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TRANSFER AND EXPRESSION OF *npt* ii AND *bar* GENES IN CUCUMBER (*CUCUMIS SATIVUS* L.)

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Summary

The generation of transgenic *Cucumis sativus* cv. Greenlong plants resistant to phosphinothricin (PPT) was obtained using *Agrobacterium tumefaciens*-mediated gene transfer. The protocol relied on the regeneration of shoots from cotyledon explants. Transformed shoots were obtained on Murashige and Skoog medium supplemented with 4.4 μ*M* 6-benzylaminopurine, 3.8 μ*M* abscisic acid, 108.5 μ*M* adenine sulfate, and 2 mg l^{-1} phosphinothricin. Cotyledons were inoculated with the strain EHA105 harboring the neomycin phosphotransferase II (*npt* II), and phosphinothricin resistance gene (*bar*) genes conferring resistance to kanamycin and PPT. Transformants were selected by using increasing concentrations of PPT (2–6 mg l^{-1}). Elongation and rooting of putative transformants were performed on PPT-containing (2 mg l^{-1}) medium with 1.4 μ*M* gibberellic acid and 4.9 μ*M* indolebutyric acid, respectively. Putative transformants were confirmed for transgene insertion through PCR and Southern analysis. Expression of the *bar* gene in transformed plants was demonstrated using a leaf painting test with the herbicide Basta. Pre-culture of explants followed by pricking, addition of 50 μ*M* acetosyringone during infection, and selection using PPT rather than kanamycin were found to enhance transformation frequency as evidenced by transient β-glucuronidase assay. Out of 431 co-cultivated explants, 7.2% produced shoots that rooted and grew on PPT, and five different plants (1.1%) were demonstrated to be transgenic following Southern hybridization.

Key words: acetosyringone; cucumber; β-glucuronidase; phosphinothricin; pre-culture; agro-transformation.

Introduction

Cucumber belongs to the genus *Cucumis* and is widely cultivated in many areas of the world. Cucumber (*Cucumis sativus* L.) cv. Greenlong is a major cultivar and an important vegetable crop in India. The fruits are generally used for slicing, pickling and juice extraction, and in cosmetics. The availability of a breeding stock resistant to disease is essential in breeding programs because agricultural yields are reduced constantly by various diseases caused by phytopathogenic fungi, bacteria, and viruses (Tabei et al., 1998). Breeding for disease resistance has become one of the most crucial objectives in cucumber cultivation.

Cucumber suffers from a number of viral diseases which seriously limit crop production (Gaba et al., 2004). Conventional crosses and transfer of desirable traits, especially disease resistance, from wild species have not been successful in *Cucumis* (Esquinas-Alcazar and Gulick, 1983). Tissue culture and biotechnology techniques offer alternative approaches to cucumber improvement. The availability of protocols to achieve high-frequency plant regeneration from cultured cells or tissue is a prerequisite for the application of tissue culture and biotechnology procedures for crop improvement.

Several procedures for transformation of cucumber (Trulson et al., 1986; Chee, 1990b; Chee and Slightom, 1991; Sarmento et al., 1992; Tabei et al., 1994, 1998; Nishibayashi et al., 1996; Raharjo et al., 1996) have been established. The gene encoding kanamycin resistance (Chee, 1990b; Sarmento et al., 1992) and a coat protein gene conferring virus resistance (Chee and Slightom, 1991), have already been transferred to cucumber. All previous selection methods in cucurbits have used kanamycin, while here for the first time we document use of phosphinothricin (PPT). The present work was undertaken to evolve an efficient protocol to produce transgenic plants in cucumber cv. Greenlong resistant to the herbicide Basta using PPT as the selection agent.

