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Transformation of Pickling Cucumber with Chitinase-encoding Genes using *Agrobacterium tumefaciens*

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Summary. Transformation of cucumber cv. Endeavor was attempted using three *Agrobacterium tumefaciens* strains (a supervirulent leucinopine type, an octopine type and a nopaline type), each harbouring one of three binary vectors which contained an acidic chitinase gene from petunia, and basic chitinase genes from tobacco and bean, respectively, driven by the CaMV 35S promoter. Petiole explants were inoculated with a bacterial suspension (10^8 cells•ml^{-1}), cocultivated for 48-96 h and placed on Murashige and Skoog (MS) medium with 5.0 µM each of 2,4-D and BA, 50 mg•l^{-1} kanamycin and 500 mg•l^{-1} carbenicillin. The frequency of embryogenic callus formation ranged from 0 to 12%, depending on strains/vectors used and length of cocultivation, with the highest being obtained using the leucinopine strain with petunia acidic chitinase gene. The kanamycin-resistant embryogenic calli were used to initiate suspension cultures (in liquid MS medium with 1.0/1.0 µM 2,4-D/BA, 50 mg•l^{-1} kanamycin) for multiplication of embryogenic cell aggregates. Upon plating of cell aggregates onto solid MS medium with 1.0/1.0 µM NAA/BA and 50 mg•l^{-1} kanamycin, calli continued to grow and later differentiated into plantlets. Transformation by the leucinopine strain and all three vectors was confirmed by PCR amplification of the *NPT II* gene in transgenic calli and plants, in addition to Southern analysis. Expression of the acidic chitinase gene (from petunia) and both basic chitinase genes (from tobacco and bean) in different transgenic cucumber lines was confirmed by Western analyses.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; BA = 6-benzyl-aminopurine; CaMV = cauliflower mosaic virus; NAA = naphthaleneacetic acid; NPT II = neomycin phosphotransferase II; PCR = polymerase chain reaction.

Introduction

An important application of cucumber (*Cucumis sativus* L.) tissue and cell culture is for plant improvement through genetic engineering (Chee and Slightom 1991; Sarmento et al. 1992; Trulson and Shahin 1986). Several procedures to regenerate plantlets from cucumber tissue and cell cultures have been developed for fresh market and pickling cultivars (Chee and Tricoli 1988; Orczyk et al. 1988; Cade et al. 1990; Chee 1990b; Gambley and Dodd 1990; Punja et al. 1990; Raharjo and Punja 1994). The application of *Agrobacterium*-based transformation systems for foreign gene transfer into cucumber has, however, only been demonstrated in a few studies. The gene encoding kanamycin resistance (Chee 1990a; Sarmento et al. 1992) and a coat protein gene conferring virus resistance (Chee and Slightom 1991) have been expressed in transgenic cucumber plants. However, except for virus resistance, other potentially useful agronomic traits have not been engineered into cucumber. The objective of this study was to introduce a heterologous acidic chitinase gene cloned from petunia (Linthorst et al. 1990), and a basic chitinase gene cloned from tobacco (Linthorst et al. 1990) and bean (Broglie et al. 1991), into pickling cucumber using *Agrobacterium*.

Chitinases are pathogenesis-related proteins which have been implicated in plant defense against fungal infection (Boller et al. 1983; Punja and Zhang 1993). The substrate is chitin, which does not occur in plants but which is an important component of fungal cell walls (Linthorst et al. 1990). The acidic and basic chitinases differ in isoelectric points, in the subcellular targeting of the enzyme, and in antifungal activity (Punja and Zhang 1993). In cucumber, the indigenous chitinase is acidic and is induced by various factors, such as pathogen infection and other biotic and abiotic stresses (Metraux 1986; Zhang and Punja 1993). The introduction of genes encoding chitinases into cucumber would be useful to elucidate the role of different chitinases in plant disease resistance to filamentous fungal pathogens. The present study describes the integration of several different chitinase genes and expression of the proteins in transgenic pickling cucumber. Three disarmed *Agrobacterium* strains, each harbouring a binary vector, were evaluated and methods are described for transformation.

