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Optimization of *Agrobacterium*-mediated genetic transformation in gherkin (*Cucumis anguria* L.)

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Abstract

The present work was aimed to study various factors influencing *Agrobacterium tumefaciens* mediated genetic transformation of gherkin (*Cucumis anguria* L). *Agrobacterium* strain LBA4404 harboring binary vector pBAL2 carrying the reporter gene β-glucuronidase intron (*gus*) and the marker gene neomycin phosphotransferase (*nptII*) was used for transformation. Factors affecting transformation efficiency, such as *Agrobacterium* concentration, effect of acetosyringone, pre-cultivation, infection and co-cultivation time of *Agrobacterium* were studied. After co-cultivation, explants were transferred into MS medium plus B5 vitamins (MSB_5) containing 1.5 µM benzylaminopurine (BAP) with 0.5 µM naphthalene acetic acid (NAA), 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin for callus induction. Regeneration of adventitious shoots from callus was achieved on MSB_5 medium containing 3.0 µM BAP, 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. Transgenic shoots were elongated in MSB_5 medium fortified with 2.0 µM gibberellic acid (GA_3), 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. The transgenic elongated shoots were rooted in MSB_5 medium supplemented with 3.0 µM indole 3-butyric acid (IBA) and 100 mg L^{-1} kanamycin. The putative transgenic plants were acclimatized in the greenhouse. A strong β-glucuronidase activity was detected in the transformed plants by histochemical assay. Integration of T-DNA into the nuclear genome of transgenic plants was confirmed by polymerase chain reaction and southern hybridization. The *nptII* gene expression in transgenic plants was confirmed by RT-PCR. A transformation efficiency of 15% was obtained. This protocol allows effective transformation and direct regeneration of *C. anguria.*

Keywords: Acetosyringone; *Agrobacterium tumefaciens*; *Cucumis anguria* L; Genetic transformation; Growth regulators; GUS.
Abbreviations: AS - Acetosyringone; BAP - 6-benzylaminopurine; GA_3 - Gibberellic acid; GUS - β-glucuronidase; IBA - Indole 3-butyric acid; NAA - Naphthalene acetic acid; npt II - neomycin phosphotransferase II; PCR - Polymerase chain reaction; RT-PCR - Reverse transcription-PCR.

Introduction

The gherkin (*Cucumis anguria* L.) is an important horticultural crop, mainly cultivated and consumed in Africa, Brazil, Cuba, India, United States and Zimbabwe. The vegetable is similar in form and nutritional value as that of cucumber (Mangan et al., 2010). The crop is popularly known as 'pickling cucumber' or 'small cucumber' among the farmers (Nabard, 2005). The fruits of gherkin are consumed as boiled, fried, stewed, pickled, fresh in salads and also in hamburgers (Dzomba and Mupa, 2012; Matsumoto and Miyagi, 2012). The fruit of the vegetable contain high amounts of protein, calcium, phosphorous, iron and vitamin C (Whitaker and Davis, 1962). The gherkin is also known for traditional importance in medicinal to treat stomach ache, jaundice, hemorrhoids and preventing stone formation in kidney (Baird and Thieret, 1988; Schultes, 1990). Phytochemists have isolated a number of potential medical components from *C. anguria*, such as cucurbitacin B, cucurbitacin D and cucurbitacins G (Sibanda and Chitate, 1990). Cucurbitacins B have potential to be used as a favorable phytochemical for cancer prevention (Promkan et al., 2013). *C. anguria* consist of many useful compounds such as flavonoids, tannins, alkaloids, saponins and steroids which contained high level of antioxidant activity (Dzomba and Mupa, 2012). Anthraquinones and saponins which are present in *C. anguria* are used for antibacterial and antifungal activity against clinical pathogens (Senthil Kumar and Kamaraj, 2010). The pickled gherkins ensure worldwide demand so many food companies have started to develop opportunities for large scale production of gherkin. The favorable growing conditions for cultivating gherkin is suitable for India which is mainly focused on exclusive exports in worldwide (Mohapatra and Deepa, 2012). Gherkin has been introduced in India in the year 1989 for commercial production mainly for exports and its cultivation is driven largely through contract farming (Venu Prasad et al., 2013). Agri export zones (AEZ) have been created for cultivation of gherkin in South India particularly in Karnataka, nearly one lakh small and marginal farmers are involved in gherkin farming and the state produced 2.65 lakh tones of gherkin in 50,000 hectares of land in 2010-11 (Venu Prasad et al., 2013). The gherkin is susceptible to bacterial, fungal and viral diseases (Alvarez et al. 2005; Srinivasulu et al., 2010; Matsumoto and Miyagi, 2012). Although new cultivars have been developed by cross breeding (Modolo and Costa, 2004), yet no cultivar has developed with resistance to all these diseases (Matsumoto et al., 2012). Genetic improvement of