Materials and Methods

Plant material, regeneration and culture conditions. Seeds of *Cucumis sativus* cv. Greenlong were provided by Indo-American Hybrid seeds (India) Pvt. Ltd., Bangalore, India. Seeds were soaked for 30 min in distilled water to render germination uniform. Seeds were surface-sterilized with 2% teepol detergent solution (Reckit Colman, India) for 10 min and rinsed three times with distilled water, followed by a wash in mercuric chloride solution (0.1% w/v) for 10 min and then washed three to four times with sterile distilled water. Seeds were inoculated into 10 × 150 mm Petri dishes (15–20 seeds per dish) (Borosil, India) containing growth regulator-free MS medium (Murashige and Skoog, 1962) with 3% sucrose and MS vitamins (pH 5.8) for germination in darkness. After 2 d of germination, the de-embryonated cotyledons were separated and cultured on MS medium containing 6-benzylaminopurine (BA; 4.4 μ*M*), abscisic acid (ABA; 3.8 μ*M*), and adenine sulfate (AdS; 108.5 μ*M*) for 10 d (pre-culture). The media used in the transformation experiments were supplemented with 87.6 m*M* sucrose solidified with 0.8% agar (Himedia Co., India). All media were autoclaved at

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121 °C for 15 min and cultures were maintained at a temperature of 25 ± 2°C under a 16 h photoperiod. These pre-cultured cotyledons were used for transformation experiments.

Kanamycin and PPT sensitivity of explants. The response of cotyledon explants to kanamycin and PPT was determined by culturing the cotyledon explants in MS medium supplemented with BA (4.4 μ*M*), ABA (3.8 μ*M*), and AdS (108.5 μ*M*) along with different concentrations of kanamycin (0, 50, 75, 100, 150, and 200 mg l^{-1}) or PPT (0, 1, 2, 4, 6, 8, and 10 mg l^{-1}). Selective concentrations of kanamycin and PPT were used throughout the selection procedures. A positive control (without kanamycin or PPT) was also maintained.

Agrobacterium strains and plasmid vector. Genetic transformation was performed using the *Agrobacterium* strains EHA105 and LBA4404. Both strains harbor the binary vector pME 504 (Fig. 1) that carries a neomycin phosphotransferase (*npt* II) gene under the control of *nos* promoter, and the *bar* and *uid*A genes separately under the control of CaMV 35S promoter (Edelman et al., 2000).

Agrobacterium culture. A single colony of *A. tumefaciens* was inoculated into 5 ml of YEP medium (Chilton et al., 1974) containing 50 mg l^{-1} kanamycin and 10 mg l^{-1} tetracycline. After 6 h, 50 μl of the above culture were transferred to 50 ml of AB minimal medium (Chilton et al., 1974), pH 7.0, containing 50 mg l^{-1} kanamycin. This culture was incubated overnight at 200 rpm on a rotary shaker at 28°C. The 1.0 $OD_{(600\,nm)}$ culture was pelleted at 5000 rpm at 28°C using a refrigerated centrifuge. The pellet was re-suspended in AB minimal medium (pH 5.6) supplemented with 50 μ*M* acetosyringone to a final density of 1.0 $OD_{600\,nm}$.

Infection, co-cultivation, and selection. After 10 d of pre-culture (when the cotyledon enlarges to four times its original size) the proximal region of the cotyledon explants was pricked with a sterile needle (Dispovan, Mumbai, India, 0.56 × 25 mm). Approximately 10 holes were made per explant in the proximal end. After pricking, the explants were immersed in an *Agrobacterium* suspension containing 50 μ*M* acetosyringone for 10 min. Infected explants were blotted dry using sterile Whatman no. 1 paper prior to co-cultivation for 3 d in the darkness at 25 ± 2°C on MS medium containing the respective growth regulators and additives used for regeneration. After co-cultivation the explants were washed three or four times with sterile distilled water and blotted dry on sterile Whatman paper. The cotyledon explants were cultured on the regeneration medium supplemented with PPT (2 mg l^{-1}) or kanamycin (100 mg l^{-1}) and cefotaxime (300 mg l^{-1}). Subsequently explants with newly initiated shoots were transferred to elongation medium containing gibberellic acid (GA_3; 1.4 μ*M*) and either PPT (2 mg l^{-1}) or kanamycin (100 mg l^{-1}). Successively shoots were transferred to increasing concentrations of PPT and were selected finally on 6 mg l^{-1} PPT. The elongated shoots (50 mm long) were rooted in half-strength MS medium containing 4.9 μ*M* indolebutyric acid (IBA) and (2 mg l^{-1}) PPT. The plants were transferred to pots in a greenhouse. Each experiment was performed with an average of 43 explants and was repeated 10 times.

