Expression of a fungal endochitinase gene in transgenic tomato and tobacco results in enhanced tolerance to fungal pathogens

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ABSTRACT

Development of transgenic *Nicotiana tabacum* and *Lycopersicon esculentum* expressing an endochitinase (*ech42*) gene from biocontrol fungus *Trichoderma virens* using *Agrobacterium*-mediated genetic transformation is reported in this paper. Integration of transgene in the genome of transgenic plants was demonstrated using polymerase chain reaction and Southern-blot hybridization, while expression was ascertained by reverse transcription polymerase chain reaction. Histochemical analysis confirmed the expression of GUS enzyme in transformed shoots. Levels of endochitinase enzyme in transgenic plants were found to be up to 10 fold higher compared to control plants. Endochitinase enzyme of 42 kDa was also visualized on SDS-PAGE gel using fluorimetric zymogram in transgenic plants. Endochitinase activity was found to be higher in leaf and stem than the root tissue in transgenic tomato plants. Transgenic lines of both plants showed enhanced resistance to fungal pathogens and a strong negative correlation was found between expression level of endochitinase enzyme and size of disease lesions. Inheritance of transgene, expression and resistance to fungal pathogens of T₁ transgenic tobacco lines was also analysed. The results of the present studies show that *ech42* is a promising candidate gene for developing fungal disease resistance in tomato plants. [Physiol. Mol. Biol. Plants 2010; 16(1) : 39-51] E-mail : eapenhome@yahoo.com/seapen@barc.gov.in.

Keywords : Chitinase, Tobacco, Tomato, Transgenic plants, *Trichoderma virens*

INTRODUCTION

Cultivated tomatoes are susceptible to attack from many fungal pathogens, which results in major losses of yield and deterioration in fruit quality. Major fungal diseases in tomato are Fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*), Verticillium wilt (*Verticillium dahliae*), early blight (*Alternaria solani*) and late blights (*Phytophthora infestans*). Transgenic plants with tolerance to fungal pathogens can be developed by transfer and expression of chitinase from homologous or heterologous sources. Although chitinase genes of plant origin were transferred to tomato (Van den Elzen *et al.*, 1993; Jongedijk *et al.*, 1995; Tabaeizadeh *et al.*, 1999); the resistance is effective only in a narrow range of pathogens and is quantitatively modest, leading to the need for using highly efficient chitinases to

achieve significant levels of plant tolerance. *Trichoderma virens* is an economically important fungus used commercially as a biofungicide (Mukhopadhyay and Mukherjee, 1996; Howell, 2003) and produces different kinds of chitinases (Kim *et al.*, 2002). The 42-kDa endochitinase produced by *Trichoderma* spp. is the most extensively studied chitinase from biocontrol point of view (Viterbo *et al.*, 2002). The involvement of the *T. virens* 42-kDa endochitinase in biocontrol of the devastating plant pathogen *Rhizoctonia solani* has been established by a gene knockout approach (Baek *et al.*, 1999). *Trichoderma* chitinases are strong inhibitors of many important plant pathogens and are able to lyse hard as well as soft structures of fungal mycelium. *Trichoderma* chitinases are more antifungal in nature than other chitinolytic enzymes purified from other sources (i.e. up to 100 times more active than the corresponding plant enzymes and effective on a much wider range of pathogens) and are nontoxic to plants and higher vertebrates even at high concentrations (Lorito *et al.*, 1998). Although there are reports on

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development of transgenic plants with fungal chitinase genes in tobacco and potato (Lorito *et al.*, 1998), grape (Kikkert *et al.*, 2000), broccoli (Mora and Earle, 2001), apple (Bolar *et al.*, 2000), petunia (Esposito *et al.*, 2000), cotton (Emami *et al.*, 2003), there are no reports on transfer of chitinase genes from *Trichoderma* into tomato. In the present paper, we report development of transgenic tobacco and tomato which express an endochitinase gene (*ech42*) from *T. virens*. Transgenic plants with fungal endochitinase gene showed enhanced resistance to infection when challenged by fungal pathogens.