Materials and methods

Plant material. The pickling cucumber cultivar Endeavor (Campbell Soup Co., Davis, CA), which has high regeneration rates in

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tissue culture (Punja et al. 1990), was used. Seeds were washed with detergent (Liqui-Nox, Alconox Inc., NY), rinsed in tap water and soaked for 30 min. Seed coats were then carefully removed using a pointed scalpel. This was followed by surface-sterilization in 70% ethanol for 30 s, a 5 min soak in a 10% solution of commercial bleach (Javex, 6.25% sodium hypochlorite) and three rinses in sterile distilled water. Seedlings were grown in Magenta culture vessels (Magenta Corp., Chicago, IL) containing 50 ml of half-strength MS basal medium (Murashige and Skoog 1962) (one-half of macro- and micro-elements, 100 mg·l^{-1} myo-inositol, 0.1 mg·l^{-1} thiamine-HCl, 0.5 mg·l^{-1} nicotinic acid, 0.5 mg·l^{-1} pyridoxine-HCl, 2 mg·l^{-1} glycine, 30 g·l^{-1} sucrose, 10 g·l^{-1} tissue culture agar). All chemicals used were tissue-culture grade (Sigma Chemical Co., St. Louis, MO). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Ampicillin was added (after filter sterilization) to a final concentration of 100 mg·l^{-1} to reduce potential bacterial contamination originating from seeds. For uniform germination, culture vessels were incubated in darkness at a temperature of 29°C for 2 days and then transferred to light (cool-white fluorescent lamps) at an ambient temperature of 25-28°C and a photoperiod of 16 h·d^{-1}. Explants for cocultivation were petiole segments (4-5 mm in length) taken from the first and second true leaves of 10- to 21-d old seedlings.

Bacterial strains. Three *A. tumefaciens* strains with different Ti helper plasmids were used: a) EHA105 (a supervirulent leucinopine type), b) MOG101 (an octopine type), and c) MOG301 (a nopaline type). The strains were provided by Dr. L. Melchers (Mogen Int. nv, The Netherlands) and harboured one of two binary vectors (pMOG196 and pMOG198) containing the petunia acidic chitinase gene and the tobacco basic chitinase gene, respectively (Linthorst et al. 1990). A third vector, pGA492-CHN, containing a bean basic chitinase gene (*CH5B*), was designed by ligation of the 2.5 kb *EcoRI-ClaI* fragment of pk35CHN-641, provided by Dr. R. Broglie (E.I. DuPont Agricultural Products, Wilmington, DE) (Broglie et al. 1991) into a *EcoRI-ClaI* linearized plasmid pGA492 (An 1987). The vector was transferred into each of the three strains by triparental mating (Turk et al. 1991). All vectors contained the *NPT II* gene as a selectable marker and the CaMV *35S* promoter for constitutive expression.

Transformation procedure. The *Agrobacterium* strains were grown on LB and Minimal media (Turk et al. 1991) with 100 mg·l^{-1} kanamycin, pH 5.4. A single bacterial colony was inoculated into 15 ml of liquid LB medium with 100 mg·l^{-1} kanamycin and incubated at 29°C on a shaker at 120 rpm for 24 h. The bacterial suspension was diluted (1:50) in minimal medium with 100 mg·l^{-1} kanamycin and grown for another 24 h. Prior to infection of explants, the bacteria were rinsed twice in MS medium (pH 5.4) and centrifuged (900 x *g*). The bacterial pellet was suspended in MS medium and adjusted to a concentration of 10^8 cells·ml^{-1} using a haemocytometer. One hour before infection of explants, acetosyringone was added at 100 µM. The petiole explants (150-240 per treatment) were dipped in bacterial suspension for 5 min (each of the three strains was evaluated in separate experiments), and blotted on sterile paper towels. The explants (12 per 100x15 Petri dish) were transferred to cocultivation medium (MS medium with 5.0/5.0 µM 2,4-D/BA, pH 5.4, without antibiotics) and incubated at 27°C in darkness for either 2 or 4 d.