β-Glucuronidase (GUS) assay. Expression of GUS in the cells was assayed histochemically as described by Jefferson et al. (1987), using the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (Sigma, St. Louis, MO, USA). Assays to score transient GUS expression in cotyledons were carried out 7 d post co-cultivation to study the effect of strain specificity, pre-culture, and acetosyringone on the frequency of transformation. Assays of GUS activity in the leaves were performed 3 wk after co-cultivation. For microscopic analysis, stem sections of the transgenic plants were stained for 8 h at 37°C in 2 m*M* X-gluc. The tissues were further cleared before observation as described by Pichon et al. (1992). The photograph was taken with a Nikon E400 microscope with HIII photographic unit (Nikon Co., Tokyo, Japan).

Southern analysis. Plant DNA was prepared from leaf tissues (control, putative transformants – T1–T12) according to Dellaporta et al. (1983). DNA (10 μg) was digested with *Hin*dIII and fractionated on a 0.8% agarose gel and blotted onto a nylon membrane (Genescreenplus, Dupont). A 0.6 kb fragment encompassing *npt* II sequence from the plasmid pME 504 was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Amersham Biosciences) and used as probe. Hybridization was performed as described in Sambrook et al. (1989). After overnight hybridization the blot was Q1 subjected to high-stringency washing conditions with 2 × SSC/0.1% sodium dodecyl sulfate (SDS), 0.5 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS. Each wash was performed for 30 min at 65°C. The blot was exposed to a X-ray film (Fujifilm, Super RX) at −80°C for 3 d.

PCR analysis. PCR analysis of the *vir* gene fragment was performed using a set of primers 5′-GCGGTGAGACAATAGGCG-3′ and 5′-GAACTGCTTGCTGTCGGC-3′ that amplified a 592 bp fragment corresponding to the *vir G* region. All PCR reactions were performed using a Peltier effect thermal cycler from MJ Research Co. (USA). Samples containing 100 ng of genomic DNA were first heated at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by 10 min final extension at 72°C. Plasmid DNA, 50 ng, was used as positive control. The PCR reactions contained 20 pmol of each primer, 200 m*M* dNTP mix, 2 m*M* $MgCl_2$, Taq polymerase 1 unit, 100 ng of template DNA in 1 × reaction mixture buffer. The PCR products were analyzed on 1% agarose gel.

Basta resistance assay. Basta leaf painting assay was performed on 3-wk-old plants growing in the greenhouse. Plant tolerance to Basta was scored after 1 wk. Plants were tested for Basta resistance by painting the whole plants with a solution containing 0.1 mg ml^{-1} Basta and 0.1% Tween-20. Resistant plants were scored after 5–10 d.

Results and Discussion

Sensitivity of cotyledon explants to kanamycin and PPT. Shoots began to differentiate from the proximal end of the explants. The shoots were normal and looked healthy. Increasing the kanamycin concentration reduced regeneration frequency. At a concentration of 100 mg l^{-1} kanamycin, 15% of the explants regenerated (data not shown). On the other hand, only 2% of the explants regenerated shoots on 2 mg l^{-1} PPT (data not shown). The onset of shoot production was delayed by PPT-containing medium. Shoots became yellow when the PPT concentration was increased above 2 mg l^{-1}, and at 2 mg l^{-1} PPT shoots were completely bleached and showed symptoms of leaf death and necrosis. This concentration of PPT (2 mg l^{-1}) was used for selecting the transformants. A strong selection agent is important for the transformation of a cucurbit variety, especially one that regenerates via direct organogenesis from cotyledon explants (Gaba et al., 1995). In muskmelon, 30%

