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Full Length Research Paper

Establishment of an efficient *Agrobacterium tumefaciens*-mediated leaf disc transformation of spine gourd (*Momordica dioica* Roxb. ex Willd)

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Spine gourd (*Momordica dioica* Roxb. ex Willd) is a medicinally and economically important plant and also used as vegetable. In this study, we established an *Agrobacterium tumefaciens*-mediated transformation procedure for *M. dioica*. Leaf explants were incubated with *A. tumefaciens* strain LBA4404 containing a binary vector pBAL2 carrying the reporter gene β-glucuronidase intron (GUS-INT) and the marker gene neomycin phosphotransferase (NPTII). Following co-cultivation, leaf explants were cultured on Murashige and Skoog (MS) + Gamborg et al., (B_5) medium supplemented with 6.6 µM 2,4-dichlorophenoxyacetic acid (2,4-D) combined with 3.3 µM 6-benzylaminopurine (BAP) containing 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. Kanamycin-resistant calluses were induced from the leaf explants after three weeks. Shoot regeneration was achieved after transferring the calluses onto fresh selection medium 8.8 µM BAP and 2.2 µM 2,4-D. Transgenic shoots were excised from callus and elongated in MS medium fortified with 3.0 µM gibberellic acid (GA_3), 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. Finally, the shoots were rooted on MS basal medium supplemented with 3.0 µM indole 3-butyric acid (IBA) and 100 mg L^{-1} kanamycin. High transformation frequency was achieved by using three-day-old precultured leaf explants. Furthermore, the presence of acetosyringone (200 µM), infection of explants for 30 min and three days of cultivation proved to be critical factors for greatly improving the transformation efficiency. Incorporation and expression of the transgenes confirmed by polymerase chain reaction (PCR), Southern blot analysis, reverse transcription (RT)-PCR and GUS histochemical assay. Using this protocol, transgenic *M. dioica* plants can be obtained in approximately three months with a high transformation frequency of 9%.

Key words: *Agrobacterium tumefaciens*, acetosyringone, growth regulators, GUS, genetic transformation, *Momordica dioica*.

INTRODUCTION

Spine gourd (*Momordica dioica* Roxb. Willd) is a dioecious and perennial cucurbitaceous climber (Trivedi and Roy, 1972) distributed in India, China, Nepal, Bangladesh, Myanmar, Pakistan and Srilanka. Immature green fruits are cooked as vegetable. Young leaves and flowers are also eaten. Fruits (100 g) contain high amounts of protein, calcium (33 mg), phosphorous (42 mg), iron (4.6 mg), highest amount of carotene (162 mg) and a high amount of vitamin C amongst the cucurbitaceous vegetables (Bhuiya et al., 1977; Ram et al., 2001; Bharathi et al., 2007). In addition, this species is valued for several medicinal and curative properties (Ram et al., 2001; Ali and Shrivastava, 1998). Fruits, leaves and tuberous roots are used as a folk remedy for diabetes. The plant was reported to posses antidiabetic, analgesic, postcoital anti-fertility (Shreedhar et al., 2001),

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Abbreviations: **AS**, Acetosyringone; **BAP**, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; **GA_3**, gibberellic acid; **GUS**, β-glucuronidase; **IBA**, indole 3-butyric acid; **LB**, Luria-Bertani; **npt II,** neomycin phosphotransferase II; **PCR**, polymerase chain reaction; **RT-PCR,** reverse transcription-PCR; **MSB_5**, Murashige and Skoog basal salt mixture + B_5 vitamins.

nematocidal, anti-allergic, anti-malarial, anti-feedant, anti-bacterial (Nabi et al., 2002), anti-oxidants and hepato-protective (Jain et al., 2008), as well as jaundice and bleeding pile properties (Deokule, 2006).

This popular vegetable has high demand in market, but still remain underutilized and underexploited (Bharathi et al., 2007; Ali et al., 1991) due to vegetative mode of propagation and dioecious nature. Commercial propagation of spine gourd largely depends on tuberous roots (Nabi et al., 2002), followed by stem cuttings, and seeds. Propagation by tuberous roots is limited due to the low multiplication rate (Mondal et al., 2006) and germination of seeds is very difficult or impossible because of its hard seed coat (Rashid, 1976). Development of an efficient gene transfer method is an absolute requirement for the genetic improvement of this plant with more desirable traits due to limitations in conventional breeding methods. We have previously reported an efficient regeneration protocol for *M. dioica*, direct organogenesis (Thiruvengadam and Jayabalan, 2001; Thiruvengadam et al., 2006a) and somatic embryogenesis (Thiruvengadam et al., 2007). Plant regeneration from leaf derived callus and somatic embryos of *M. charantia* (Thiruvengadam et al., 2006b; Thiruvengadam et al., 2010) has also been reported.