MATERIAL AND METHODS

Construction of the plant expression vector with *ech42* gene

Trichoderma virens (IMI 304061) *ech42* cDNA (GenBank Acc. No. EU035808) coding for the 42-kDa endochitinase was amplified by RT-PCR using the primer pair chitafl (5' ACA TGT TGA GCT TCC TCG GAA ATC 3') and chitr1 (5' GAA TTC AGT TGA GAC CCC TTC CTG AT 3') and cloned in BlueScript KS(+) vector (J. Latha and P.K. Mukherjee, unpublished work). The resulting plasmid was designated as pTv-*ech42*. The *EcoRI* site of pTv-*ech42* was destroyed using Klenow fill-in reaction. For this, pTv-*ech42* was digested with *EcoRI*, sticky *EcoRI* ends were filled with Klenow enzyme and resultant blunt end fragment was self ligated. After destroying the *EcoRI* site, the resulting plasmid was restriction digested with *NcoI-XbaI* to isolate the *ech42* ORF. The *ech42* ORF from *T. virens* was subcloned into pBI525 plasmid using cloning sites *AflIII-XbaI*, which places the gene between the control of double 35S cauliflower mosaic virus (CaMV) promoter that contained alfalfa mosaic virus (AMV) 5' untranslated RNA leader sequence as translation enhancer element and nopaline synthase (*nos*)

terminator. This resulting plasmid was digested with *HindIII-EcoRI* restriction enzymes allowing the release of 5'-35S-35S promoter-AMV-*ech42-nosT*-3'. This fragment was finally cloned into *HindIII-EcoRI* sites of binary vector pCambia1301 (purchased from Cambia, Australia). The resulting vector was named as pCambia-*ech42* (Fig. 1) and introduced into *Agrobacterium tumefaciens* LBA 4404 cells via electroporation.

Tobacco transformation

The expression cassette was initially tested for the functionality and effect on *in planta* expression of *ech42* using tobacco as a model system. *Nicotiana tabacum* cv. Havana 425 obtained from Central Tobacco Research Institute, Rajmundry, India was used for the experiments. Transformation of *N. tabacum* was done according to the method of Horsch *et al.* (1988). Leaf explants of size 10 mm x 10 mm from *in vitro* grown shoot cultures of *N. tabacum* were used for transformation using *Agrobacterium tumefaciens* LBA4404-pCambia-*ech42*. After co-culture, explants were transferred to Murashige and Skoog's (Murashige and Skoog, 1962) basal medium supplemented with benzyl adenine (2.0 mg l⁻¹) and indole acetic acid (0.1 mg l⁻¹) (MSR). The medium was supplemented with 25 mg l⁻¹ hygromycin B for selection of transgenic shoots and 500 mg l⁻¹ of cefotaxime to eliminate the *Agrobacteria*.

Tomato transformation

Seeds of *Lycopersicon esculentum* cv. Arka Alok obtained from Dr. B. S. Konkan Krishi Vidhyapeeth, Dapoli, Dist Ratnagiri, Maharashtra were germinated on moistened cotton under aseptic conditions after sterilization. Cotyledons devoid of cotyledonary nodes (approximately 10 mm in size) were excised from 7-8 day old seedlings and transferred to pre-culture medium

Fig. 1. Linear representation of T-DNA of pCambia-*ech42*, having *ech42* under double 35S promoter, *hph* as selectable marker and *uidA* as reporter gene. For Southern hybridization genomic DNA was digested with *EcoRI* enzyme which gives minimum 5.2 kb band. Size of T-DNA is 7.2 kb. Arrow indicates the region amplified and used as probe.