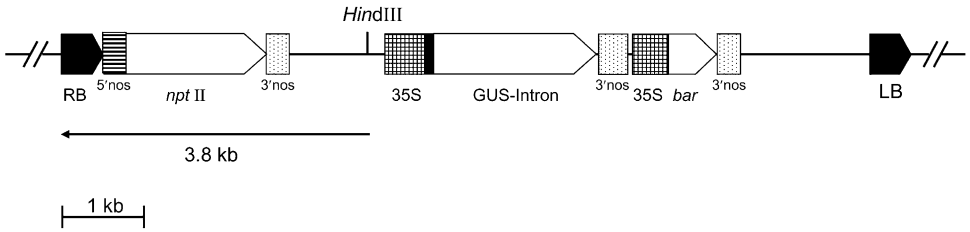

FIG. 1. Schematic representation of the T-DNA region of the binary plasmid pME 504. This plasmid carries the *npt* II gene, conferring resistance to kanamycin, under the control of the nopaline synthase (NOS) promoter. The *bar* gene, conferring resistance to the herbicide Basta, and the *uid*A gene coding the GUS reporter, were each under the control of the 35S-CaMV promoter and terminated by the 3nos region.

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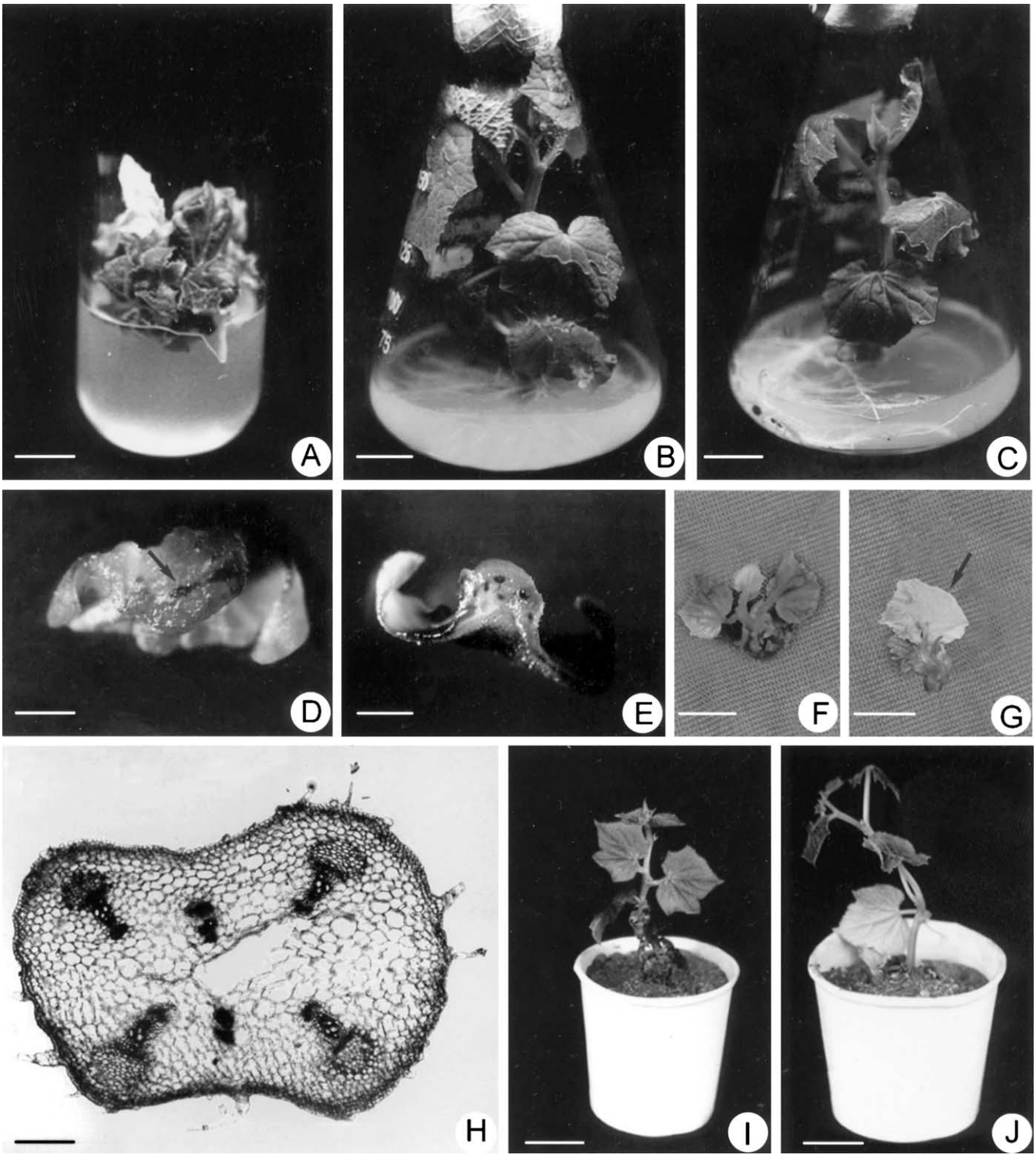