C. anguria has been achieved mainly by traditional breeding methods, but recent advances in gene transformation techniques have opened new avenues for crop improvement. *Agrobacterium*-mediated transformation is a widely used and powerful tool for introducing foreign DNA into many plant species. It has several advantages over physical transformation methods including its tendency to generate single or a low copy number of transgenes with defined ends and preferential integration into transcriptionally active regions of the chromosomes (Birch, 1997). *Agrobacterium*-mediated transformation in Cucurbitaceae has been reported to be successful with *Cucumis melo* (Fang and Grumet, 1990; Guis et al., 2000; Curuk et al., 2005; Rhimi et al., 2007) and *Cucumis sativus* (Chee, 1990; Sarmento et al., 1992; Nishibayashi et al., 1996; Vengadesan et al., 2005). The availability of an efficient *in vitro* regeneration system is the primary requirement for genetic transformation of most plants. We have established an efficient reproducible protocol for high frequency regeneration via organogenesis from leaf explants of gherkin. In this paper, we describe for the first time a protocol for *Agrobacterium*-mediated genetic transformation via direct regeneration using leaf explants in *C. anguria*. This optimized transformation system could be used to transform features with desirable characteristics such as disease resistance, stress resistance and high yield of gherkin.

Results and discussion

Organogenesis from leaf explants

Different plant growth regulators can directly influence on callus induction, shoot bud formation, shoot elongation and rooting for leaf explants of *C. anguria*. Callus induction on MSB_5 medium supplemented with 1.5 µM BAP and 0.5 µM NAA resulted in a 90.51% induction frequency (Table 1). When calluses were transferred to fresh medium containing 3.0 µM BAP, shoot regeneration was induced (Table 1). 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 (35 shoots). The shoots were excised from callus and elongated (75.4%) in MSB_5 medium fortified with 2.0 µM GA_3 (Table 1). The elongated shoots were rooted (92.0%) in MSB_5 medium supplemented with 3.0 µM IBA (Table 1). Rooted plants were acclimatized in the greenhouse (85%). The transformation can be effective only if we have a robust tissue culture protocol.

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, 90.5% callus induction was attained in explants cultured on CIM lacking kanamycin (Fig. 1). On medium containing kanamycin, the highest percentage (35.0%) 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 (Fig. 1). To minimize escape and prevent necrosis, we have selected 100 mg L^{-1} kanamycin for the transformation experiments. The present investigations are in accordance with *Colocynthis citrullus* (Ntui et al., 2010), *Cucumis melo* (Choi et al., 2012; Bezirganoglu et al., 2013) and *C. sativus* (Rajagopalan and Perl-Treves, 2005; Selvaraj et al., 2010). Carbenicillin was used to kill *Agrobacterium* after co-cultivation with leaf explants. The obtained results were supported in *C. sativus* (Wang et al., 2013) and *Citrullus colocynthis* (Dabauza et al., 1997).

