100 mg/l rifampicin, 100 mg/l carbenicillin and 50 mg/l kanamycin. A single colony of *Agrobacterium* was cultured in 25 ml of LB broth medium with the above antibiotics at 28˚C on a rotary shaker (200 rpm) for about 18 h until an optical density (OD) 600 of approximately 0.5 was reached. Aliquots of the culture were centrifuged at 3000 rpm for 5 min. The pellets were suspended in MS medium containing 3% sucrose in 1:10 dilution and used for infecting the explants.

2.3. Tissue Culture Selection and Plant Regeneration

MS medium with 3% sucrose and 0.8% agar was used for inducing shoot proliferation and root induction from shoots. The shoot meristems excised from healthy shoots were cultured on MS medium with 0.5 mg/l BAP supplemented with a range of concentration of kanamycin (10 - 100 mg/l) to evaluate its effect on shoot bud differentiation. In addition, the sensitivity of the shoots to root induction was tested by culturing healthy shoots on MS medium with 0.1 mg/l IBA or 0.1 mg/l 2,4-D and 10 - 100 mg/l kanamycin.

Healthy shoot meristems were infected with the *Agrobacterium* by exposing the cut end of the shoot meristem to bacterial suspension for 10 min, blotted on sterile filter paper and transferred to MS medium with 0.5 mg/l BAP. After 2 days of co-cultivation on MS medium with 0.5 mg/l BAP at 22˚C in the dark, the explants were placed on MS medium with 0.5 mg/l BAP and 250 mg/l cefotaxime for 2 days for eliminating the *Agrobacterium*. The shoot meristems were then transferred to MS medium with 0.5 mg/l BAP, 250 mg/l cefotaxime and 100 mg/l kanamycin as the selection agent. The shoots meristems that have responded for bud differentiation were subsequently transferred to MS medium with 0.5 mg/l BAP and 100 mg/l kanamycin. The multiple shoots that were induced were subcultured on the same medium for five times to avoid the possible escapes. Healthy shoots (2 - 3 cm) derived from the cultures at the end of 5th subculture on selection medium were transferred to MS medium

with 0.1 mg/l IBA or 0.1 mg/l 2,4-D with 50 mg/l kanamycin for root induction. The shoots that developed healthy roots were transferred to plastic pots containing soil and organic manure and the humidity was maintained by covering with polythene cover for 10 - 15 days. Subsequently, the plants were transferred to earthen pots and maintained in the green-house.

2.4. GUS Analysis

GUS gene expression in the leaves and callus cultures of the untransformed and putative transformed plants was detected using the method of [9]. Tissues were incubated for 12 hr at 37˚C in 50 mM Sodium phosphate buffer (pH 7.0) containing 1mM X-gluc. Following overnight incubation the tissues were rinsed in 70% ethanol and the development of blue colour was monitored.

2.5. Characterization of Putative Transformed Plants

Genomic DNA was isolated from leaf tissues by the procedure based on CTAB (Cetyl trimethyl ammonium bromide) method, which is a modification of the method of [10]. Leaves were collected from the putative transformants and untransformed plants (controls) established in the field. Presence of *npt* II gene was confirmed by PCR amplification. The primer sequences for *npt* II gene are: *npt II* Left 5' GAG GCT ATT CGG CTA TGA CTG 3' *npt II* Right 5' ATC GGG AGC GGC GAT ACC GTA 3'. PCR was performed on a Perkin Thermal cycler, with DNA extracted from putative transformants and untransformed control plants under the following conditions. Each reaction (50 µl) contained 0.2 µg DNA, primers, 1.25 units of Taq polymerase (GIBCO BRL Life Technologies, Burlington, Ontario), 1.5 mM $MgCl_2$, 10 mM dNTP mix and primers, covered with mineral oil. The samples were heated to 94˚C for 3 min, followed by 35 cycles of 94˚C (1 min), 55˚C for 1 min and 72˚C for 1 min, with a final 10 min extension at 72˚C. The amplified products were electrophoresed on 0.8% agarose gel and visualized by ethidium bromide staining photographed under ultraviolet light.

3. Results

Shoot meristems from the *in vitro* multiplied shoots (MS medium with 0.5 mg/l BAP) were used for the genetic transformation studies. Shoot meristems of 0.5 cm were cultured on medium containing 10 - 100 mg/l kanamycin to determine the concentration that is lethal for multiple shoot induction and root induction from the regenerated shoots.