(MSR) for 3 days with abaxial surface of cotyledon in contact with the medium. Overnight grown *Agrobacterium* cultures were harvested by centrifugation at 4000 g for 15 min and suspended in equal volume of MSR medium, wounded with a sterile needle and exposed to *Agrobacterium* suspension, supplemented with 200 μ M acetosyringone and kept in contact with *Agrobacterium* suspension on a rotary shaker at 70–80 rpm. After this, explants were blotted dry on sterile filter paper and transferred to the same pre-culture medium for 3 days and then transferred to selection medium-MSR containing 7.5 mg l⁻¹ hygromycin B. Explants were sub-cultured at the end of 21 days on the MSR medium containing 7.5 mg l⁻¹ hygromycin B. Putative transformed shoots were selected at the end of 40 days and rooted on MS medium containing hygromycin B (2.5 mg l⁻¹).

A set of non-transformed leaf disc explants of *N. tabacum* and cotyledon explants of *L. esculentum* were also regenerated on MSR medium and shoots rooted on the same medium without any antibiotics and maintained as negative control.

Histochemical GUS assay

Histochemical assay for the reporter gene product was conducted according to the procedure of Jefferson (1987). Three to four days after co-culture, leaf discs of tobacco and cotyledons of tomato were incubated in GUS buffer. Putatively transgenic shoots were also subjected to histochemical GUS assay to detect the stable GUS expression.

Molecular analysis

Total genomic DNA from the leaves of putatively transformed and control plants was isolated and subjected to PCR amplification using the *ech42* gene specific primers EchF (5' GTT GAG CTT CCT CGG AAA ATC 3') and EchR (5' GTT GAG ACC CTT CTT CCT GAT GTT GTC 3'). This primer pair amplified a 1293 bp domain. PCR amplification conditions used were: initial denaturation of 94 °C for 3 min followed by 30 cycles of amplification consisting of 94 °C for 45 sec, 59 °C for 45 sec, 72 °C for 1 min 20 sec followed by final extension at 72 °C for 10 min. The transcription of *ech42* gene in transgenic plants was studied by RT-PCR. One μ g of total RNA was used for cDNA synthesis in a final volume of 20 μ l reaction using random primers (first strand cDNA synthesis kit-Stratagene) according to manufacturer's instructions. Two μ l of sample from first strand reaction mix was used for PCR amplification. For *ech42* amplification

from cDNA EchF' (5' CCG ATC TCA GCT GGA TGC CTA TG 3'; internal primer of gene) and EchR primers were used. PCR amplification conditions used were: initial denaturation of 94 °C for 3 min followed by 35 cycles of amplification consisting of 94 °C for 45 sec, 59 °C for 45 sec, 72 °C for 45 sec followed by final extension at 72 °C for 10 min. For actin (housekeeping gene) actfor (5' GTC TGG TGA TGG TGT TAG C 3') and actrev (5' CCT ATC AGC AAT TCC AGG AAA C 3') primers were used.

Southern blot hybridization was carried out for genomic DNA of control and transformed tomato lines (LE1-LE8). Thirty μ g genomic DNA of putatively transformed tomato lines and control were digested with *Eco*RI enzyme. PCR amplified product of *ech42* from plasmid was used for probe preparation using DIG-DNA labeling kit (Roche Biochemicals, Germany). Pre-hybridization, hybridization, washing and detection were carried out using chemiluminescent detection system as per manufacturer's protocols (Roche Biochemicals, Germany).

Endochitinase activity assay

For assaying the endochitinase expression in transgenic tobacco and tomato, approximately 300 mg leaf tissue from 3 week old cultures was ground in 100 μ l of 0.1 M sodium acetate buffer (pH 5.8) and centrifuged at 9000 g at 4 °C for 2 min. Twenty five μ l of extract was incubated with 5 μ l of 4-methyl umbelliferyl- β -D-N, N', N''-triacylchitotrioside (4 MU- β -(glcNAc)₃, stock 1 mg ml⁻¹) for 20 min at 40 °C and 1.97 ml of 0.2 M Na₂CO₃ was added to stop the reaction. The fluorescence was measured at excitation 350 nm and emission 440 nm in a Perkin Elmer Luminescence Spectrometer LS 50B (Baek *et al.*, 1999). Fluorescence of known concentration of 4-methyl umbelliferon sodium salt was used to plot a standard curve to determine the activity of endochitinase present in the sample tested. Total protein in the samples was measured by Lowry's method (Lowry *et al.*, 1951). The activity was defined as pM 4-MU released min⁻¹ μ g⁻¹ of total protein. All the chemicals used for fluorimetric assay and growth media were purchased from Sigma Chemical Co. and assay was repeated at least twice with three replicates of all samples. Endochitinase activities were also measured in different parts i.e. root, leaf and stem of transgenic tomato plants.