Following cocultivation, explants were rinsed three times in MS medium, with the last rinse containing 500 mg·l^{-1} ampicillin, and blotted on sterile paper towels. The explants were placed on selective medium (MS medium with 5.0/5.0 µM 2,4-D/BA, 50 mg·l^{-1} kanamycin and 500 mg·l^{-1} carbenicillin). The kanamycin concentration of 50 mg·l^{-1} was sufficient to prevent callus growth on non-transformed tissues (authors, unpublished). Dishes were incubated in darkness at 24-26°C for 4 wk. Explants and calli were subcultured to fresh medium 4 to 5 wk after infection. Two controls were included: a positive control (no cocultivation, no antibiotics in the medium) and a negative control (no cocultivation, on selective medium with kanamycin and carbenicillin). After 8 wk, observations were made on the extent of callus formation, diameter, and presence of bacterial ooze. Transformation efficiencies were calculated as the number of explants producing embryogenic calli on kanamycin per total explants plated after cocultivation. To enhance plantlet regeneration frequency, embryogenic calli proliferating after one or two subcultures were transferred to a liquid suspension culture (Raharjo and Punja 1994) containing 1.0/1.0 µM 2,4-D/BA and 50 mg·l^{-1} kanamycin, and shaken continuously (120 rpm) at 24-28°C. The suspension cultures were maintained by subcultures every two wk.

Plantlet regeneration. Embryogenic aggregates of various sizes (1 to 12 mm in diameter) from the suspension culture were rinsed in MS medium and then plated onto solid MS medium with 1.0/1.0 µM 2,4-D/BA or 1.0/1.0 µM NAA/BA, and 50 mg·l^{-1} kanamycin. When shoots developed, they were excised and transferred to MS medium with no growth regulators but with kanamycin, on which they developed roots after 2-3 transfers onto the same medium. These plantlets were transferred to sterile soil and maintained in a growth chamber at 26°C and 16 h·d^{-1} photoperiod.

Confirmation of transformation. Total nucleic acids were extracted from putatively transgenic plants and from untransformed (negative) controls using a procedure modified from Mettler (1987). Leaf tissue (100-300 mg) was cut and frozen in liquid nitrogen and ground to a fine powder in a small mortar. The powder was homogenized in buffer (1% SDS, 0.25 mM glucose, 50 mM NaCl, 20 mM EDTA, and 50 mM Tris, pH 8.0) and transferred to 1.5 ml centrifuge tubes. Following a 30-min incubation at room temperature, the DNA was extracted with an equal volume of TE saturated phenol (pH 8.0). The aqueous phase was repeatedly extracted with chloroform: isoamylalcohol (24:1) (Sevag) until the interface was clear. The genomic DNA was precipitated at -20°C with 0.3 M sodium acetate (pH 5.3) and two volumes of 95% ethanol. The nucleic acids were recovered by centrifugation for 20 min at 16,000 x *g*, at 4°C. The pellet was resuspended and incubated for 2 h at 37°C with 50 µg·ml^{-1} of RNAse. The DNA was then re-extracted with phenol : chloroform : isoamylalcohol (25 : 24 : 1), ethanol precipitated and resuspended in TE buffer. DNA concentrations were estimated by comparing with standards of Herring Sperm DNA.

Two specific primer sequences for the *NPT II* coding region, obtained from Dr. M.M. Moloney (University of Calgary, AB), were used for PCR amplification of this gene in genomic DNAs isolated from putatively transformed plants. Oligomer A, a 17mer with 5'-3' sequence GATGGATTGCACGCAGG, is located 15 bp upstream from the start codon and oligomer B, a 17mer with 5'-3' (bottom strand) sequence GAAGGCGATAGAAGGCG shares identity with the 3' region of the *NPT II* gene, 17 bp 5' of the stop codon. Each PCR reaction (25 µl overlaid by 50 µl of mineral oil) consisted of 1x *Taq* buffer ($MgCl_2$-free), 1 mM $MgCl_2$, 200 µM of dNTPs, 0.5 µM of each oligo-nucleotide primer, and 100-200 ng of template DNA. Each PCR reaction was incubated at 92°C for 5-10 min and 1.25 units of *Taq* polymerase (Promega) was added and quickly placed on ice. The temperature cycling for the PCR was as follows: 29 cycles of 94°C for 1 min, 54°C for 2 min and 72°C for 3 min. The 30th cycle was the same with the exception that DNA extension at 72°C was carried out for 10 min. The PCR products were then analysed by electrophoresis on 2.0% agarose gels. The oligomers were first tested by amplifying the characteristic 800 bp region of the *NPT II* gene by using 10 ng of pMOG196 as positive control template.