The response of shoot multiplication decreased (66.2% - 6.2%) as the concentration of kanamycin increased (10.0 - 70.0 mg/l) with complete inhibition of shoot proliferation at 100 mg/l kanamycin (**Table 1**, **Figure 1(a)**). The rooting phase was very sensitive to kanamycin compared to shoot multiplication. The percentage of shoots that rooted decreased (53.8% - 4.8%) with increase in the concentration of kanamycin (10.0 - 70.0 mg/l) on IBA and 2,4-D supplemented medium.

Kanamycin had a marked effect on root development with induction of 1-2 roots of 1.0 - 1.5 cm after 30 days of culture in comparison to control shoots which rooted profusely on IBA or 2,4-D supplemented medium with induction of roots of 6.7 - 8.2 cm after 30 days of culture. Initially, a single colony of *Agrobacterium* strain harbouring p'GUSINT' was cultured in LB broth medium overnight on a orbital shaker at 200 rpm and a temperature of 28˚C until the turbidity reached OD of 0.5 at 600 nm. Shoot tips were infected with *Agrobacterium* strain for 5 min and then were placed on MS medium with 0.5 mg/l BAP. After 48 hr of co-cultivation, the shoot tip explants were incubated on MS medium with 0.5 mg/l BAP and 250 mg/l cefotaxime for 2 days for eliminating the *Agrobacterium*. Subsequently, the shoot tips were placed on selection medium containing MS medium with 0.5 mg/l BAP and 100 mg/l kanamycin and scored for the putative transformed shoots. An average of 62.2% of shoot tips developed shoot buds from the base and the shoots reached a length of 0.5 - 1.0 cm at the end of 30 days of culture on the selective medium (**Table 2** and **Figure 1(b)**). In contrast, the non-transformed shoot tips (controls) did not show any shoot bud induction and ultimately died after 30 days of culture on kanamycin supplemented medium. Shoots induced from co-cultivated shoot tips were subjected to repeated selection on MS medium supplemented with 0.5 mg/l BAP and 100 mg/l kanamycin. Shoots that attained a height of 2 - 3 cm at the end of 5th subculture on selection medium were separated and transferred to rooting medium containing 0.1 mg/l IBA and 0.1 mg/l 2,4-D individually with 50 mg/l kanamycin (**Figures 1(c)** and **(d)**). Selection for transformants using kanamycin in the rooting medium was very effective as a high frequency of shoots that rooted (69.5% - 50.5%) on IBA or 2,4-D containing medium showed GUS expression. Leaves excised from the putative transformed shoots/plants were placed on medium with 2.0 mg/l 2,4-D for callus induction for histochemical GUS assay. An average of 66.7% of the regenerated plants showed GUS expression in the frequency of 43.2% and 65% in the leaves and callus respectively. Leaves and callus induced from the controls did not show GUS activity (**Figures 1(e)** and **(f)**).

Genomic DNA was isolated from the leaves that were collected from the putative transformants and non-trans-