Fluorimetric zymography

For visualization of enzyme activity, fluorimetric zymogram was performed. SDS-PAGE (Laemmli, 1970)

was conducted with 100 µg of total protein isolated from transgenic and control leaves. Samples were prepared after ammonium sulphate precipitation to 90 %, followed by overnight dialysis against 0.1 M sodium phosphate buffer (pH 6.0). Detection of the endochitinase band on the gel was done by fluorescence method as described by Ren *et al.* (2000). After electrophoresis, gel was probed with the substrate 4 MU-β-(glcNAc)₃ and incubated at 37 °C for 15 min and visualized under UV transilluminator.

Bioassay of transgenic tobacco lines for disease resistance

Culture of *B. cinerea* (ITCC No. 6011) was obtained from the Indian Type Culture Collection, Division of Plant Pathology, Indian Agriculture Research Institute, New Delhi, maintained on PDA slants. Third leaf from the top of transformed and control tobacco plants was excised, sterilized with 70 % alcohol for 10–20 sec, washed with sterile water for 2–3 times and placed on moist sterile Whatman no 3 paper in petri plates under sterile conditions in dark. Fungal plugs of 5 mm diameter were cut from culture growing on PDA plates and placed upside down on the leaf. The plates were sealed and incubated at 30 °C. Percentage leaf area infected was calculated after 5 days of infection. In another experiment, one month old hardened plants were spray inoculated with 10⁸ CFU ml⁻¹ of *B. cinerea* culture suspended in 0.85 % NaCl. Plants were kept in dark for initial 24 h and then shifted to light. Disease symptoms and severity were assigned as follows 0= no visible symptoms; 1= slight chlorosis, 0–5 necrotic flecks; 2= chlorosis, 6–10 necrotic flecks; 3= total inoculation site covered with necrotic flecks, more than 10 necrotic flecks; 4= coalescing necrotic flecks, necrotic area expand beyond original inoculation site; 5= 50 % or more leaf area covered with necrotic lesion. Disease severity scores were used to calculate disease index as described by Powell *et al.* (1971). Plants were also inoculated with *Sclerotinia sclerotiorum* under *in vivo* condition, 5 mm agar discs were excised from the region close to the edge from 4 days old culture of *Sclerotinia sclerotiorum* (ITCC No 5482). The agar plugs were placed on upper surface of the leaves of 3 week old hardened transgenic tobacco plants grown in chambers at 25 °C under a 16-h light and 8-h dark period. Inoculated plants were maintained at 25 °C and 95 % relative humidity, favourable for fungal growth. Percentage leaf area with necrosis was calculated after 5 days. Three independent experiments with three replica of each sample were conducted and the data taken after five days were analyzed by Analysis of variance and

Pearson's correlation coefficient (using Microcal™ Origin Pro 6.1).

Screening of transgenic tomato lines for disease resistance

Transgenic tomato lines were challenged with two pathogenic fungi i.e. *Alternaria alternata* (ITCC No. 5582) and *Sclerotinia sclerotiorum*. The crude protein extracted from leaf of transgenic tomato plants was used for *in vitro* inhibitory effect on the *S. sclerotiorum*, while the protein from non-transgenic plant and protein extract buffer served as controls. A fungal plug from the margin of actively growing fungal plates were taken and kept in the centre of each plate. Plates were incubated overnight at 25 °C and 80–90 % humidity in the dark. After 1 day, two wells were made in PDA plates and wells were filled with 100 µl protein extract containing 80–100 µg total protein of each sample. The observations were recorded in each sample.