Approximately 12 µg of genomic DNA was digested with *HindIII* and the fragments were separated by electrophoresis on 0.7% agarose gels. DNA fragments were transferred from the gels to positively-charged nylon membranes (Boehringer Mannheim, Laval, PQ) by capillary blotting with 20 X SSC and fixed by UV crosslinking. The probe was made by amplification of the 800 bp fragment from the vector which contained the *NPT II* coding region using the PCR conditions specified above, and was labeled with Digoxigenin-UTP. Hybridization was conducted using about 15 ng of the probe per ml of hybridization solution containing 2% blocking buffer (Boehringer Mannheim) and 50% formamide and incubated at 40-42°C for at least 15 h. Hybridization filters were washed and detected using chemi-luminescence according to manufacturer's instructions. The blots were finally exposed to Kodak OMAT-K X-ray film for 24 h.

Young leaves from transgenic cucumber plants were frozen in liquid nitrogen immediately after collection, and finely ground into a powder using a mortar and pestle. All extractions were performed at 4°C. The powder was extracted with 0.1 M sodium citrate buffer (pH 5.0), and the crude homogenate was filtered through four layers of Miracloth and centrifuged at 20,000 x *g* for 30 min. The supernatant was transferred onto an ultrafiltration unit (Amicon YM10 filter) for adjustment of protein concentrations. Protein samples were loaded and separated by SDS-PAGE. The separated samples were transferred to 0.45 µm nitrocellulose membranes at 5 mA·cm^{-2} for 30 min in Bjerrum and Schafer-Nielsen transfer buffer with a Bio-Rad Trans-Blot semi-dry electrophoretic transfer cell as described in the instruction manual. Western blots were performed following the procedure described in the Bio-Rad Immuno-Blot GAR-AP assay kit instruction manual. The antiserum raised against petunia chitinase, provided by Dr. L. Melchers (Mogen International nv, The Netherlands) was used at 1 : 5,000 dilution for assay of transgenic plants with a petunia chitinase transgene. The

antisera raised against tobacco chitinase, provided by Dr. B. Fritig (Institute de Biologie Moleculaire des Plantes, France) and bean chitinase, provided by Dr. R. Broglie (E.I. DuPont Agricultural Products, USA) were used at 1 : 2,000 dilution for assays of transgenic plants expressing tobacco and bean chitinase transgenes, respectively. A quantitative assay of endochitinase activity in transgenic and control plants was conducted using a modified colorimetric method as described by Legrand et al. (1987). In this assay, samples from four transgenic plants transformed using pMOG196 with a tobacco acidic chitinase gene, i.e. plants #2, #14, #19 and #21, and from one control plant, were used.

Results

Development of kanamycin-resistant calli.

Cocultivated petiole segments swelled and developed callus after 3-4 weeks on selective callus initiation medium containing 2,4-D/BA (5.0/5.0 µM), kanamycin (50 mg.l^{-1}) and carbenicillin (500 mg.l^{-1}) (Fig. 1a). Calli which survived this selection step were subcultured to

