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An efficient *Agrobacterium tumefaciens* -mediated genetic transformation of bitter melon (*Momordica charantia* L.)

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Abstract

A simple and efficient protocol for *Agrobacterium tumefaciens*-mediated genetic transformation of bitter melon (*Momordica charantia* L.) has been developed. Pre-cultured leaf explants were transformed by co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 harbouring a binary vector pBAL2 carrying the reporter gene β-glucuronidase intron (*gus*) and the marker gene neomycin phosphotransferase (*nptII*). After co-cultivation, explants were transferred in to a callus induction medium containing 7.7 µM naphthaleneacetic acid (NAA) with 2.2 µM thidiazuron (TDZ), 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. Regeneration of adventitious shoots from callus was achieved on MS medium containing 5.5 µM TDZ, 2.2 µM NAA, 3.3 µM silver nitrate ($AgNO_3$), 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. Transgenic shoots were excised from callus and elongated in MS medium fortified with 3.5 µM, gibberellic acid (GA_3), 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. The transgenic elongated shoots were rooted in MS medium supplemented with 4.0 µM indole 3-butyric acid (IBA) and 100 mg L^{-1} kanamycin. The putative transgenic plants were acclimatized in the greenhouse and seeds were subsequently collected from mature fruits. Further, the presence of acetosyringone (300 µM) in the co-cultivation medium, infection of explants for 30 min and 3 days of co-cultivation proved to be critical factors for greatly improving the transformation efficiency. Histochemical GUS assay and polymerase chain reaction of field-established transgenic plants and their offspring confirmed the presence of the *gus* and *nptII* genes, respectively. Integration of T-DNA into the genome of putative transgenics was further confirmed by Southern blot analysis. The *nptII* gene expression in transgenic plants was confirmed by RT-PCR. A transformation efficiency of 7% was obtained.

Keywords: *Agrobacterium tumefaciens*; Acetosyringone; Growth regulators; GUS; Genetic transformation; *Momordica charantia* L.
Abbreviations: $AgNO_3$ - Silver nitrate; AS - Acetosyringone; GA_3 - Gibberellic acid; GUS - β-glucuronidase; IBA - Indole 3-butyric acid; NAA - Naphthaleneacetic acid; npt II - neomycin phosphotransferase II; PCR - Polymerase chain reaction; RT-PCR - Reverse transcription-PCR; TDZ - Thiadiazuron.

Introduction

Bitter melon (*Momordica charantia* L.) is an important horticultural as well as medicinal crop in tropical and subtropical areas of Asia, East-Africa and South America (Grover and Yadav, 2004). The fruits of bitter melon contain rich amount of vitamins C, E, carotenoids, lycopenes and flavonoids that prevent free radical damages (Semiz and Sen, 2007). Bitter melon has some interesting biological and pharmacological activities such as anticancer, antiviral, antibacterial, analgesic, anti-obesity, anti-inflammatory, hypotensive, anti-fertility, hepatotoxicity and antioxidant (Beloin et al., 2005; Grover and Yadav, 2004; Ng et al., 1992; Zafar et al., 1991). In addition, cucurbitane-type triterpenoids have been reported to exhibit various pharmacological and biological activities including anticancer, anti-HIV, antidiabetic, antifeedant and antioviposition activities (Raman and Lau, 1996; Grover and Yadav, 2004; Beloin et al., 2005). More recent techniques of genetic engineering have many potential applications in fields such as medicine, agriculture and industries. In agriculture, the new application of genetic engineering includes the development of transgenic plants. The transgenic plants carry desirable traits like disease resistance, insect resistance and herbicide resistance. To date, the genetic improvement of bitter melon has been mainly achieved by conventional plant breeding methods. Increasing demand for these crops will necessitate improving their agronomic characteristics, such as disease resistance, environmental tolerance and fruit quality. Genetic engineering can be used to produce desirable agronomic characteristics quickly and efficiently. Most plant transformation procedures require a plant regeneration system for efficient gene transfer, selection, and regeneration of transgenic plants (Thiruvengadam and Yang, 2009). We have previously reported an efficient regeneration protocol for *Momordica*, direct organogenesis (Thiruvengadam and Jayabalan, 2001; Thiruvengadam et al., 2006a) and somatic embryogenesis (Thiruvengadam et al., 2006b, 2007). Among the available gene transfer systems, *Agrobacterium*-mediated gene transfer is considered as more efficient for the stable integration of genes into plant genome. So far, a few reports are available on gene transfer studies in cucurbits such as *Cucumis melo* (Curuk et al., 2005; Rhimi et al., 2007), *C. sativus* (Nishibayashi et al., 1996; Soniya and Das, 2002) and *Colocynthis citrullus* (Ntui et al., 2010). *Agrobacterium*-mediated β-glucuronidase expression was detected in explants of immature cotyledonary nodes in *M. charantia* (Sikdar et al., 2005). Recently tumor research centre has reported *Agrobacterium* mediated genetic transformation from cotyledonary nodes of bitter melon. However, to our knowledge, no report exists to date on the production of a transgenic bitter melon via indirect organogenesis. For genetic transformation of bitter melon, it is necessary to established stable plant regeneration protocol from leaf explants of *M.*