For detached leaf assay, cultures of *A. alternata* (ITCC No. 5582) were grown on PDA plates at 30 °C. When the fungal mycelia reached the edge of the plate, 5.0 mm diameter agar plug with mycelium was removed from a region close to the edge and used for inoculations. Healthy and young leaves were collected from 1 month old hardened control and transgenic tomato plants and placed on wet Whatmann paper in Petri dishes. Agar plugs containing *A. alternata* cultures were placed directly on the adaxial side of the leaves and incubated in the dark and moist conditions. Leaves were observed daily and after two weeks, percentage of leaf area with necrosis was determined as described for detached leaf assay in tobacco.

Analysis of T₁ generation of transgenic tobacco lines

To investigate the inheritance of hygromycin resistant trait, six independent lines were selected and self pollinated. Seeds were harvested and germinated on MS medium for one month. Almost 95 % seeds were found viable. One month old germinated seedlings were then transferred to MS medium containing hygromycin B (25 mg l⁻¹). After 4 weeks, number of hygromycin resistant seedlings with well developed roots and leaves which were clearly distinguished from the sensitive seedling with pale green cotyledons and less developed root system were counted. At 25 mg l⁻¹ concentration of hygromycin B, T₁ generation seedlings of non-transformed plants were found sensitive. Leaf tissue of PCR confirmed T₁ generation plants from each of the four T₀ lines (highly expressing endochitinase) were assayed for the expression of *ech42* gene using

endochitinase activity assay as described above. These T_1 plants were multiplied on MSR medium containing hygromycin B (25 mg l^{-1}). The regenerated shoots were transferred to rooting medium and well rooted shoots were transferred to pots. The transgenic plants were challenged with *B.cinerea* and *S. sclerotiorum* in the same way as described for T_0 plants.

RESULTS

Production of transgenic tobacco and tomato plants and their molecular characterization

After transformation by *Agrobacterium*-mediated co-culture method, transgenic plants were selected on medium containing hygromycin B, since transgenic plants would have *hph* gene conferring resistance to hygromycin B. Histochemical assay of GUS enzyme when checked, putatively transgenic shoots showed the presence of blue coloration (Fig. 2), while the control leaf explants did not show any color. PCR amplification using specific primers for *ech42* gene showed amplification of expected size of 1.3 kb (Figure not shown). RT-PCR results showed that in PCR confirmed transgenic plants *ech42* gene is being transcribed (Fig. 3a, b). Results of Southern hybridization of genomic DNA of putatively transgenic tomato plants suggested that there is at least single copy insertion in the genome of all transgenic tomato lines (Fig. 4). The minimum band size that can be obtained is 5.3 kb, as there is single *EcoRI* site in the T-DNA of pCAMBIA-*ech42*. *EcoRI* and *HindIII* digested fragment of pCAMBIA-*ech42* having 'double 35S promoter-*ech42*-AMV-nos T' of 2.2 kb size was used as positive control. There was

Fig. 2. Histochemical GUS assay. Stable GUS expression in regenerating tomato shoots after co-culture with *A. tumefaciens*

no signal found in control plants which suggest the absence of homologous DNA sequence in the genome of control plants. The results of PCR, RT-PCR and Southern blot hybridization demonstrated that *ech42* gene integrated into the genome of transgenic lines of tomato and tobacco and expressed

Endochitinase activity assay

Endochitinase activity in the leaves of transgenic and control plants when measured by fluorimetric method, was found to be 2-10 fold higher in transgenic plants as compared to control tobacco plants (Fig. 5). Out of the thirty putatively transgenic tomato shoots, eight showed

Fig. 3. RT-PCR amplification of *ech42* mRNA amplified with primer *chitfor* and *chitrev*. Lane 1, 100 bp ladder; lane 2, control plant; lane 3-7, Transformed tobacco lines NT1-NT6 (a) lane 3-11; transformed tomato lines LE1-LE8 (b); lower panels actin as loading control (450 bp).