Evaluation of factors influencing transformation

Effect of pre-cultivation of leaf explants

Pre-culture of explants is a critical factor to achieve high frequency of transformation. It makes the tissues competent enough to withstand the bacterial infection and increased the production of GUS positive. Pre-culturing of explants prior to infection with *Agrobacterium* enhanced the transformation frequency in *C. sativus* (Vengadesan et al., 2005). In contrast, Cervera et al. (1998) stated that the pre-culture of explants drastically reduced the regeneration of GUS positive. Higher frequency of GUS positive (80%) were noted in 3 days pre-cultured explants (Fig. 2a). However pre-culture of leaf explants for 4 days and 5 days showed reduction in recovery of GUS positive (66%). The control experiments without pre-culture of leaf explant observed 35% of GUS positive (Fig. 2a). Similarly, pre-cultivation period of 3 days have showed higher frequency of GUS expression in *Momordica dioica* (Thiruvengadam et al., 2011) and *Momordica charantia* (Thiruvengadam et al., 2012). In contrary to our observation, Vengadesan et al. (2005) and Selvaraj et al. (2010) demonstrated that 5 days of pre-culture in *C. sativus*.

Effect of bacterial density and infection time

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). Selection of appropriate OD is an important factor of concern for *Agrobacterium* genetic transformation. Lower densities (OD600 0.2 and 0.6) were not effective for transformation, whereas the highest density (OD600 1.0) decreased infection efficiency (Fig. 3a). GUS expression was significantly high (80%) at 0.8 OD600 (Fig. 3a). Similarly, Miao et al. (2009) demonstrated that *Agrobacterium* densities OD600 0.8 was induced higher frequency of GUS expression. In contrary, Nanasato et al. (2013) and Rajagopalan and Perl-Treves (2005) demonstrated that higher frequency of GUS expression OD600 0.5 and 1.0 respectively in *C. sativus*. A density of more than 1.0 was not manageable and overgrowth of *Agrobacterium* affected growth of calli adversely. GUS expression was significantly high (80%) when infection was allowed for 30 min (Fig. 3b). Similar results were reported in *M. dioica* (Thiruvengadam et al., 2011). In contrast to these reports, Selvaraj et al. (2010) reported higher GUS expression was observed in 10 min infection of *Agrobacterium*. Overgrowth of *Agrobacterium* was the real problem, when infection time increased to 45 min (data not shown). Similarly, increase of infection time to more than 35 min caused browning of the target tissue and did not allow it to flourish. Our observations are in agreement with the previous studies (De Clercq et al., 2002; Kumria et al., 2001). Therefore, optimized OD (0.8) was used with 30 min of infection time in further experiments.

Effect of acetosyringone and co-cultivation

Acetosyringone is a phenolic compound produced during wounding of plant cells, and in previous studies has been shown to induce transcription of the virulence genes of

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

Growth regulators (μM)	Callus per leaf explant (%)	No. shoots from leaf calli	Shoot elongation (%)	Root induction (%)
BAP + NAA				
0.5 + 0.5	49.00±0.76^{c}			
1.0 + 0.5	70.00±1.15^{b}			
1.5 + 0.5	90.51±1.12^{a}			
2.0 + 0.5	51.42±1.21^{c}			
1.0		12.52±0.57^{d}		
2.0		24.00±1.15^{b}		
3.0		35.00±0.57^{a}		
4.0		17.21±0.75^{c}		
GA_3				
1.0			40.53±0.28^{c}	
2.0			75.41±0.98^{a}	
3.0			49.64±0.92^{b}	
IBA				
1.0				55.23±1.01^{c}
2.0				70.00±1.15^{b}
3.0				92.00±1.00^{a}
4.0				69.52±0.95^{b}

Data represents mean values ± standard error (SE) of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