charantia via organogenesis (Thiruvengadam et al., 2010). In this paper, we describe an efficient protocol for *Agrobacterium* mediated genetic transformation of bitter melon using leaf disc as explants. This optimized transformation system could be used for the genetic improvement of bitter melon.

Results and discussion

In vitro regeneration from leaf explants

To set up an effective regeneration system suitable for *Agrobacterium* mediated transformation. Calli were induced from leaf explants grown on MSB_5 containing 30 g L^{-1} sucrose, 2.2 g L^{-1} gelrite, and 7.7 µM NAA with 2.2 µM TDZ. Regeneration of adventitious shoots from callus was achieved on MS medium containing 5.5 µM TDZ, 2.2 µM NAA, and 3.3 µM $AgNO_3$. The shoots were excised from callus and elongated in MS medium fortified with 3.5 µM GA_3. The elongated shoots were rooted in MS medium supplemented with 4.0 µM IBA. Rooted plants were acclimatized in the greenhouse.

Influence of antibiotics on shoot regeneration from leaf explants

In order to determine the appropriate concentration of selection agent to effectively screen transformed shoots, we cultured leaf explants on CIM supplemented with different concentrations of kanamycin. After 3 weeks of culture, 95% callus induction was attained in explants cultured on CIM lacking kanamycin. On medium containing kanamycin, the highest percentage (38.6%) of callus induction was at 25 mg L^{-1}. At 75 mg L^{-1}, 90% of explants bleached and died. Further increase in the level of kanamycin to 100 and 150 mg L^{-1} totally inhibited callus induction (Table 1). To minimize escape and prevent necrosis, we have selected 100 mg L^{-1} kanamycin for the transformation experiments. Similar results were observed in *C. citrullus* (Ntui et al., 2010) and *C. sativus* (Selvaraj et al., 2010). 300 mg L^{-1} carbenicillin was used to kill *Agrobacterium* after co-cultivation with leaf explants. This result was corroborated with *Eustoma grandiflorum* (Thiruvengadam and Yang, 2009).