FIG. 2. Stages in the development and confirmation of transgenic cucumber plants. *A*, Transgenic shoots regenerating from co-cultivated cotyledon explants in the presence of 2 mg l^{-1} PPT (*bar* = 2 mm). *B*, Elongation of shoots in MS medium containing 1.4 μ*M* GA_3 and 2 mg l^{-1} PPT (*bar* = 3 mm). *C*, Transgenic shoots rooted in MS medium containing 4.9 μ*M* IBA and 2 mg l^{-1}PPT (*bar* = 3 mm). *D*, GUS foci (*arrow*) in the proximal region of the co-cultivated cotyledon explants in kanamycin-containing (100 mg l^{-1}) medium (*bar* = 2 mm). *E*, GUS foci in the proximal region of the co-cultivated explants in PPT-containing (2 mg l^{-1}) medium (*bar* = 2 mm). *F*, GUS-positive shoots obtained in the presence of 2 mg l^{-1} PPT (*bar* = 2 mm). *G*, Chimeric shoots obtained in the presence of 100 mg l^{-1} kanamycin showing non-stained regions (*arrow*) along with GUS-positive regions (*bar* = 2 mm). *H*, Cross-section of stem of transgenic shoot showing histological localization of GUS activity (*bar* = 50 μm). *I*, Transformed shoots surviving the Basta paint treatment (*bar* = 3 mm). *J*, Non-transformed shoots showing leaf necrosis and abscission after Basta treatment (*bar* = 3 mm).

(Fang and Grumet, 1990) and 75–90% (Dong et al., 1991) non-transgenic (escapes) shoot development has been noticed after selection on 75–100 mg l^{-1} kanamycin. Also, in an attempt to transform cucumber through suspension culture, a large number of escapes were encountered using kanamycin as selection agent (Schulze et al., 1995).

Plant transformation and regeneration. Co-cultivated explants regenerated shoots after transfer to regeneration medium. On 2 mg l^{-1} PPT or kanamycin (100 mg l^{-1}) and 300 mg l^{-1} cefotaxime small shoots began to develop. In PPT-containing medium, shoots died after 10 d of culture due to their sensitivity. However, continued culture in the same medium resulted in proliferation of new shoots that survived the selecting concentration (Fig. 2*A*). We co-cultivated 431 cotyledon explants in 10 separate experiments, of which 19.0 ± 1.3% explants (mean ± SE for the experiment) developed shoots on MS medium containing 4.4 μ*M* BA, 3.8 μ*M* ABA, 108.5 μ*M* AdS, and 2 mg l^{-1} PPT. Subsequently, 7.2 ± 0.8% shoots (mean ± SE for the experiment) grew and rooted on PPT (i.e., were PPT-resistant). No shoots developed from the control without *Agrobacterium* inoculation ($n = 50$ explants). Shoots were healthy and exhibited normal growth. However, shoots were short and the leaves smaller in size than compared to the control. The elimination of escapes was a major problem solved by continuous culture in regeneration medium containing increasing concentrations of PPT. Elongation and rooting in the presence of PPT favored selection of transformed plants (Fig. 2*B*, *C*). Although complete inhibition of shoot regeneration occurred at 100 mg l^{-1} kanamycin, a large number of escapes was observed when kanamycin was used as a selection agent. Therefore, compared to kanamycin (100 mg l^{-1}), PPT (2 mg l^{-1}) is an efficient selection agent in *C. sativus* cv. Greenlong. Similar problems were encountered in the studies of Trulson et al. (1986) and Tabei et al. (1994) in cucumber, and Fang and Grumet (1990), Dong et al. (1991) and Valles and Lasa (1994) in muskmelon. However, Chee (1990a) and Sarmento et al. (1992) used kanamycin as the selection marker to regenerate transformants in cucumber. Examination of GUS activity in co-cultured explants after 7 d showed numerous scorable GUS foci (Fig. 2*D*, *E*), and varied little with the selection agent used (Fig. 3).