Agrobacterium tumefaciens-mediated transformation is an effective and widely used approach to introduce desirable genes into plants. Limited successes have been obtained in cucumber transformation protocols using agronomic traits, such as virus resistance (Chee and Slightom, 1991; Wu et al., 2009) and fungal resistance (Raharjo et al., 1996; Tabei et al., 1998) expressed in transgenic cucumber plants. To date, there is no report on genetic transformation of spine gourd. For genetic transformation of spine gourd, it is necessary to establish a plant regeneration system for this species. In this paper, we described for the first time a protocol for stable plant regeneration and *Agrobacterium*-mediated genetic transformation of this important crop using leaf explants. This optimized transformation system could be used to transform features with desirable characteristics such as disease resistance, stress resistance and high yield.

MATERIALS AND METHODS

Tubers of *M. dioica* Roxb. Willd (one year old) were collected from the Semmalai hills and the plants were raised in the Botanical Field Evaluation Garden at Tiruchirappalli in India. Leaf explants were collected and washed in running tap water for 5 min and surface-sterilized in 70% (v/v) ethanol for 1 min. Further, explants were treated in 1.0% (v/v) sodium hypochlorite solution for 10 min with occasional agitation. Finally, the leaf explants were rinsed with sterile distilled water for seven times and sliced into explants of approximately 0.3 to 0.5 cm^2.

Plant regeneration

After sterilization, the explants were cut into approximately 1-cm^2 sections which included the midrib portion and cultured *in vitro* with their adaxial sides on the semi-solid callus induction medium in culture tubes (25 × 150 mm, LCM, Lake Charles, LA.) plugged with non-absorbent cotton plugs. The callus induction medium comprised of MS (Murashige and Skoog, 1962) basal salt mixture + B_5 vitamins (Gamborg et al., 1968) (MSB_5) plus 30 g L^{-1} sucrose, 8.0 g L^{-1} agar (Sigma-Aldrich, Inc., St. Louis, Mo, USA) and different concentrations (0.0 to 8.8 µM) of 2,4-dichlorophenoxyacetic acid (2,4-D) with (0.0 to 5.5 µM) N6 - benzyl amino purine adjusted to pH 5.8 prior autoclaving. After three weeks of inoculation, well developed calli were produced from the cut ends of leaf. The leaf calli were transferred to a shoot induction medium (SIM) MSB_5 containing 30 g L^{-1} sucrose, 8.0 g L^{-1} agar and different concentrations of 6-benzylaminopurine (BAP) (0.0 to 1.2 µM) and 2,4-D (0.0 to 5.5 µM).

The regenerated shoots were harvested and transferred to shoot elongation medium (SEM) containing MSB_5 supplemented with (0.0 to 4.0 µM) gibberellic acid, 30 g L^{-1} sucrose and 8.0 g L^{-1} agar. All the cultures were maintained at 24 ± 2°C under 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 45 µmol m^{-2} s^{-1} provided by 40 W cool white fluorescent lamps (Sylvania, USA) and with 60 to 65% relative humidity. Two weeks later, shoots were placed on a root induction medium (RIM) MSB_5 containing 30 g L^{-1} sucrose, 8.0 g L^{-1} agar with different concentrations of (0.0 to 4.0 µM) indole 3-butyric acid. After 3 weeks, rooted plantlets were transferred to potting soil.