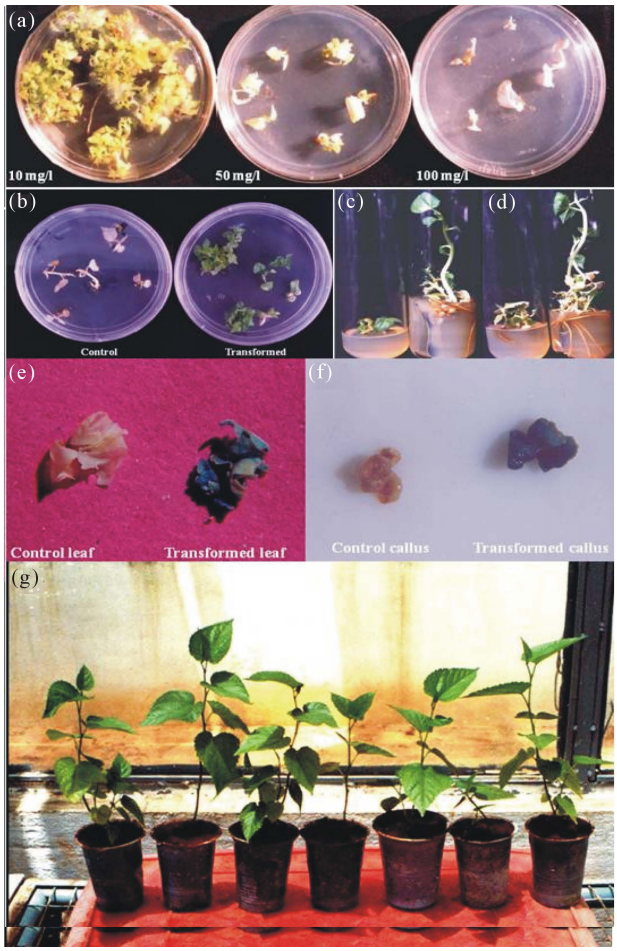

Figure 1. (a) Kanamycin sensitivity of shoot tip explants of S-36 cultivar to shoot proliferation on MS medium with 0.5 mg/1 BAP; (b) Response of shoot proliferation from non-transformed and transformed shoot tips on selection medium containing 0.5 mg/1 BAP and 100 mg/1 kanamycin; (c) Comparison of root induction from the non-transformed and transformed shoots of S-36 cultivar on MS medium with 0.1 mg/1 IBA and 50 mg/1 kanamycin after 30 days of culture; (d) Comparison of root induction from the non-transformed and transformed shoots of S-36 cultivar on MS medium with 0.1 mg/1 2,4-D and 50 mg/1 kanamycin after 30 days of culture; (e) X-gluc reaction of leaves excised from non-transformed and transformed plantlets of S-36 cultivar: only transformed leaves stained blue; (f) GUS gene expression in callus derived from non-transformed and transformed leaves of S-36 cultivar, only calli from transformed leaves stained blue; (g) Transgenic plants of S-36 cultivar established in greenhouse after 30 days after transfer.

formed plants established in the field (Figure 1(g)). The putative transformed plants were subjected to PCR analysis to confirm the presence of the *npt* II gene using specific primers for *npt* II gene. A DNA fragment of 700 bp corresponding to the expected size was amplified in 7 transgenic plants with slight difference in the amplification whereas no amplification was observed in non-transformed plants (Figure 2).

4. Discussion

Plant regeneration frequency plays a crucial role in the success of genetic transformation endeavours. Major limitations of most of the regeneration and transformation protocols have been the low regeneration frequency and their genotype dependence [11]. The present study aimed at the development of rapid, high-frequency, genotype-independent regeneration and transformation of mulberry using *in vitro* regenerated shoot meristems as explants for *Agrobacterium*-mediated transformation.

With recent developments in the field of molecular biology and gene manipulations, the meristem tip culture has been adopted as a tool for gene transfer in higher plants [12,13]. The use of the shoot apex as the explant for *Agrobacterium* mediated transformation has been reported in petunia [12], pea [14], sunflower [15], *Zea-mays* [13] and cotton [16]. In *Acacia mangium* a protocol was described for *Agrobacterium* mediated transformation using rejuvenated shoots as the explants. Thirty-four percent of the stem segments produced resistant multiple adventitious shoot buds, of which 30% expressed the β-glucuronidase gene [17].

Table 1. Effect of kanamycin on survival of various explants.

Explant	Type of hormone (mg/l)	Concentration of kanamycin (mg/l)	% explants survived	Type of response
Meristem	0.5 BAP	0.0	90.0 ± 1.5	Multiple shoot induction
		10.0	66.2 ± 1.5	
		30.0	42.7 ± 2.1	
		50.0	13.6 ± 2.0	
		70.0	6.2 ± 1.7	
		100.0	0.0	
Shoot	0.1 IBA	0.0	89.7 ± 1.5	Rooting from the shoots
		10.0	53.8 ± 1.8	
		30.0	22.7 ± 1.5	
		50.0	11.6 ± 1.5	
		70.0	6.1 ± 0.8	
		100.0	0.0	
	0.1 2,4-D	0.0	72.7 ± 1.2	Rooting from the shoots
		10.0	51.7 ± 1.5	
		30.0	20.2 ± 1.5	
		50.0	10.6 ± 1.2	
		70.0	4.9 ± 0.7	
		100.0	0.0	

The average values of 3 to 5 experiments are represented as ±SE. The changes were all statistically significant ($P < 0.05$).

Table 2. Selection of the putative transformants on MS medium supplemented with kanamycin.

Explant	Type of hormone (mg/l)	Kanamycin (mg/l)	% response	Type of response
Meristems	0.5 BAP	50.0	13.6 ± 2.0 (Control)	Multiple shoot induction
		100.0	0.0 (Control)	
		50.0	86.7 ± 2.6	
		100.0	62.2 ± 1.3	
Shoot	0.1 IBA	50.0	11.6 ± 1.5 (Control)	Root induction from the shoots
		100.0	0.0 (Control)	
		50.0	69.5 ± 2.3	
		100.0	5.6 ± 0.9	
Shoot	0.1 2,4-D	50.0	10.6 ± 1.3 (Control)	
		100.0	0.0 (Control)	
		50.0	50.5 ± 0.9	
		100.0	4.9 ± 0.7	

The average values of 3 to 5 experiments are represented as ±SE. The changes were all statistically significant ($P < 0.05$).