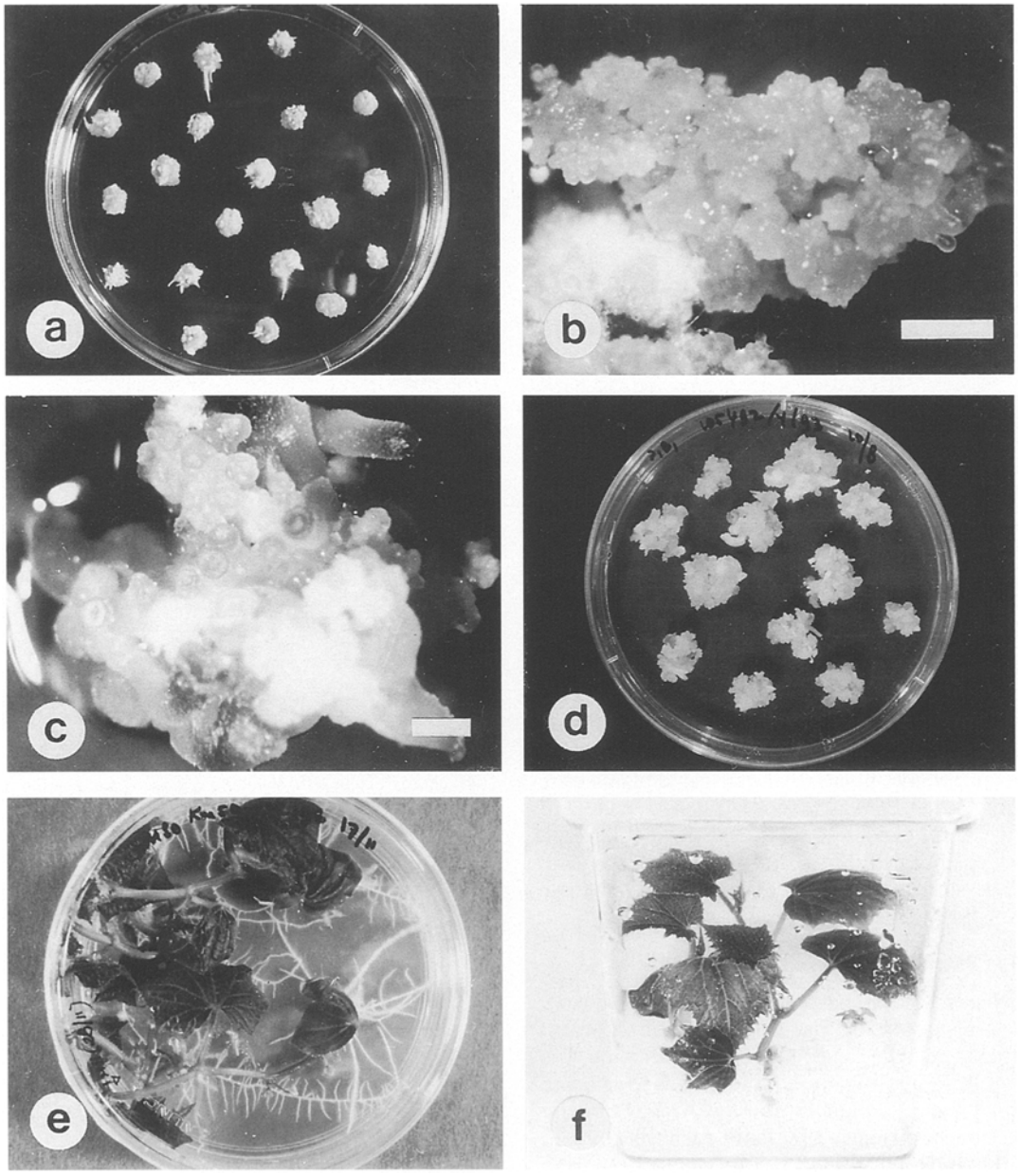

Fig.. 1. Development of transgenic cucumber plants from cocultivated petiole explants. **a)** Calli developing from petiole explants on MS medium with (5.0/5.0 µM) 2,4-D/BA, 50 mg·l^{-1} kanamycin and 500 mg·l^{-1} carbenicillin (selective medium), 4 wk after cocultivation with strain EHA105/pMOG196; **b)** Embryogenic calli development after several subcultures onto fresh selective medium 12 wk after cocultivation; **c)** and **d)** Shoot formation from embryogenic cell suspensions plated onto solid MS medium containing a combination of 1.0/1.0 µM 2,4-D/BA or NAA/BA, with 50 mg·l^{-1} kanamycin; **e)** Plantlets developing roots after 2-3 subcultures on MS0 medium containing 50 mg·l^{-1} kanamycin; **f)** Further development of transgenic plantlets. Bars = 2*mm*.

fresh medium of the same composition where they developed embryogenic (yellow and friable) sectors (Fig. 1b). By comparison, most non-cocultivated explants were bleached and did not develop further. The frequency of embryogenic calli which developed further and grew on kanamycin-containing medium, recorded 8 wk after cocultivation, ranged from 0 to 12%, depending on *Agrobacterium* strain and vector used. The highest frequency (12%) was achieved when strain EHA105 (supervirulent leucinopine) with pMOG196 was used. Strains MOG101 and MOG301 yielded a maximum frequency of around 5% (data not shown). The length of cocultivation, either 2 or 4 d, did not appear to consistently affect the frequency of development of kanamycin-resistant embryogenic calli. No direct shoot formation via embryogenesis or organogenesis was obtained from these calli over a 3-mo period.