Plasmid and bacterial strains used for transformation

The *A. tumefaciens* strain LBA 4404 harboring the binary vector pBAL2 was used for spine gourd transformation. Binary vector pBAL2 harbors neomycin phosphotransferase II (*npt II*) driven by the nopaline synthase (NOS) promoter and terminator, which confers resistance to the antibiotic kanamycin as a plant selection marker, and the β-glucuronidase (GUS) gene interrupted with a plant intron (GUS-INT) (Vancanneyt et al., 1990) driven by the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator as reporter gene (Franklin and Lakshmi Sita, 2003). *Agrobacterium* was maintained on Luria-Bertani (LB) agar plate containing 50 mg L^{-1} kanamycin sulphate (Sigma-Aldrich, USA) and 25 mg L^{-1} rifampicin (Sigma-Aldrich, USA). A single colony was grown overnight in liquid LB broth with appropriate antibiotics at 28°C on a rotary shaker (180 rpm) until the optical density (OD_{600}) reached 0.6 to 0.9. Bacterial cells were harvested by centrifugation at 3,000 rpm for 10 min in a 50 ml sterile centrifuge tube (Corning, USA) and then resuspended in 30 ml of liquid inoculation medium [Half-MS medium augmented with 1.5% sucrose and 100 µM of acetosyringone (3',5'-dimethoxy-4'-hydroxy-acetophenone; Sigma-Aldrich, St Louis, MO, USA)] in the 50 ml tube.

Determination of antibiotic sensitivity

Since the pBAL2 contains the *npt* gene that confers kanamycin resistance, we selected kanamycin as selection agent in our transformation experiments. In order to determine the appropriate concentration of selection agent to effectively screen transformed shoots, we cultured leaf explants on MSB_5, 3% sucrose, 8.0 mg L^{-1} agar supplemented with 6.6 mg L^{-1} 2,4-D combined with 3.3 mg L^{-1} BAP supplemented with different concentrations of kanamycin (0, 50, 75, 100 and 150 mg L^{-1}). Explants were cultured on each selection medium for three weeks at 25°C under 16-h photoperiod. This experiment was performed with three replications. After three weeks, the number of explants producing callus was calculated. In addition, bacterial cells of *A. tumefaciens* were cultured on MS medium containing different concentrations of carbenicillin,

including 100, 200, 250, 300 and 400 mg L^{-1} to determine the appropriate concentration for inhibiting bacterial growth. All antibiotics were filter-sterilized prior to their addition to the autoclaved medium (121°C, 20 min) cooled to 45°C.

Transformation procedure and plantlet formation

Leaf explants were cultured on callus-inducing medium (CIM) medium for pre-cultivation (0 to four days). The pre-cultivated leaf explants were inoculated with the *Agrobacterium* suspension in the 50 ml tube for 30 min at 25°C in darkness with gentle shaking. The explants were then blotted dried on sterile filter paper 3 min to remove excess solution before transferring to a fortified co-cultivation medium (MSB_5, 3% sucrose supplemented with 6.6 µM 2,4-D, 3.3 µM BAP, 200 µM AS and 0.8% agar) and incubated for three days at 25°C in darkness. After co-cultivation, the explants were washed repeatedly in sterile distilled water and in 300 mg L^{-1} carbenicillin, and then transferred to selection medium MSB_5, (3% sucrose augmented with 6.6 µM 2,4-D, 3.3 µM BAP and 300 mg L^{-1} carbenicillin containing 100 mg L^{-1} kanamycin) for the induction of transgenic callus.

After three weeks of culture, transgenic calli developing from the explants were separated and subcultured for shoot induction medium (MSB_5, 3% sucrose augmented with 8.8 µM BAP, 2.2 µM 2,4-D and 300 mg L^{-1} carbenicillin containing 100 mg L^{-1} kanamycin), and transferred to shoot elongation medium (MSB_5 + 3.0 µM GA_3 and 300 mg L^{-1} carbenicillin) containing 100 mg L^{-1} kanamycin. The elongated shoots were then transferred to rooting medium containing MS media supplemented with (3% sucrose, 3.0 µM indole 3-butyric acid (IBA) and 100 mg L^{-1} kanamycin). The rooted plantlets were washed in sterile distilled water to remove traces of medium and then transferred to plastic pots (5 cm diameter) containing sterile soil, sand and vermiculite mixture (1:1:1). After two weeks, the plants were transferred to pots containing soil and grown in a green house. Later the plants were transferred to the field.

Histochemical GUS assay

Histochemical GUS assay (Jefferson et al., 1987) was used to assess GUS staining of putative transformed plants using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide (X-Gluc) as the substrate. Leaves of putative transformed plants grown on kanamycin were subjected to GUS assay. Tissue samples were incubated in sodium phosphate buffer containing X-Gluc for 18 h at 37°C after vacuum infiltration with the buffer solution for 10 min. Following incubation, tissues were bleached with 70% ethanol.