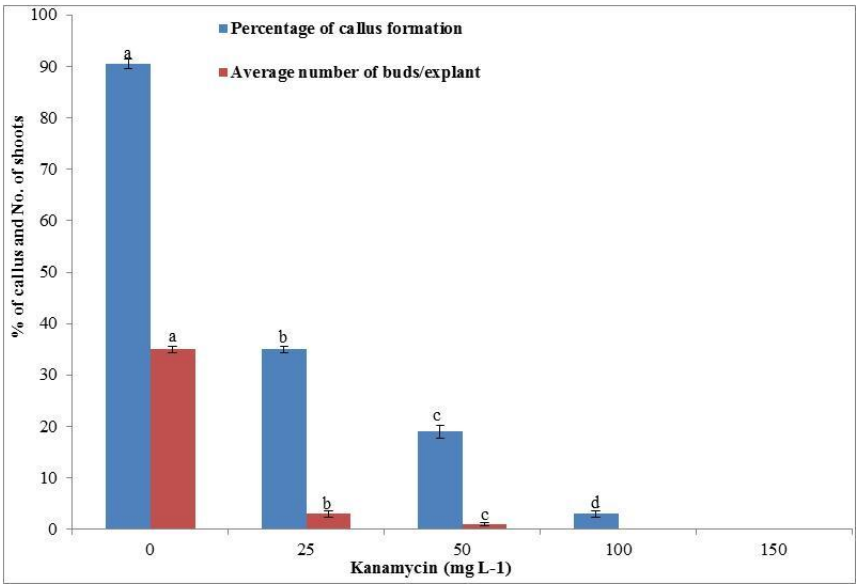

Fig 1. Effect of kanamycin concentrations on the callus formation and shoot bud induction of *Cucumis anguria*. Twenty explants were taken for the experiment. Data represents mean values ± standard error (SE) of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

Agrobacterium tumefaciens (James et al., 1993). Figure 2b shows that addition of 200 μM acetosyringone to *Agrobacterium* culture had significant improvement in number of GUS positive (85%) over the *Agrobacterium* culture without acetosyringone. Several fold increase in transformation efficiency has been noted when 200 μM acetosyringone is used in cucumber transformation (Gal-on et al., 2005; Nanasato et al., 2013). Previous cucumber transformation groups used lower concentrations of acetosyringone such as 20 μM (Vasudevan et al., 2007) and 100 μM (He et al., 2006; Miao et al., 2009; Nishibayashi et al., 1996). To determine the most suitable duration of co-cultivation, explants were co-cultivated with *A. tumefaciens* for 0 to four days in the dark (Fig. 3c). The highest frequency of transient GUS expression (80%) was obtained for explants co-cultivated for three days at dark conditions (Fig. 3c). Similar results were observed in *C. sativus* (Vasudevan et al., 2007; Wang et al., 2013). Co-cultivation in dark condition increased the transformation frequency in *C. sativus* (Chee, 1990; Tabei et al., 1994). Further, the majority of the explants perished due to bacterial overgrowth when the co-cultivation period exceeded three days.

Genetic transformation of leaf explants

Callus initiation was observed in selective medium (MSB_5 medium containing 1.5 μM BAP plus 0.5 μM NAA with 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin) within three weeks of culture (Fig. 4a and b), while untransformed control explants on selective medium turned yellow and did not

produce calli (Fig. 4a). Putative transgenic callus were transferred to MSB_5 medium containing 3.0 µM BAP with 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin for shoot bud initiation (Fig. 4c) within two weeks. The putative transgenic shoots were transferred to MSB_5 medium containing 2.0 µM GA_3 with 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin for shoot elongation for three additional weeks (Fig. 4d). Shoots that survived this selection stage were transgenic and were transferred to MSB_5 medium supplemented with 3.0 µM IBA and 100 mg L^{-1} kanamycin for rooting medium cultured in three weeks (Fig. 4e). The rooted plantlets were transferred to pots, acclimated for two weeks in the culture room and were moved to the greenhouse (Fig. 4f).

Analysis of putative transformants

Histochemical GUS assay

The expression of the GUS gene with introns is a reliable indicator of successful plant transformation as GUS with introns can be expressed efficiently in plant cells but not in *Agrobacterium* (Vancanneyt et al., 1990). 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, while calli pieces infected with *Agrobacterium* displayed varying GUS response (data not shown). The strong GUS expression was observed in two-week-old young leaves (Fig. 4g). 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. Figure 5 depicts the results from a few representative plants. PCR analysis conducted with genomic DNA showed the amplification of the predicted 0.7 kb fragments corresponding to the *nptII* genes, respectively, in kanamycin-resistant transformed plants (Fig. 5a; lanes 1 to 4). No specific amplification products were detected in the non-transgenic control plant (Fig. 5a; 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 *C. anguria* plants (Fig. 5b, lanes 1 to 4). Results indicated 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, two and three copies of the transgene integrated into their genome, while no signal was detected in the untransformed control (Fig. 5b, 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 confirm that *nptII* was expressed in these transgenic plants (Fig. 5c). 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.