Regeneration of plantlets.

The embryogenic sectors from kanamycin-resistant calli were dissected and transferred into liquid medium containing 1.0/1.0 µM 2,4-D/BA, and 50 $mg \cdot l^{-1}$ kanamycin, to initiate the suspension culture. Kanamycin-resistant embryogenic aggregates could be maintained by a subculture every 7-10 d over a 15-mo period (Raharjo and Punja 1994). Embryogenic calli obtained from non-cocultivated explants did not develop further when transferred into the liquid medium with kanamycin. Shoots were obtained by plating kanamycin-resistant embryogenic aggregates onto solid medium containing 1.0/1.0 µM NAA/BA, and 50 $mg \cdot l^{-1}$ kanamycin (Fig. 1c, 1d); the frequency of regeneration was 11-24%. When the shoots were excised after 4-5 wk and transferred onto MS medium without growth regulators and with 50 $mg \cdot l^{-1}$ kanamycin, they elongated and developed roots after two to three subcultures onto the same medium (Fig. 1e, 1f). Upon transfer to soil and maintaining in a growth chamber at 26°C and a photoperiod of 16 $h \cdot d^{-1}$, the rooted plantlets developed into plants. All transgenic plants were obtained using strain EHA105 (supervirulent). A total of 32 plants were obtained following transformation with pMOG196, 9 plants with pMOG198, and 44 plants with pGA492-CHN. Plants transformed using each of the three vectors were all generated from same original callus.

Confirmation of transformation.

DNA was isolated from plants that were regenerated from kanamycin-resistant calli as well as from control (untransformed) plants. Evidence of transformation was confirmed by PCR amplification of the *NPT II* gene using two specific primer sequences for the *NPT II* coding region. Three randomly selected plants, each transformed with one of pMOG196, pMOG198 and pGA492-CHN, and each originating from one transformation event, produced bands of the expected size of 800 bp for the *NPT II* fragment at the same position as the binary vector positive control (pMOG196) (Fig. 2). Southern hybridization following *HindIII* digestion produced a single band for DNA from a plant transformed using EHA105 with pMOG196, two bands for DNA from a plantlet transformed with pMOG198, and no band for DNA from a control plant (Fig. 3). The bands also indicated the minimal copy number of the transgene in these plants.

Nine plants transformed using pMOG196 (acidic chitinase gene) which originated from the same cell suspension culture and two negative controls (untransformed plants) were subjected to Western analysis using an antibody which was raised against the petunia chitinase. The protein, approximately 26 kD, was shown to be present in these 9 transformed plants, but not in the 2 untransformed ones (Fig. 4a). The bands at the bottom of the gel migrated very close to the dye front. They were probably degraded proteins. Western analysis using protein extracted from callus and leaf tissue also showed that plants transformed using pMOG198 and pGA492-CHN expressed the expected tobacco (Fig. 4b) and bean chitinase proteins (Fig. 4c), respectively.

The chitinase activity in transgenic plants expressing the petunia acidic chitinase gene and in control plants was

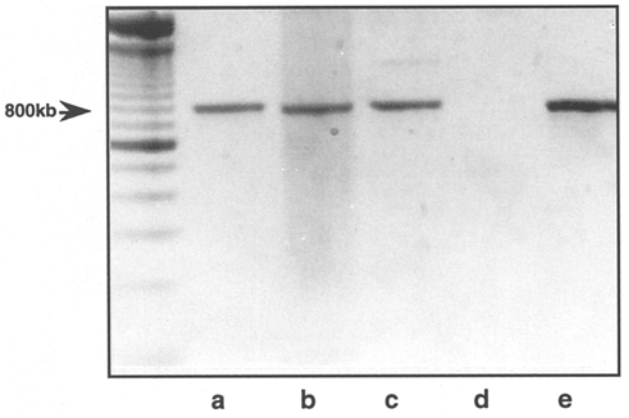

Fig. 2. PCR amplification of the *NPT II* gene from genomic DNA isolated from transgenic plants of EHA105/pMOG196 (lane **a**), EHA105/pMOG198 (lane **b**), EHA105/pGA492-CHN (lane **c**) and negative control (untransformed plant, lane **d**) using two specific primer sequences of the *NPT II* coding region. Lane **e** is the positive vector control.