Fig. 4. Southern blot hybridization of genomic DNA of transgenic tomato lines LE1-LE8 digested with *EcoRI* enzyme.

the high expression (6-11 times as compared to control) and these plants were selected for further analysis (Fig. 6). When endochitinase activities were measured in different parts of transgenic tomato lines, it was observed that the transgene *ech42* expressed at higher level in leaves compared to stem and roots. As concluded from table 1, in the leaf tissue, average activity in all transgenic is 8.43 times, in stem 7.68 times and in root 2.53 times higher as compared to control.

Fluorimetric zymogram

Total plant proteins of confirmed tobacco and tomato transgenic lines were separated by polyacrylamide gel electrophoresis in the presence of SDS. After renaturation, the enzyme was probed with the

Fig. 5. Endochitinase activity and resistance of transgenic tobacco plants against *Botrytis cinerea*. A Endochitinase activity and average percentage leaf area infected in control and transformed tobacco lines NT1-NT6. Error bars indicate standard error of means. The value for the transgenic line is significantly different from the control plants at $P < 0.05$ (*) and $P < 0.01$ (**). Mean separation was performed using the method of Tukey. The number above the data point in linear graph showed the endochitinase activity compared to control.

fluorogenic substrate 4-methyl umbelliferyl- β -D-N, N', N''-triacetylchitotriose (4 MU- β -(glcNAc)₃). When the gel was probed with the fluorogenic substrate, fluorescence of 42 kDa was detected under UV transilluminator (Fig. 7 a, b).

Table 1. Endochitinase activities in different tissue of transgenic tomato lines.

Lines	Leaf $\pm$ SE**	Average activity*	Stem $\pm$ SE**	Average activity*	Root $\pm$ SE**	Average activity*
Control	47.27 $\pm$ 19.8		50.78 $\pm$ 7.65		40.67 $\pm$ 5.67	
LE1	328.67 $\pm$ 7.86	8.43 x	312.78 $\pm$ 1.13	7.68 x	78.19 $\pm$ 1.35	2.53 x
LE2	253.40 $\pm$ 5.52		218.97 $\pm$ 2.34		50.12 $\pm$ 5.64	
LE3	351.20 $\pm$ 7.61		320.67 $\pm$ 6.57		76.56 $\pm$ 9.87	
LE4	564.30 $\pm$ 2.34		520.98 $\pm$ 8.97		129.87 $\pm$ 1.52	
LE5	453.60 $\pm$ 6.57		450.67 $\pm$ 8.97		110.65 $\pm$ 9.8	
LE6	490.34 $\pm$ 6.66		467.87 $\pm$ 9.87		116.87 $\pm$ 1.52	
LE7	435.67 $\pm$ 7.68		429.43 $\pm$ 7.68		98.76 $\pm$ 5.43	
LE8	312.40 $\pm$ 5.67		400.78 $\pm$ 5.64		105.65 $\pm$ 7.86	

*Average activity of all transgenic lines compared to control plants.

**Endochitinase activity in pmol MU released min⁻¹ μ g⁻¹ of total protein.

Fig. 6. Endochitinase activities of transgenic tomato lines LE1-LE8. The number above the bar shows the higher endochitinase activity compared to control tomato plants.

Bioassay of transgenic tobacco plants

In detached leaf assay, transgenic lines showed reduction in lesion diameter compared to control plants. The analysis of variance for the percentage leaf area infected in control and transformed tobacco lines indicated that resistance to fungal infection was significant in NT1, NT2, NT4, NT5 and NT6 at $P < 0.05$. However, at $P < 0.01$ NT1 line was found to be not significant (Fig. 8a, b). Percentage leaf area infected in control plants observed was $42.09 \pm 2.7 \text{ mm}^2$, while in transgenic lines it ranged from 4.65 ± 2.08 to $18.15 \pm 8.1 \text{ mm}^2$ showing 14-89 % reduction as compared to control plants. When these lines were compared for their endochitinase expression

Fig. 7. Fluorimetric gel based detection of 42 kDa endochitinase from *T. vires* in transgenic lines lanes M- protein marker (Sigma); C- control plants; 1, 2, 3, 4- transgenic tobacco lines NT2, NT4, NT5 and NT6 respectively (a) and tomato lines (b) Lanes 1-8 lines LE1-LE8. Marker lane was cut and overlapped with the zymogram.