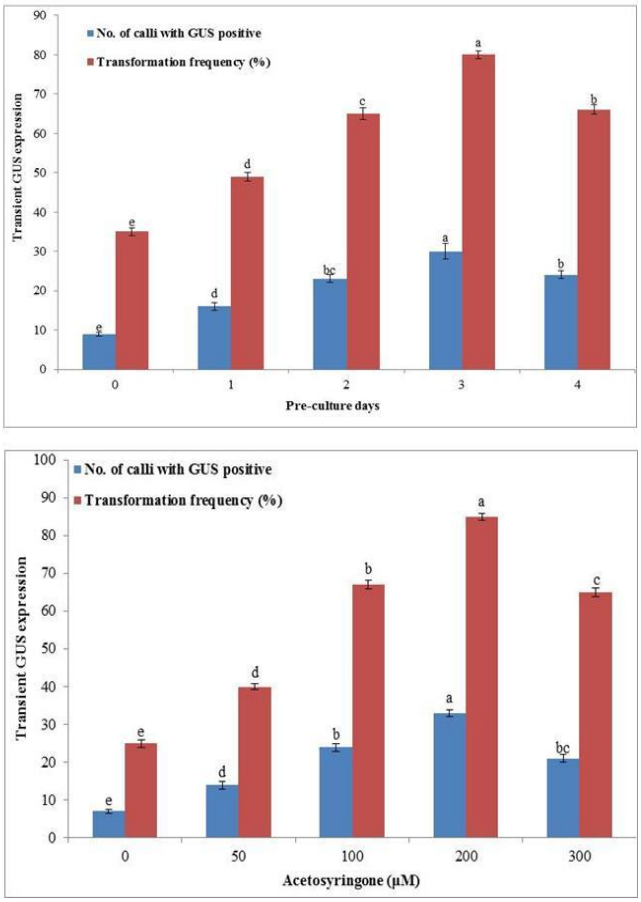

Fig 2. Factors influencing GUS expression and transformation frequency of *C. anguria*. **a,** Effect of pre-culture days on transformation frequency. **b,** Effect of AS on transformation frequency. Data represents mean values ± standard error (SE) of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

Materials and methods

Establishment of *in vitro* cultures

Plant materials

Seeds of gherkin (*Cucumis anguria* L.) were obtained from Nunhems Seeds Pvt., Ltd., Bangalore, 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 (Murashige and Skoog, 1962) basal salt mixture + B5 vitamins (Gamborg et al., 1968) (MSB_5) supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose without any growth regulators. The seedlings were grown under white fluorescent light (35 µmol $m^{-2}s^{-1}$) at a photoperiod of 16/8 h of light/dark and temperature 25 ± 2˚C. The leaf explants were trimmed into appropriate sizes (0.5 cm^2) to obtain from 10-day-old *in vitro* seedlings.

In vitro plant regeneration

Leaf explants were cultured on semi-solid callus induction medium (CIM) in culture tubes (25 × 150 mm, LCM, Lake Charles, LA.) plugged with non-absorbent cotton plugs. The callus induction medium comprised of MSB_5 plus 30 g L^{-1} sucrose, 8.0 g L^{-1} agar (Sigma-Aldrich, Inc., St. Louis, Mo, USA) and different concentrations of 0.5 - 2.0 µM benzylaminopurine (BAP) with 0.5 µM Naphthaleneacetic acid (NAA) 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 1.0 - 4.0 µM BAP. The regenerated shoots were harvested and transferred to shoot elongation medium (SEM) containing MSB_5 supplemented with 1.0 - 3.0 µM gibberellic acid (GA_3), 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 1.0 - 4.0 µM indole 3-butyric acid (IBA). After 3 weeks, rooted plantlets were transferred to potting soil and acclimatized in green house.