PCR assay

For polymerase chain reaction (PCR) analysis, the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) was used to isolate DNA from fresh leaves (100 mg) of putative transgenic and non-transgenic plants from the greenhouse. The presence of the NPT II gene was confirmed by PCR using NPT II gene–specific primer sequences. The NPT II primer sequences (5'- 3') were GAG GCT ATT CGG CTA TGA CTG and ATC GGG AGG GGG GAT ACC GTA. The total volume of reaction mixtures was 20 µL, including 20 ng genomic DNA, 0.5 µL of each primer (20 µM), 1.5 µL of dNTP mix (2.5 mM), 2.0 µL buffer (10 ×) with magnesium chloride ($MgCl_2$) (15 mM) and 0.5 µL Taq DNA polymerase. The cycling parameters began with an initial hot start at 95°C for 5 min, then 35 cycles of denaturation (95°C; 1 min), annealing (56°C; 1 min), and extension (72°C; 1 min), followed by a final extension of 20 min at 72°C. The expected PCR products were 700 bp for NPT gene. PCR amplification products were analysed by electrophoresis in 1% agarose gels.

Southern blot analysis

For Southern blot analysis, genomic DNA (15 µg) extracted from fresh leaves of PCR positive transgenic plants, as well as non-transgenic control plants, was digested with the restriction enzyme *Hind*III separated on 1% (w/v) agarose gels. Following gel electrophoresis, DNA was transferred to Hybond N+ (Amersham, Buckinghamshire, UK) nylon membrane as described by Sambrook et al. (1989). A PCR generated *NPTII* gene fragment (700 bp) was used as a probe. The probe was radiolabelled with αP^{32} dCTP according to the manufacturer's instructions (DECAprime™ II, Random Primed DNA labeling kit, Ambion) and used for hybridization. Pre-hybridization, hybridization and washing were performed according to standard methods (Sambrook et al., 1989). The membranes were washed at 60°C twice with 2x SSC and 0.5% SDS (20 min each) and twice with 1x SSC and 0.1% SDS for 20 min. The washed blots were exposed to X-ray film (Kodak X-Omat) with intensifying screens for signal detection at -80°C.

Reverse transcription (RT) -PCR assay

Total RNA (100 mg L^{-1}) was isolated from leaves of different putative transgenic plants and non-transformed plants using the Trizol method according to the manufacturer's instructions. For cDNA synthesis, total RNA (1 µg) was reverse-transcribed in a 20-µL reaction mixture using the BcaBEST™ RNA PCR system (TaKaRa Shuzo Co., Shiga, Japan). A 5.0-µL cDNA sample from the RT reaction was used for PCR. The NPT II primer sequences (5'- 3') were GAG GCT ATT CGG CTA TGA CTG and ATC GGG AGG GGG GAT ACC GTA. The *NPTII* fragment was amplified under the following conditions: one cycle of 95°C for 5 min; 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final elongation at 72°C for 20 min. The RT-PCR products were separated on a 1% agarose gel by electrophoresis and photographed using a Kodak EDAS 290 electrophoresis documentation system.

Experimental design and data analysis

The data were collected after three weeks of callus induction, five weeks of shoot regeneration and three weeks of rooting. All the experiments were conducted with a minimum of 20 replicates per treatment. The experiments were repeated three times. The data were analysed statistically using SPSS ver. 14 (SPSS Inc., Chicago, USA). The significance of differences among means was determined using Duncan's multiple range test at P = 0.5. The results are expressed as a means ± standard error (SE) of three experiments.

RESULTS AND DISCUSSION

Callus induction from leaf explants

Plant growth regulators are important factors that can selectively influence the genes to trigger differentiation of cells in culture (Thorpe, 1983). We examined the effects of different plant growth regulators on callus induction, shoot bud formation, shoot elongation and rooting for leaf explants of *M. dioica*. Callus induction on MS medium supplemented with 6.6 mg L^{-1} 2,4-D combined with 3.3

Table 1. Effect of growth regulators on callus induction, shoot formation, shoot elongation and root induction of *Momordica dioica*.