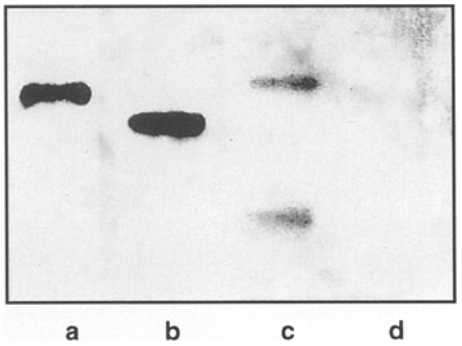

Fig. 3. Southern blot hybridization of *HindIII* restricted DNA (10 µg per lane) from transgenic cucumber plants transformed using EHA105/pMOG196 (lane **b**) and EHA/pMOG198 (lane **c**), and an untransformed (negative) control plant (lane **d**). Lane **a** is the positive control (vector DNA). The filter was hybridized to a 800 bp Digoxigenin-UTP-labelled fragment containing the NPT II coding region. The blot was exposed to X-ray film for 24 h following chemiluminescent detection.

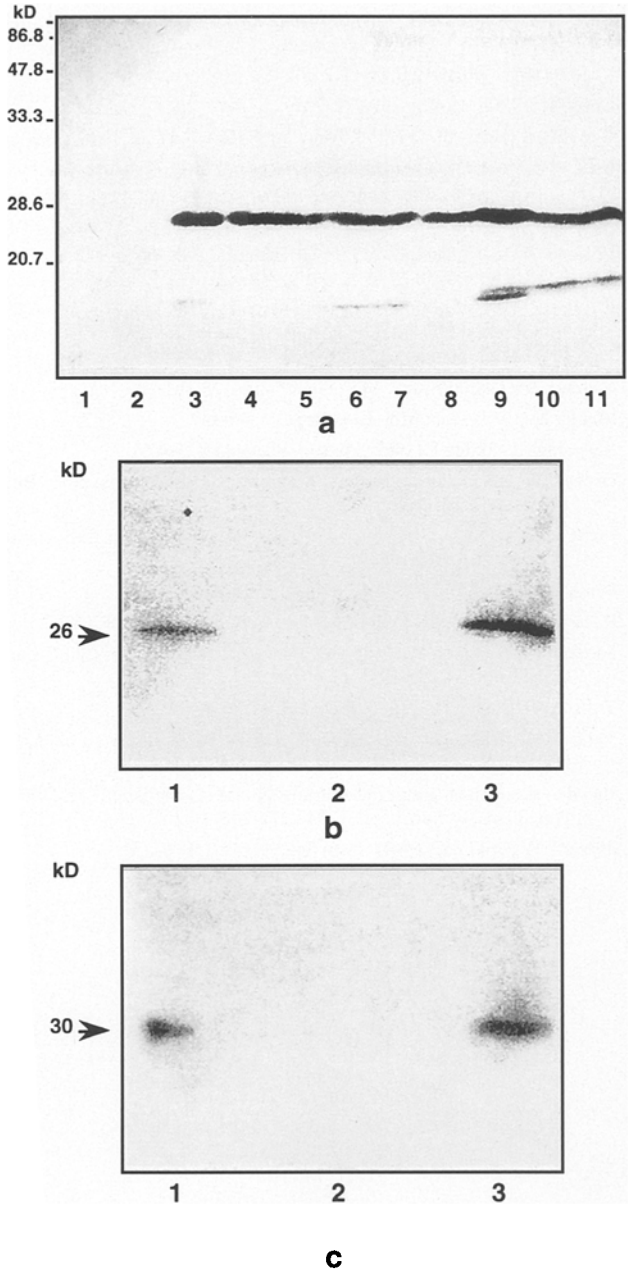

Fig. 4. Western analysis showing expression of heterologous chitinases in transgenic cucumber plants. Samples extracted from leaves were seperated by SDS-PAGE, each probed with an antiserum raised against the respective chitinase. Ten µg protein was loaded per lane. **a)** Transgenic plants (lanes 3-11) transformed using pMOG196 with petunia acidic chitinase gene, all originating from one transformation event; lanes 1-2 are negative controls (untransformed plants); **b)** Transgenic plant transformed using pMOG198 with tobacco basic chitinase gene (lane 3); lane 1 is the positive control (tobacco plant) and lane 2 the negative control (untransformed cucumber plant). **c)** Transgenic plant transformed using pGA492-CHN with bean basic chitinase gene (lane 3); lane 1 is the positive control (bean plant) and lane 2 the negative control (untransformed plant).

measured using a colorimetric assay. There was some variation among the four transgenic plants (Fig. 5); however, plants #2 and #14 showed higher levels of activity when compared to the control.