Optimization of transformation parameters

In order to optimize conditions for bitter melon transformation, the effects of several parameters known to influence *Agrobacterium*-mediated DNA transfer were compared. We examined the infection frequency, based on transient GUS expression. Preculturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in chinese cabbage (Lee et al., 2000) plum (Mante et al., 1991) and apricot (Laimer et al., 1992) but decreased in citrus (Costa et al., 2002). The effect of preculture on the frequency of transformation in *M. charantia* was examined using leaf explants cultured on MS medium supplemented with 7.7 µM NAA and 2.2 µM TDZ. About 30% transformation frequency from non-precultured leaf explants tested showed GUS-positive features following coculture for 4 days with *Agrobacterium*. However, following preculture for 1-day the proportion of GUS-positive explants had 48%, 2-day 52% 3-day 76% and 4-day pre-culture resulted in 47% GUS-positive explants (Table 2). We therefore concluded that the 3-day pre-cultivation of explants had favorable condition for transformation in *M. charantia*. Bacterial cell density as measured by the optical density (OD) of bacterial suspension is directly related to their cell mass or cell number (Dutt and Grosser, 2009). From the different concentrations, 0.8 OD culture showed a superior response (73.0%) and unfortunately above 1.0 OD culture showed a decline in the percentage of response and the explants turned to necrotic and devoid of callus induction due to excessive growth of the bacteria (Fig. 2a). No significant GUS expression was noticed when infection was allowed for 5 min (Fig. 2b). Overgrowth of *Agrobacterium* was the real problem, when infection time increased to 45 min (data not shown). GUS expression was significantly high (70%) at 0.8 OD_{600} when infected for 25 min (Fig. 2a and b). Therefore, optimized OD (0.8) was used with 25 min of infection time in further experiments. The duration of the co-cultivation period with bacteria affected the infection frequency. The optimum length of the co-cultivation period was 1-5 days, resulting in an infection frequency of 20-75%. The blue area indicating the transient expression of the *gus* gene was initially observed after 2 days of co-cultivation; after 3 days of co-cultivation the number of GUS-positive explants dramatically increased (Fig. 2c). We have selected 4 days as the optimum co-cultivation period for transformation. Similarly, in melon (Dong et al., 1991), rice (Li et al., 1992) and citrange (Cervera et al., 1998), extended co-cultivation increased the transformation efficiency and longer co-cultivation periods frequently resulted in *Agrobacterium* overgrowth and subsequent death of explants. Bacterial density has been previously shown to influence transformation efficiency of target plant cells (Suzuki and Nakano, 2002). Kondo et al., (2000) demonstrated the optimal duration was no longer than 4 days in many species. However, prolonged co-cultivation caused an overgrowth of *Agrobacterium* and decreased the transformation efficiency in *Datura* (Curtis et al., 1999). Co-cultivation is one of the most important steps for genetic transformation of plants. In this step, T-DNA is incorporated into plant genomic DNA (Han et al., 2005). The use of acetosyringone during co-cultivation has been shown to increase *Agrobacterium*-mediated transformation frequencies (Sheikholeslam and Weeks, 1987). Table 3 shows that the addition of acetosyringone to co-cultivation medium increased the transformation frequency five fold in *M. charantia* explants (72.5%). Acetosyringone is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulence genes of *A. tumefaciens*. Its beneficial role has been demonstrated in the genetic transformation of some plants, such as apple (James et al., 1993) and kiwifruit (Janssen and Gardner, 1993). We have shown here that *M. charantia* explants responded positively to the presence of acetosyringone in the co-cultivation medium.

Genetic transformation of leaf explants

Agrobacterium-mediated genetic transformation of bitter melon leaf disc was performed. After 4 days of co-cultivation, the explants were transferred onto the callus induction medium (CIM) composed of MSB_5 containing 7.7 µM NAA, 2.2 µM TDZ, 300 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin. Callus initiation was observed in selective medium within 3 weeks of culture (Fig. 3b), while untransformed control explants on selective medium turned yellow and did not produce calli (Fig. 3a). A total of 3-5 weeks was needed from callus initiation to the formation of excisable shoots (2-3 mm), which were then transferred to shoot initiation (Fig. 3c) with MS medium containing 5.5 µM TDZ, 2.2 µM NAA and 3.3 µM $AgNO_3$ within two weeks (Fig. 3d). The shoots were cultured on elongation medium (MSB_5 medium containing 3.5 µM GA_3 with 300 mg L^{-1}

Table 1. Effect of kanamycin concentrations on the callus formation and bud induction of bitter melon.

Kanamycin (mg L^{-1})	Number of explants used	Percentage of callus formation	Average number of buds/explant
0	50	95.0±1.0a	37.0±0.5a
25	50	38.6±2.0b	3.0±0.2b
50	50	22.4±1.0c	1.0±0.1c
75	50	10.2±0.5d	0.0
100	50	0.0	0.0
150	50	0.0	0.0

The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different by different letters.