Growth regulator (µM)	% of callus per leaf explant	No. shoots from leaf calli	% of shoot elongation	% of root induction
2,4-D + BAP				
2.2 + 3.3	45.4^{c}	-	-	-
4.4 + 3.3	71.0^{b}	-	-	-
6.6 + 3.3	97.5^{a}	-	-	-
8.8 + 3.3	60.1^{bc}	-	-	-
2.2 + 4.4	-	7.0^{c}	-	-
2.2 + 6.6	-	13.0^{b}	-	-
2.2 + 8.8	-	39.0^{a}	-	-
2.2 + 1.2	-	12.1^{bc}	-	-
GA_3				
1.0	-	-	30.5^{c}	-
2.0	-	-	45.0^{bc}	-
3.0	-	-	84.2^{a}	-
4.0	-	-	48.0^{b}	-
IBA				
1.0	-	-	-	40.8^{c}
2.0	-	-	-	58.5^{b}
3.0	-	-	-	85.2^{a}
4.0	-	-	-	52.0^{bc}

Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters. BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA3, gibberellic acid; IBA, indole 3-butyric acid.

mg L^{-1} BAP resulted in a 97.5% induction frequency (Table 1). When calluses were transferred to fresh medium containing 8.8 mg L^{-1} BAP and 2.2 mg L^{-1} 2,4-D, shoot regeneration resulted. This indicated that BAP was sufficient for the callus induction and shoot bud regeneration. The calluses induced in the compact were green and showed higher shoot regeneration frequency (39 shoots). The shoots were excised from callus and elongated in MS medium fortified with 3.0 µM GA_3. The elongated shoots were rooted in MS medium supplemented with 3.0 µM IBA. Rooted plants were acclimatized in the greenhouse. The transformation can be effective only if we have a robust tissue culture protocol.

Influence of antibiotics on shoot regeneration from leaf explants

After three weeks of culture, 92% callus induction was attained in explants cultured on callus induction medium but lacking kanamycin. On medium containing kanamycin, the percentage of explants with at least one or more shoots (50.4%) was obtained at 25 mg L^{-1}. At 75 mg L^{-1}, 90% of explants bleached and died (Figure 1a). Further increase in the level of kanamycin to 100 and 150 mg L^{-1} totally inhibited shoot production (Table 2). To minimize escape and prevent necrosis, we chose 100 mg L^{-1} kanamycin for the transformation experiments. Similar results were observed in *Colocynthis citrullus* (Ntui et al., 2010) and *Cucumis sativus* (Selvaraj et al., 2010). Carbenicillin at 300 mg L^{-1} was used to kill *Agrobacterium* after co-cultivation with leaf explants. This result corroborates with the result of *Eustoma grandiflorum* (Thiruvengadam and Yang, 2009).

Optimization of transformation parameters

In order to optimize conditions for spine gourd transformation, the effects of several parameters known