Comparing the two *A. tumefaciens* strains, EHA105 consistently produced a significantly greater transformation response than LBA4404 at 1.0 OD concentration (9% versus 3%, $P < 0.01$), a difference commonly noted (Hood et al., 1986, 1993).

GUS assay. Young shoots (7 cm) from each clone were assayed for GUS activity. Shoots which developed from non-transformed explants did not show any GUS activity when stained with X-Gluc, while shoots obtained from co-cultivated explants stained blue. The first indication of transformation was obtained from the GUS assay in shoots cultured in 2 mg l^{-1} PPT (Fig. 2*F*). The occurrence of pale zones in leaf tissues may be due to lower GUS activity in cells that divide less frequently (Nagata et al., 1987). Chimeric shoots were obtained when kanamycin (100 mg l^{-1}) was used as a selection agent (Fig. 2*G*). The cross-section of the stem revealed GUS activity in vascular bundles following selection on 2 mg l^{-1} PPT (Fig. 2*H*). Although CaMV 35S is a constitutive promoter, localized expression in actively dividing cells can be a characteristic feature (Jefferson et al., 1987; Valles and Lasa, 1994).

PCR analysis. Molecular analysis through PCR amplification confirmed the absence of any *Agrobacterium* contamination in the developing shoots, following the use of *vir G* specific primers which did not amplify the expected 592 bp *vir G* fragment (Fig. 4*A*, lanes 4–15).

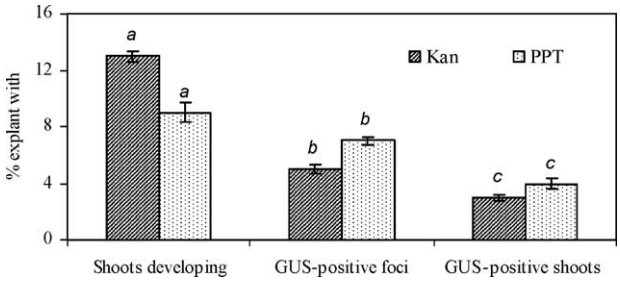

FIG. 3. GUS activity in explants co-cultivated with EHA105: pME 504 along with 50 μ*M* acetosyringone in regeneration medium. After transformation, explants were cultured in regeneration medium with 100 mg l^{-1} kanamycin (Kan) or 2 mg l^{-1} PPT and subcultured three times on the same medium at 20-d intervals. Results are five individual experiments with 100 explants per selection process per experiment. The mean was calculated for each experiment and then the mean ± SE of the different experiments was calculated and is shown here. Different *letters* show significant difference among treatments according to Fisher's least significant difference test ($P = 0.05$).