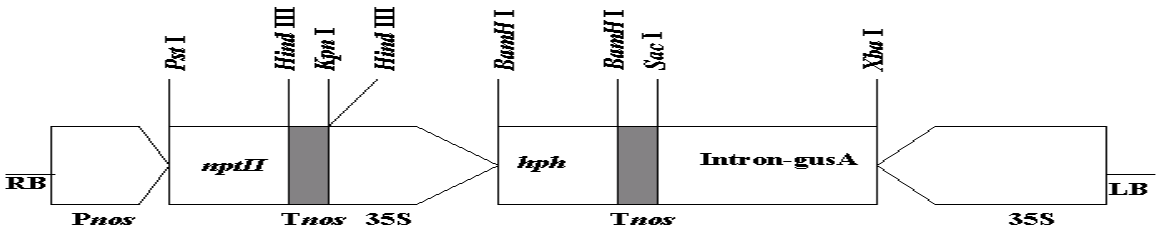

Fig 1. TDNA portion of pBAL2 map showing sites. LB, RB left and right borders, respectively.

carbenicillin and 100 mg L^{-1} kanamycin) for three additional weeks. Shoots that survived this selection stage were putatively transgenic and were transferred to rooting medium (MSB_5 medium supplemented with 4.0 µM IBA and 100 mg L^{-1} kanamycin) and cultured for 3 weeks (Fig. 3e). The rooted plantlets were transferred to pots, acclimated for 2 weeks in the culture room and were moved to the greenhouse (Fig. 3f).

Histochemical GUS assay

Expression of the *gus* gene was assessed after the addition of X-Gluc to leaves of the untransformed control and transgenic plants. GUS activity was not detected in any of the untransformed tissues (Fig. 3h). In contrast, 76% of the plants regenerated on medium containing kanamycin showed GUS activity. High level of GUS staining was observed entirely on the leaves of transgenic plants (Fig. 3g).

Molecular analyses of transformants

To confirm that these plants were transformants, total DNA and RNA were isolated and analysed from the leaves of both non-transformed and six transgenic plants. Specific fragments of the *nptII* gene (700 bp) were detected by PCR in the leaves of transgenic plants (T_0- lanes 1-4 and T_1 lanes 5-6) (Fig. 4a). Leaves from non-transformed plants did not have this 700-bp fragment (Fig. 4a, lane 7). Foreign gene integration into the transgenic plant was confirmed by Southern blot analysis. Southern blot analysis was performed on genomic DNA from six independent PCR positive transgenic plants (T_1 lanes) (Fig. 4b). Results show that all six transgenic lines (T_1) have a single copy of the transgene and transgenic lines (T_0) transgenic plants had a one and two copies of the transgene integrated into their genome, while no signal was detected in the untransformed control (Fig. 4b, lane 7). 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 confirmed that *nptII* was expressed in these transgenic plants (T_0 and T_1) (Fig. 4c). According to the results of GUS assay, PCR, Southern blot analysis and RT-PCR, we conclude that the *gus* and *nptII* gene was introduced into these transformed plants. In summary, a highly efficient *Agrobacterium*-mediated leaf disc transformation method was established for bitter melon using leaf explants. This is the first report on the genetic transformation of *M. charantia* via indirect organogeneis, and this protocol may be adopted for transferring any character genes of agronomic interest.