Evaluation of factors influencing transformation

Agrobacterium strains and binary vector

The *Agrobacterium tumefaciens* strain LBA 4404 harbouring the binary vector pBAL2 was used for gherkin 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 (Vancanneyt et al., 1990) driven by the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator as reporter gene (Thiruvengadam et al., 2011, 2012).

Effect of acetosyringone, bacterial density and infection time

Selected *Agrobacterium* strain LBA 4404 harbouring the binary vector pBAL2 was grown to different OD_{600} (0.2, 0.4, 0.6, 0.8 and 1.0). Pre-cultured leaf explants were infected with *Agrobacterium* suspension of varying OD_{600} for 5, 10, 20, 30 and 40 min. The infected leaf explants were transferred on co-cultivation medium containing different concentrations of AS (0, 50, 100, 200 and 300 µM). Twenty explants were taken for the experiment.

Sensitivity to kanamycin

Sensitivity testing to kanamycin was carried out in order to determine the inhibitory concentration which arrests growth of leaves. The leaf explants were placed with abaxial side on CIM medium containing MSB_5 plus 30 g L^{-1} sucrose, 8.0 g L^{-1} agar, 1.5 µM BAP with 0.5 µM NAA and different concentrations of kanamycin (0, 25, 50, 100 and 150 mg L^{-1}). 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.

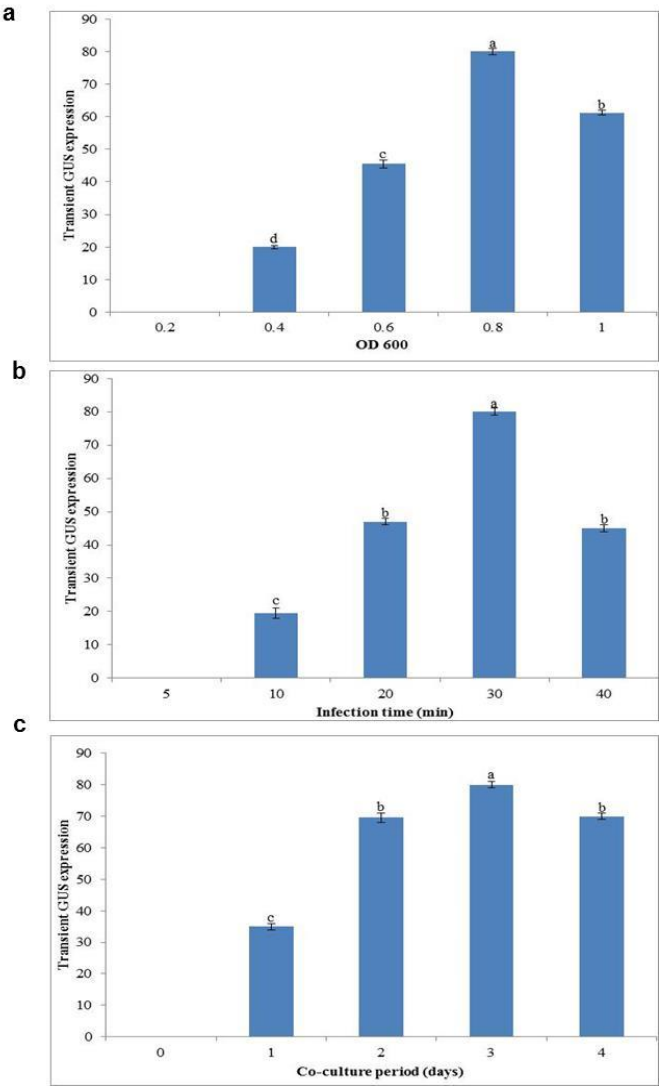

Fig 3. Factors influencing GUS expression and transgenic plant regeneration in *C. anguria*. **a,** The effect of bacterial concentration. **b,** Infection time. **c,** Co-culture duration. Data represents mean values ± standard error (SE) of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT).