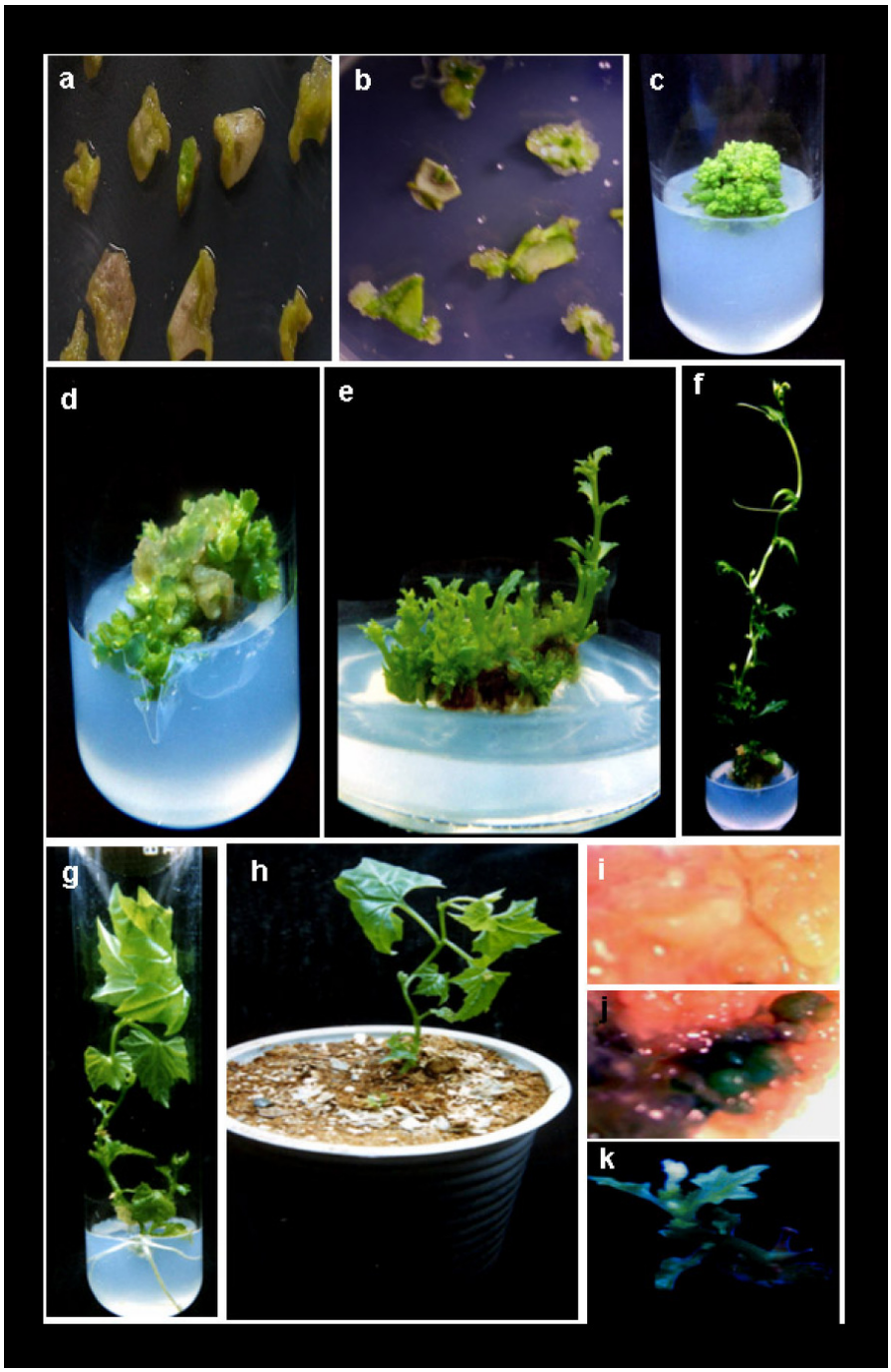

Figure 1. Regeneration of transgenic plants from leaf explants of *Momordica dioica*. (a) Non-transgenic leaf cultured on MSB_5 + 6.6 µM 2,4-D with 3.3 µM BAP and 100 mg L^{-1} kanamycin; (b and c) Callus initiation from leaf explants MSB_5 + 6.6 µM 2,4-D with 3.3 µM BAP, 300 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin; (d and e) Multiple shoot induction from transgenic leaf callus (MSB_5 + 8.8 mg L^{-1} BAP and 2.2 mg L^{-1} 2,4-D, 300 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin); (f) An elongated transformed shoots (MSB_5 + 3.0 µM GA_3, 300 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin); (g) transgenic rooted plant (MSB_5 + 3.0 µM IBA and 100 mg L^{-1} kanamycin); (h) hardening of transformed plant in pot; (i) GUS assay for the non-transformed callus two weeks after infection; (j) GUS expression as observed in leaf callus two weeks after infection. (k) GUS expression in two-week-old transformed shoots. MSB_5, Murashige and Skoog basal salt mixture + B_5 vitamins; 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; GA_3, gibberellic acid; GUS, β-glucuronidase.

Table 2. Effect of kanamycin concentrations on the callus formation and bud induction of *Momordica dioica*.

Kanamycin (mg/L)	Number of explant used	Callus formation rate (%)	Average number of bud/explant
0	50	92.0[a]	39.0[a]
25	50	50.4[b]	2.0 ± 0.2[b]
50	50	24.6[c]	1.0 ± 0.1[c]
75	50	14.2[d]	0.0
100	50	7.5[e]	0.0
150	50	0.0	0.0

Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters.

to influence *Agrobacterium*-mediated DNA transfer were compared. To determine the optimum conditions for *Agrobacterium* infection of spine gourd leaf explants, we examined the infection frequency, based on transient GUS expression. Transient GUS expression varied with duration of preculture of explants prior to co-cultivation. It was observed that preculturing the explants for three days proved to enhance the transient expression frequency (75.0%). This was the highest transient GUS expression seen compared with 0, one, two and four days preculture (Table 3). Sarmento et al. (1992) and Rajagopalan and Perl-Treves (2005) found efficient DNA delivery following two days pre-culture of cucumber hypocotyls. However, in the present study, three days pre-culture was observed as effective for highest transient GUS expression. To determine the most suitable duration of co-cultivation, explants were co-cultivated with *A. tumefaciens* for 0 to four days. The highest frequency of transient GUS expression (75.5%) was obtained for explants co-cultivated for three days (Table 3). Further, the majority of the explants perished due to bacterial overgrowth when the co-cultivation period exceeded three days.