Materials and methods

Collection of seeds and germination

Seeds of bitter melon (*M. charantia* cv. Coimbatore-1) were obtained from Arignar Anna farm, Kudimianmalai, Pudukkottai, Tamil Nadu, India. The seeds were surface-sterilized first with 70% (v/v) ethanol for 1 min and then with 25% (v/v) commercial bleach (with sodium hypochlorite as the active agent) containing 0.05% (w/v) of Tween-20 (polyethylene sorbitan monooleate; Nutritional Biochemical, Cleveland, OH) for 20 min and then rinsed thoroughly (three times) with sterile distilled water. Disinfected seeds were germinated in the dark for 48h in a jar containing half-strength MS medium (Murashige and Skoog, 1962) plus B_5 (Gamborg et al., 1968) vitamins (MSB_5) supplemented with 8.0 g L^{-1} agar and 30 g L^{-1} sucrose without any growth regulators. The seedlings were grown under white fluorescent light (30 µmol $m^{-2}s^{-1}$) at a photoperiod of 16/8 h of light/dark and temperature 25 ± 2˚C. Two week old leaf explants were used for transformation.

Plasmid and bacterial strains used for transformation

The *Agrobacterium tumefaciens* strain LBA 4404 harbouring the binary vector pBAL2 was used for bitter melon transformation. Binary vector pBAL2 harbours neomycin phosphotransferase II (*nptII*) driven by the nopaline synthase 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

Table 2. Effect of preculture days on transformation frequency.

Pre-culture days	No. of calli cultured	No. of calli with GUS positive	Transformation frequency (%)
0	32	9.0±0.5e	30.5±1.0d
1	30	14.0±0.7cd	48.0±0.9bc
2	36	19.0±1.0b	52.5±1.0b
3	36	24.0±1.0a	76.0±2.0a
4	32	15.0±0.5c	47.0±1.2c

The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different by different letters.

(Vancanneyt et al., 1990) driven by the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator as reporter gene (Fig.1). *Agrobacterium* was maintained on 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 - 0.9. Bacterial cells were harvested by centrifugation at 3000 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 50 ml tube.

Determination of antibiotic sensitivity

A dose-response assay was conducted to determine the optimal concentrations of kanamycin and carbenicillin in the selection medium. Different concentrations of kanamycin (0, 50, 75, 100 and 150 mg L^{-1}) were added to the CIM medium (MSB_5, 3% sucrose supplemented with 7.7 µM NAA, 2.2 µM TDZ and 8.0 g L^{-1} agar). As a control, explants were cultured on CIM medium without any antibiotics. Explants were cultured on each selection medium for 3 weeks at 25 ± 2˚C under 16-h photoperiod. This experiment was performed with three replications. After 3 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.

Transformation procedure and plantlet regeneration

Leaf explants were cultured on CIM medium for pre-cultivation (0-4 days). The pre-cultivated leaf explants were inoculated with the *Agrobacterium* suspension in 50 ml tube for 25 min at 25±2˚C in darkness with gentle shaking. The explants were then blotted, dried on sterile filter paper for 3 min to remove excess bacterial culture before transferring to co-cultivation medium (MSB_5, 3% sucrose supplemented with 7.7 µM NAA, 2.2 µM TDZ, 300 µM AS and 0.8% agar) and incubated for 4 days at 25±2˚C in darkness. After co-cultivation, the explants were washed repeatedly in sterile distilled water containing 500 mg L^{-1} carbenicillin, then transferred to callus induction medium (MSB_5, 3% sucrose supplemented with 7.7 µM NAA, 2.2 µM TDZ, 300 mg L^{-1} carbenicillin, 100 mg L^{-1} kanamycin and 0.8% agar) for three weeks. Later, the callus were transferred to shoot induction medium (MSB_5, 3% sucrose augmented with 5.5 µM TDZ, 2.2 µM NAA, 3.3 µM $AgNO_3$, 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin). After 5 weeks of culture, multiple shoots developing from the explants were separated and transferred to shoot elongation medium (MSB_5 + 3.5 µM GA_3 and 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin). The elongated shoots were transferred to rooting medium containing MS medium supplemented with 3% sucrose, 4.0 µM IBA and 100 mg L^{-1} kanamycin. The rooted plantlets