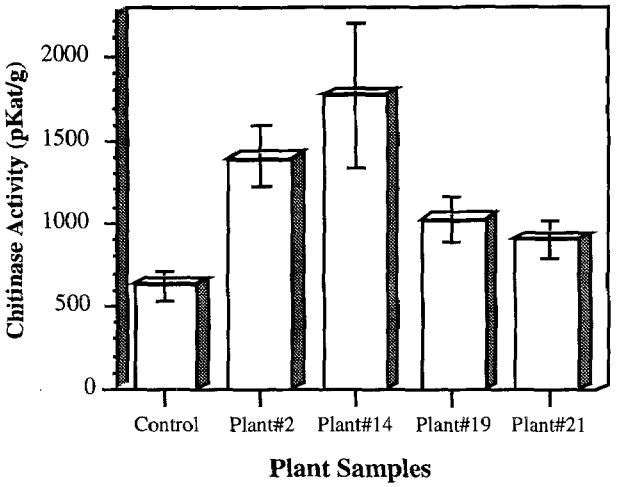

Fig. 5. Total chitinase activity levels in leaves of four transgenic plants following transformation with pMOG196. One Kat is defined as the enzyme activity catalyzing the formation of 1 mol of N-acetyl-D-glucosamine (or its equivalent) per second. Bars represent standard error of the mean (n=6).

Discussion

This work has demonstrated the introduction of three chitinase genes into pickling cucumber and expression of the proteins in the transgenic leaves. Other reports of plant chitinase gene introduction into tobacco, canola (Broglie et al. 1991; Broglie and Broglie 1993), and tomato (van den Elzen et al. 1993), have demonstrated similar results.

In this study, the duration of cocultivation, either 2 or 4 d, did not alter the frequency of kanamycin-resistant callus development, although calli free of *Agrobacterium* contamination were more difficult to obtain after a 4-d cocultivation. Shoot or embryo formation from these calli was not observed even after several subcultures onto fresh selective medium over a 3-mo period. Therefore, the kanamycin-resistant embryogenic calli and cell aggregates were multiplied using a suspension culture system (Raharjo and Punja 1994). The suspension cultures proved to be useful for multiplication of embryogenic aggregates and for production of cell clumps capable of shoot formation when later transferred to MS medium without plant growth regulator (PGR). The frequency of plantlet formation was 11-24%, which was lower than that reported for nontransformed calli using similar plant growth regulators (42-46%) (Raharjo and Punja 1994). Direct shoot regeneration from kanamycin-resistant cucumber calli following *Agrobacterium*-mediated transformation has previously been reported to occur at a lower frequency (Chee 1990a, Sarmento et al. 1992) than that achieved using the suspension culture system in this study.

To prove the integration of the foreign DNA into the cucumber genome, PCR amplification of the *NPTII* gene was conducted and yielded the correct size of the band (800 kb) corresponding to the *NPTII* gene in the vectors. In addition, Southern blot analysis of *HindIII* restriction digests of genomic DNA from leaves of the two transgenic lines (each transformed using pMOG196 or

pMOG198), which was performed using an *NPT II* probe, yielded a hybridizing signal that corresponded to the inserted fragment(s). Hybridization with the probe revealed one or two bands in the respective transgenic lines, indicating the integration of one or two copies of the *NPT II* gene, respectively. Protein extracts from the transgenic plants showed varying but enhanced levels of chitinase activity in leaves compared to the untransformed control. The presence of the heterologous chitinase enzymes was distinguished from background cucumber chitinase levels using antisera corresponding to each chitinase in Western analyses. The results from this study demonstrate the feasibility of transforming pickling cucumber with potentially useful agronomic genes.

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