In the present study, the influence of acetosyringone (0, 100, 200 and 300 µM) on T-DNA delivery was also evaluated. The number of explants expressing GUS transient activity (blue spots) was higher in the presence of acetosyringone than in its absence (Table 3). This study indicates that a concentration of 200 µM acetosyringone is the optimum concentration in terms of highest GUS expression (72.5%). However, increasing the concentration of acetosyringone to 300 µM did not enhance the percentage of transient GUS expression. Improved transformation rate was observed when acetosyringone was added to the *Agrobacterium* culture/ co-cultivation medium (Nishibayashi et al., 1996; Mohiuddin et al., 1997; Boase et al., 1998; Wu et al., 2003). Sarmento et al. (1992) reported that exposing the explants to a bacterial suspension containing 20 µM acetosyringone for 5 min had no significant effect in enhancing the frequency of kanamycin resistant calli. In our protocol, transformation improved when 100 µM acetosyringone for 30 min had significant effect in enhancing the frequency of kanamycin resistant calli.

Genetic transformation of leaf explants

Callus initiation was observed in selective medium within three weeks of culture (Figures 1b and c), while untransformed control explants on selective medium turned yellow and did not produce calli (Figure 1a). A total of three to five weeks was needed from callus initiation to the formation of excisable shoots (2 to 3 mm), which were then transferred to shoot bud initiation (Figure 1d) with MS medium containing 8.8 µM BAP and 2.2 µM 2,4-D within two weeks (Figure 1e), shoot elongation medium (MSB_5 medium containing 3.0 µM GA_3 with 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin) and cultured for three additional weeks (Figure 1f). Shoots that survived this selection stage were transgenic and were transferred to rooting medium (MSB_5 medium supplemented with 3.0 µM IBA and 100 mg L^{-1} kanamycin) and cultured for three weeks (Figure 1g). The rooted plantlets were transferred to pots, acclimated for two weeks in the culture room and were moved to the greenhouse (Figure 1h).

Analysis of putative transformants

Histochemical GUS assay

Histochemical analysis of GUS activity was carried out in putative kanamycin-resistant transgenic lines to confirm the transformation events. The control of leaf callus did not show any blue coloration (Figure 1i), while calli pieces infected with *Agrobacterium* displayed varying GUS

Table 3. Influence of various transformation factors on frequency of transient GUS expression (%) in leaf explants of *M. dioica*.

Factor studied	Variable	Transient GUS expression frequency (%)
Preculture (days)	0	31.2[e]
	1	45.0[d]
	2	63.5[b]
	3	75.0[a]
	4	60.1[c]
Infection duration (min)	15	20.5b[c]
	30	65.4[a]
	45	30.2[b]
Co cultivation period (days)	0	38.5[d]
	1	55.0[c]
	2	63.5[b]
	3	75.5[a]
	4	60.8[bc]
Acetosyringone (µM)	0	41.0[d]
	100	60.0[b]
	200	72.5[a]
	300	55.3[bc]

For each variable studied, experiments were repeated three times with 20 explants. Values represent mean percentage of observations from three replications for each treatment. Transient GUS expression frequency (%) = (number of explants producing GUS-positive spots on the leaf explants/total number of explants inoculated) x 100. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters. GUS, β-glucuronidase.

response (Figure 1j). The strong GUS expression was observed in two-week-old young leaves (Figure 1k). Since the GUS-INT gene was used, we can safely conclude that the blue colour is due to the actual integration of this gene after splicing and not due to *Agrobacterium* contamination.