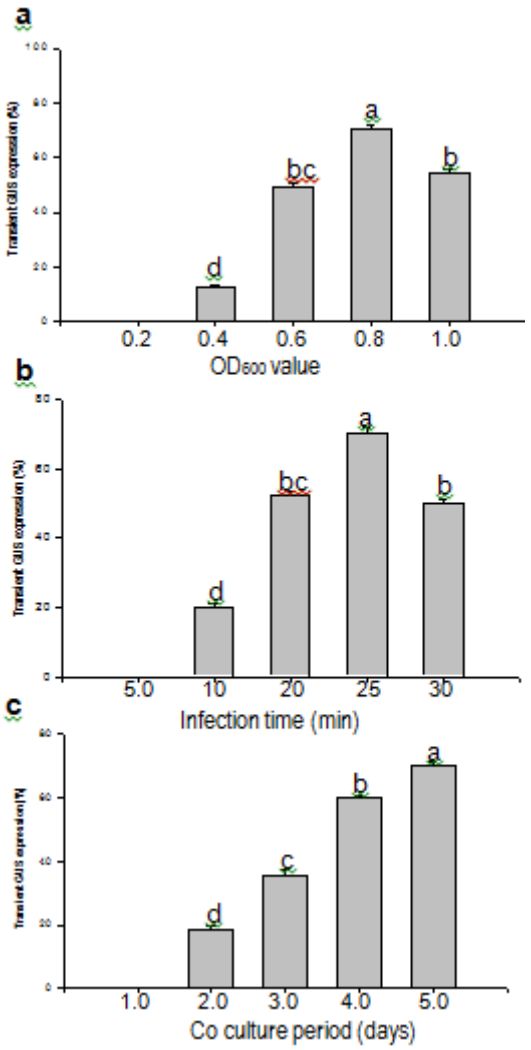

Fig 2. Factors influencing GUS expression and transgenic plant regeneration in *M. charantia* (**a**) The effect of bacterial concentration. (**b**) Infection time. (**c**) Coculture duration. The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different by different letters.

were washed in sterile distilled water to remove traces of medium and then transferred to plastic pots (5 cm diameter)

Table 3. Effect of acetosyringone concentration in co-culture media on transformation frequency.

Acetosyringone (μM)	No. of calli cultured	No. of calli with GUS positive	Transformation frequency (%)
0	20	3.0±0.4d	15.0±1.0e
100	18	7.0±1.0c	38.5±1.5cd
200	20	9.0±0.5bc	59.6±1.2b
300	21	15.0±1.0a	72.5±1.5a
400	22	10.0±1.0b	47.0±1.0c

The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different by different letters.

Fig 3. Regeneration of transgenic plants from leaf explants of *Momordica charantia*. (**a**) Non-transgenic leaf cultured on MSB_5+7.7 μM NAA with 2.2 μM TDZ, and 100 mg L^{-1} kanamycin. (**b**) Callus initiation from leaf explants MSB_5 +7.7 μM NAA with 2.2 μM TDZ, 300 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin. (**c** and **d**) Multiple shoot induction from transgenic leaf callus (MSB_5 +5.5 μM TDZ, 2.2 μM NAA, 3.3 μM $AgNO_3$, 300 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin). (**e**) Rooting of elongated transformed shoots (MSB_5 + 4.0 μM IBA and 100 mg L^{-1} kanamycin). (**f**) Hardening of transformed plant in pot. (**g**) GUS expression as observed in leaf callus two weeks after infection. (**h**) GUS assay for the non-transformed leaf callus two weeks after infection.

containing sterile soil, sand and vermiculite mixture (1:1:1). After 2 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

The presence of the *gus* gene in the putative transformants was detected histochemically following the procedure of Jefferson et al. (1987). The putative transgenic plant leaves were immersed in the staining solution (50 mM sodium phosphate buffer, pH 7.0, Triton X-100, 1 mM-5-bromo-4-chloro-3-indole-β-D-glucuronide) overnight (16 h) at 37˚C. These were rinsed successively with 70% ethanol and 30% acetic acid for 24h, and numbers of blue-stained explants were counted. Untransformed explants cultured under identical conditions served as control.