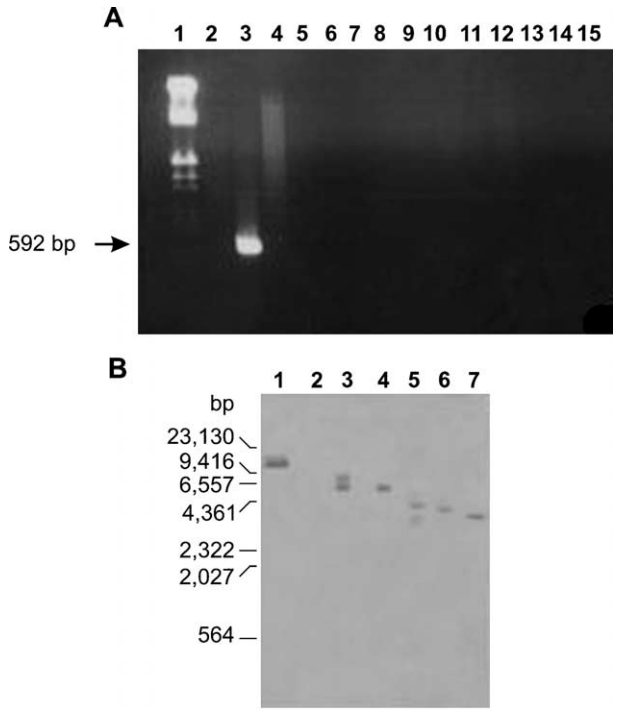

FIG. 4. Molecular analysis of putatively transformed *C. sativus* cv. Greenlong plants by PCR (*A*) and Southern (*B*). *A*, Total DNA from putatively transformed plants was subjected to PCR amplification with *Vir G* primers to detect the *Agrobacterium* contamination. *A. tumefaciens* (EHA105: pME 504) total DNA was subjected to PCR with (*lane 3*, positive control) or without (*lane 2*, negative control) *Vir G* primers. *Lanes 4–15* contain total DNA from putative transformants (T1–T12). Lamda DNA digested with *Hin*dIII was used as the size marker (*lane 1*). *B*, Total DNA (10 μg) from untransformed (*lane 2*) and five transformed (*lane 3–7*) plants was digested with *Hin*dIII, fractionated with 0.8% agarose gel, and blotted onto a nylon membrane. The blot was hybridized to a 0.6 kb *npt* II fragment (radiolabeled with [α-^{32}P]dCTP) as the probe. Plasmid DNA (pME 504) was also digested with *Hin*dIII and labeled as positive control (*lane 1*).

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Southern blotting. Genomic DNA was digested with *Hin*dIII and subjected to Southern hybridization using *npt* II as probe to detect right border junction fragments. As the distance between the *Hin*dIII site in the T-DNA and the right border is 3.8 kb (Fig. 1), the sizes of the junction fragments are expected to be greater than 3.8 kb. The results of the hybridization revealed the integration of the *npt* II genes into the genome of *C. sativus* cv. Greenlong. The sizes of the bands obtained in lanes 3–7 (Fig. 4*B*) (putative transformants) were greater than 3.8 kb as expected. A similar result was observed in the case of plasmid DNA pME 504 (lane 1) whereas no hybridization occurred in the case of DNA isolated from control plants (non-transformed, lane 2).

Herbicide painting. Successful expression of the introduced *bar* gene was confirmed by performing herbicide leaf-painting tests on shoots that rooted and grew on 2 mg l^{-1} PPT. Putative transformants were examined after 7 d. Plants devoid of any damage were referred to as stable transformants (Fig. 2*I*), whereas leaf death and necrosis occurred in non-transformed (control) plants (Fig. 2*J*). Thirty one percent of the regenerated T_0 plants were resistant to a spray of 0.1 mg ml^{-1} (0.01%) commercial Basta herbicide. Possibly the non-resistant plants at this stage did not express enough phosphinothricin acetyl tranferase protein in the leaves to confer resistance, or were transgenic-chimeric, or were escapes.

Our results demonstrated that most of the green adventitious shoots examined were transgenic. In any transformation system, the risk of producing chimeric plants containing both transformed and non-transformed cells needs to be minimized (Christou and McCabe, 1992). In the present work, shoots obtained from transformed cotyledon cells were selected routinely in PPT-containing medium and this avoided the generation of chimeras. Transformed shoots were more efficiently selected at 6 mg l^{-1} PPT. We believe that this transformation method will contribute to the production of useful transgenic cucumber plants.

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Author Queries

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Q1 SSC in full (sodium chloride and sodium citrate?)