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 MSB_5 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.

Genetic transformation, selection and regeneration of transformants

Agrobacterium cultures were initiated from glycerol stock and grown overnight in liquid Luria-Bertani (LB) medium (Maniatis et al., 1982) containing 50 mg L^{-1} kanamycin and 25 mg L^{-1} rifampicin at 28ºC, on continuous shaking (180 *g*) and were used at appropriate OD_{600} (0.8). Bacterial cells were centrifuged at 3,000 *g* for 5 min and resuspended in liquid MS to maintain desired OD (0.8). Pre-cultivated leaf explants

were cultured on infection medium (liquid MS + 200 µM AS) incubated for 30 min on mild shaking (50 *g*). Leaf explants were transferred to co-cultivation medium containing MSB_5 containing 30 g L^{-1} sucrose supplemented with 1.5 µM BAP plus 0.5 µM NAA, 200 µM AS and 8.0 g L^{-1} agar at 26 ± 1ºC in the dark for 3 - 4 days. Infected leaf explants were washed repeatedly in sterile distilled water containing 500 mg L^{-1} carbenicillin and then transferred to selection medium MSB_5 containing 30 g L^{-1} sucrose augmented with 1.5 µM BAP plus 0.5 µM NAA, 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin for the induction of transgenic callus. As a control, uninfected explants were cultured on selective medium. After 3 weeks of culture, leaf calli were transferred to shoot induction medium MSB_5 containing 30 g L^{-1} sucrose supplemented with 3.0 µM BAP, 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin and transferred to shoot elongation medium MSB_5 supplemented with 2.0 µM GA_3, 300 mg L^{-1} carbenicillin and 100 mg L^{-1} kanamycin for 5 weeks. Elongated shoots were transferred to rooting medium containing MSB_5 media supplemented with 30 g L^{-1} sucrose, 8.0 g L^{-1} agar, 3.0 µM IBA and 100 mg L^{-1} kanamycin. Rooted plantlets were washed in sterile distilled water to remove traces of medium and were transferred to plastic pots (5 cm diameter) containing a mixture of sterile soil, perlite and vermiculite (3:1:1). After 3 weeks, the plants were transferred to pots containing soil and grown in a greenhouse. Later, the plants were transferred to the field.

Analysis of transgene integration

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 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 0.7 kb for *nptII* gene. PCR amplification products were analysed by electrophoresis in 1% agarose gel.