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 (T_0 and T_1) and non-transgenic plants from the greenhouse. The presence of the *nptII* gene was confirmed by the polymerase chain reaction (PCR) using *nptII* 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 mixture 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 (10X) with $MgCl_2$ (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 product was 700 bp for *nptII* gene. PCR amplification products were analysed by electrophoresis in 1% agarose gel.

Southern blot analysis

For Southern blot analysis, genomic DNA (15 μg) isolated from fresh leaves of PCR positive transgenic plants (T_0 and T_1), as well as non-transgenic control plants, was digested with the restriction enzyme *Hind*III, separated on 1% (w/v)

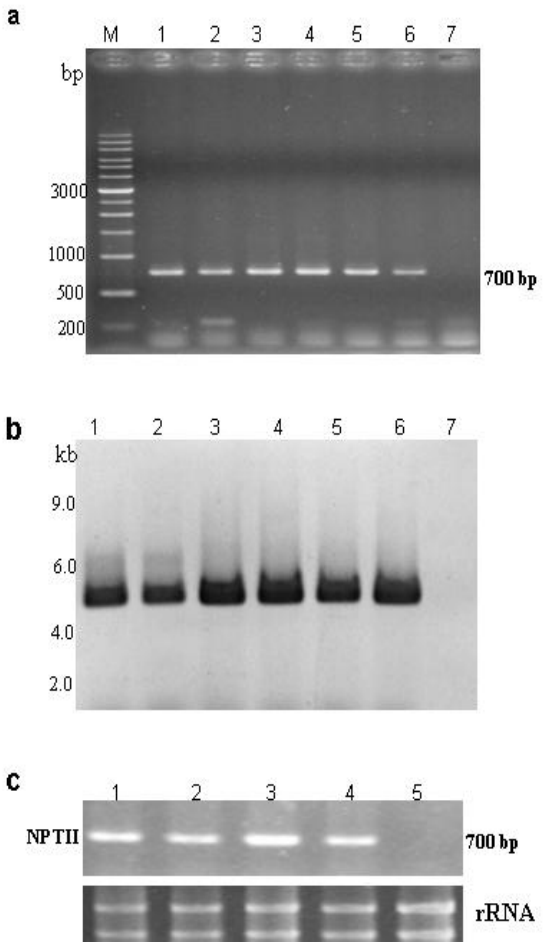

Fig 4. Molecular analysis of transformants (**a**) PCR analysis of DNA isolated from leaves of transgenic lines (T_0 and T_1) and Non-transformed plants of *M. charantia*. Agarose gel electrophorogram of PCR amplification performed with primers for the *nptII* gene. A 700 bp DNA fragment was amplified. Lanes M- marker; Lanes 1 - 4 Transgenic lines (T_0); Lanes 5 – 6 Transgenic lines (T_1) of *M. charantia*; Lane 7 - Non-transformed plants of *M. charantia*. (**b**) Southern blot analysis of transgenic plants. Lanes 1 - 6 Transgenic lines (T_1) of *M. charantia*; Lane 7 - Non-transformed plants of *M. charantia*. 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 plants (Lanes 1 - 2 Transgenic lines (T_0); Lanes 3 - 4 Transgenic lines (T_1) of *M. charantia*; Lane 5 - non-transgenic plant of *M. charantia*.

agarose gel. 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. Prehybridization, 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 0.1% 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.

RT-PCR assay

Total RNA (100 mg) was isolated from leaves of different putative transgenic plants (T_0 and T_1) and non-transformed plants using the Trizol method according to the manufacturer's instructions. For cDNA synthesis, total RNA (1µg) 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 extension 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.

Inheritance analysis

Seeds collected from five transformed plants (T_0) were sterilized and germinated on MS basal medium supplemented with 100 mg L^{-1} kanamycin. The plates were incubated under the conditions as described for *in vitro* culture. After 3-4 weeks, kanamycin sensitive seedlings germinated but bleached quickly, whereas resistant seedlings were green and formed true leaves and roots. The plants (T_1) were analyzed for the presence of the *nptII* gene by the PCR, Southern blot analysis and RT-PCR assays, respectively.

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