Molecular analyses of transformants

To confirm that these plants were transformants, total DNA and RNA were extracted and analysed from the leaves of both non-transformed and transgenic plants. Putative transgenic plants were initially screened by PCR using *nptII* gene-specific primers to detect the presence of transgenes in the transgenic and non-transgenic plants and were later subjected to Southern blot and RT-PCR analysis. Figures 2a to c depicts the results from a few representative plants. PCR analysis conducted with genomic DNA showed the amplification of the predicted 700 bp fragments corresponding to the *nptII* genes, respectively, in kanamycin-resistant transformed plants (Figure 2a; lanes 1 to 4). No specific amplification products were detected in the non-transgenic control plant (Figure 2a; lane 5). Foreign gene integration into the transgenic plant was verified by Southern blot analysis.

Southern blot analysis was performed on DNA from four independent PCR positive transgenic *M. dioica* plants (Figure 2b, lanes 1 to 4). Results indicate the integration of TDNA into the genome of all four transgenic lines when probed with the *nptII* gene. Since the number of hybridizing bands is an indication of the number of copies integrated, all the transgenic plants had a one and two copies of the transgene integrated into their genome, while no signal was detected in the untransformed control (Figure 2b, lane 5). The presence of DNA for the *nptII* gene in transgenic leaves indicated that the plants were actually transgenic. Furthermore, RT-PCR was performed and the results confirms that *nptII* was expressed in these transgenic plants (Figure 2c).

According to the results of GUS assay, PCR, Southern blot analysis and RT-PCR, we believed that the *gus* and *nptII* gene was introduced into these transformed plants. In conclusion, a simple and efficient gene transfer protocol was reported herein for *M. dioica*. This is the first

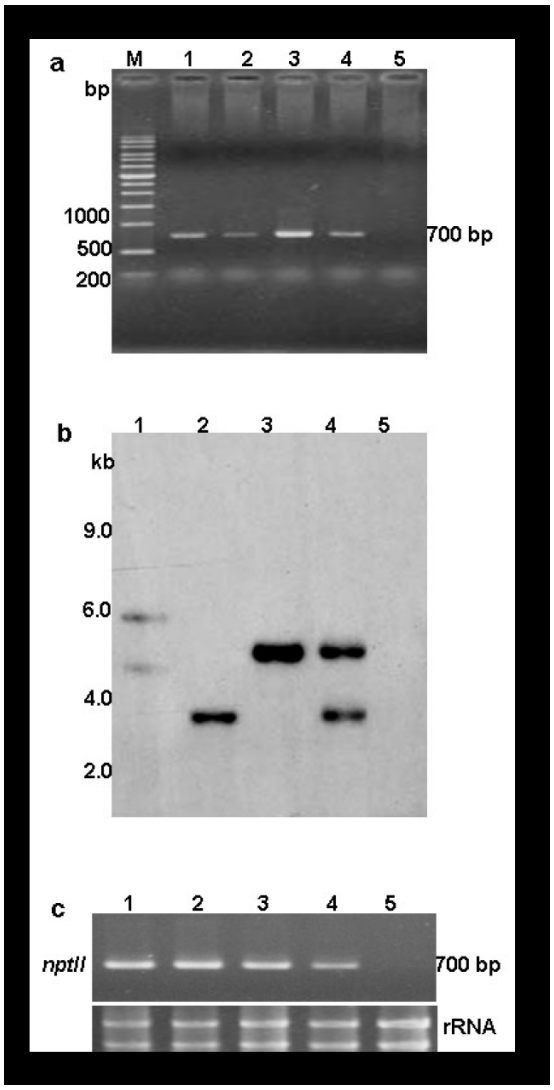

Figure 2. Molecular analysis of transformants. (a) PCR analysis of DNA isolated from leaves of transgenic lines and non-transformed plants of *M. dioica*. Agarose gel electrophorogram of PCR amplification performed with primers for the *NPTII* gene. A 700 bp DNA fragment was amplified; Lane M, marker; lanes 1 to 4, transgenic lines of *M. dioica*; lane 5, non-transformed plants of *M. dioica*. (b) Southern blot analysis of transgenic plants. Lanes 1 – 4, transgenic lines of *M. dioica*; lane 5, non-transformed plants of *M. dioica*; Genomic DNAs were digested with *Hind*III and hybridized to a 700 bp *NPTII* -probe. (c) RT-PCR assay of *NPTII* gene expression using primers of NPTII. A 700 bp cDNA fragment was amplified; Total RNA was isolated from four *NPTII* transgenic spine gourd plants in (a) (lanes 1 to 4) and one non-transgenic plant (lane 5). PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

report on the genetic transformation of *M. dioica*, and this protocol may be adopted for transferring any character genes of agronomic interest.

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