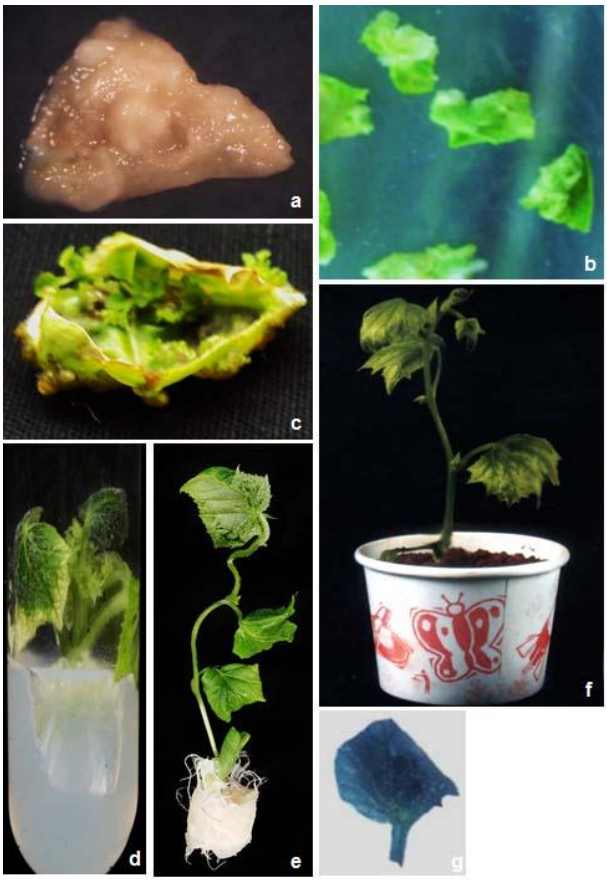

Fig 4. Regeneration of transgenic plants from leaf explants of *C. anguria*. **a,** Non-transgenic leaf cultured on MSB_5 + 1.5 µM BAP with 0.5 µM NAA and 100 mg L^{-1} kanamycin. **b,** Callus initiation from leaf explants MSB_5 + 1.5 µM BAP with 0.5 µM NAA, 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin. **c,** Multiple shoot induction from transgenic leaf callus (MSB_5 + 3.0 µM BAP, 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin). **d,** Transgenic shoot elongation (MSB_5 + 2.0 µM GA_3, 100 mg L^{-1} kanamycin and 300 mg L^{-1} carbenicillin). **e,** Rooting of elongated transformed shoots (MSB_5 + 3.0 µM IBA and 100 mg L^{-1} kanamycin). **f,** Hardening of transformed plant in pot. **g,** GUS expression as observed in leaf.

Southern blot analysis

For Southern blot analysis, genomic DNA (15 µg) isolated 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 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 (0.7 kb) 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).

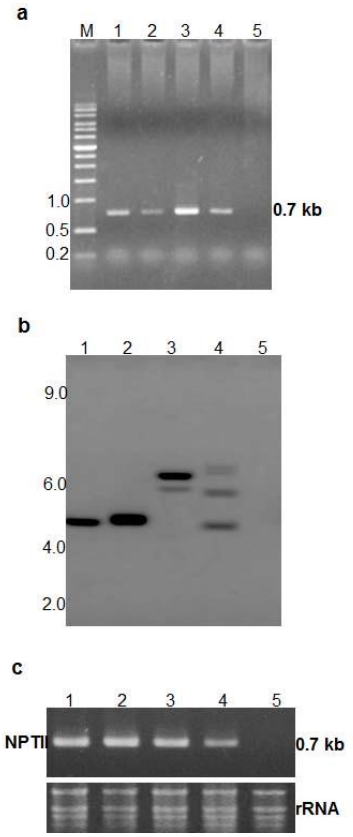

Fig 5. Molecular analysis of transformants. **a,** PCR analysis of DNA isolated from leaves of transgenic lines and non-transformed plants of *C. anguria*. Agarose gel electrophorogram of PCR amplification performed with primers for the *nptII* gene. A 0.7 kb DNA fragment was amplified. Lanes M- marker; Lanes 1 - 4 transgenic lines of *C. anguria*; Lane 5 non-transformed plants of *C. anguria*. **b**, Southern blot analysis of transgenic plants. Lanes 1 - 4 transgenic lines of *C. anguria*; Lane 5 non-transformed plants of *C. anguria*. Genomic DNAs were digested with *HindIII* and hybridized to a 0.7 kb NPTII - probe. **c,** RT-PCR assay of *nptII* gene expression using primers of NPTII. A 0.7 kb cDNA fragment was amplified. Total RNA was isolated from four *nptII* transgenic plants (Lanes 1 - 4) of *C. anguria*; Lane 5 non-transgenic plant of *C. anguria*.

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 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.

Experimental design and data analysis

All the experiments were performed in triplicates and each experiment was repeated twice. The data was expressed as means ± standard error. One way ANOVA analysis followed by the Duncan's test was used to determine significant ($P \leq 0.05$) differences. All the statistical analyses were done by using SPSS Ver. 20 (SPSS Inc., Chicago, IL, USA) statistical software package.

Conclusions

The protocol for delivery of T-DNA using *A. tumefaciens* has been developed and subsequent regeneration of transformed shoots has been achieved in *C. anguria*. Transgenic plants were confirmed by GUS, PCR, RT-PCR and Southern blotting. This is the first report of successful transformation of gherkin using *A. tumefaciens* and this robust protocol has the potential to facilitate work on functional analysis of agronomically important genes.

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