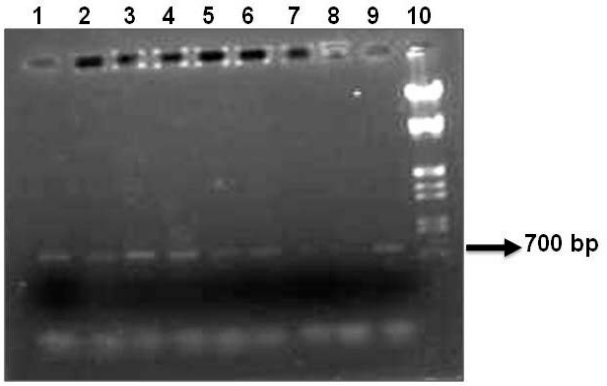

Figure 2. PCR analysis using npt II primers showing amplification of 700 bp fragment in transformants. Lane 1 - 7 transgenic plants, lane 8 negative control, lane 9 positive control, lane 10 molecular marker.

Successful transformation using *Agrobacterium* depends not only on the efficiency of plant regeneration system but also on the sensitivity of the cultured tissues to antibiotic. In the present study, the sensitivity of the tissues at different stages of plant regeneration, *i.e.* multiplication of shoots and root induction from the shoots was determined by supplementing kanamycin at 10 - 100 mg/l in the respective medium. The concentration of kanamycin in the selective medium had a significant effect on the survival of explants. Multiple shoots were induced with frequency of 13.6% - 66.2% from the shoot tips cultured on medium with 10 - 50 mg/l kanamycin. Shoot induction and root induction were completely suppressed from the shoots when kanamycin was used at 100 mg/l in the respective medium. The visible effect of kanamycin at 50 mg/l on root induction was delayed root induction along with reduction in the number and the growth of the roots. It is well known that the roots are very sensitive to antibiotics used in plant transformation experiments [18, 19]. Range of kanamycin was used to observe its effect on regeneration capacity of cotyledon explants in *Morus alba* L [6]. Kanamycin at 50 $mg \cdot L^{-1}$ was found to be optimum for selection of cotyledons inducing adventitious shoot buds directly. Concentration above 50 $mg \cdot L^{-1}$ turned the explants completely necrotic with negligible increase in the explants fresh weight. In mulberry, [20] examined the suppressive effects of antibiotics on adventitious bud formation and found that at 50 $mg \cdot L^{-1}$ kanamycin concentration the percentage of adventitious shoot bud induction was 25% which dropped to 8 at 100 $mg \cdot L^{-1}$ kanamycin.

In our study, about 62.2% co-cultivated shoot tips yielded resistant shoots on medium containing BAP and kanamycin (100 mg/l). Regenerated shoots from the putative transformed explants were propagated on the same medium for five passages to eliminate the possibility of chimeras subsequently; the putative transformed shoots were transferred to rooting medium containing kanamycin for root induction. Leaves and calli of kanamycin resistant plants were evaluated using the GUS histochemical assay. An average of 66.7% of the regenerated plants showed GUS expression in contrast to those selected at the shoot multiplication stage where only 43.2% of shoots showed GUS expression in the leaves and callus. These results suggested that there are escapes on kanamycin selection medium and that selection for transformants was more effective with the use of kanamycin at the rooting stage. Regeneration of escapes could be explained by the loss of foreign gene expression or by the ineffective kanamycin selection where non-transformed cells are protected from the selective agent by the surrounding transformed cells [21]. Although there is a possibility of escapes using shoot tips, repeated proliferation of shoots on selection medium followed by the use of kanamycin in the rooting medium will minimize the possibility of escapes.

In the present study, examination of the GUS positive transgenic plants from seven separate transformation events by PCR confirmed that all transgenic plants contained the expected DNA fragment with a slight difference in the amplification. No amplified fragment was observed in the non-transformed plants. Thus our study presents an efficient regeneration system, combined with *Agrobacterium transformation*, a method for routine genetic transformation of mulberry which can be exploited for transferring biotic as well as abiotic resistant genes for its improvement.

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