Fig. 8. (a) Resistance of transgenic tobacco lines to *B. cinerea* infection. Each bar represents mean disease index $\pm$ SE. The value for the transgenic lines is significantly different from the control value at $P < 0.01$ (*). (b) Left: non-transgenic and right: transgenic tobacco leaves inoculated with *B. cinerea*.

Fig. 9. (a) Resistance of transgenic tobacco lines to *S. sclerotiorum* infection. Each bar represents percentage leaf area infection on single leaf. The numbers above each set of bar represent mean of percentage lesion area ± SE. The value for the transgenic lines is significantly different from the control value at $P < 0.05$ (*) and $P < 0.01$ (**). (b) Left: non-transgenic and right: transgenic tobacco leaves inoculated with *S. sclerotiorum*.

level and resistance to pathogen, a strong negative correlation between endochitinase activity and percentage of leaf area infected was observed ($R = -0.83$) in all transgenic lines, which suggests that there is significant reduction in the amount of disease with increase in endochitinase activity. In case of *in vivo* bioassay with *Botrytis cinerea* also, transformed lines showed fewer amounts of disease lesions. The mean disease index value for control plants was 91.25 ± 0.94 and in transformed plants was from 37.59 ± 5.77 to 74.10 ± 2.40 . A significant ($p < 0.01$) reduction in disease index value was observed in transgenic lines except NT3 (Fig. 8 a,b). Transformed lines NT2, NT4, NT5 and NT6 showed significant ($P < 0.05$) reduction in percentage leaf area infected as compared to non-

transformed plants in *in vivo* fungal infection assay with *S. sclerotiorum*. However, at $P < 0.01$ NT2 and NT4 were not significantly different in showing resistance to fungal pathogen as compared to non-transformed plants. The average percentage leaf area infected in transformed lines was $16.925 \pm 3.12 \text{ mm}^2$ and in control plants $35.02 \pm 5.22 \text{ mm}^2$ (Fig. 9) i.e. 25-75 % reduction in infection of transgenic lines.

Bioassay of transgenic tomato lines

Few highly expressing, independent transgenic tomato lines LE1, LE2, LE4 and LE5 were assayed for their efficacy in inhibiting the growth of *S. sclerotiorum* under *in vitro* condition. Leaf extract of transgenic lines tested showed inhibition of fungal mycelium and a clear

Fig. 10. *In vitro* inhibitory activity of leaf extract obtained from transgenic tomato lines after 5 days of incubation. The numbers on the plates represent the line number, C- control plant, B- buffer as blank control.

inhibition zone was visible (Fig. 10), while no inhibition was detected in the presence of buffer as well as in control leaf extract. The assay indicated the inhibitory effect was due to the presence of endochitinase in the sample tested, which inhibited the hyphal growth. Table 2 shows the results of inhibitory assay. When transgenic tomato lines (LE1-LE8), which were expressing high endochitinase levels, were challenged with the pathogenic fungus *Alternaria alternata* using detached leaf assay, they showed significant reduction in lesion diameter as compared to control plants. The analysis of variance for the percentage leaf area infected in control and transformed tomato lines indicated that resistance to fungal infection was significantly higher in all transgenic lines at $p < 0.01$ (Fig. 11). Percentage leaf

Table 2. *In vitro* growth inhibitory assay of leaf extracts obtained from transgenic tomato lines.

Sample	diameter of Inhibition zone in mm $\pm$ SE
Control	0
LE1	5.0 $\pm$ 0.1
LE2	3.9 $\pm$ 0.1
LE4	2.53 $\pm$ 0.47
LE5	2.9 $\pm$ 0.05

area infected in control plants observed was 41.17 ± 0.45 mm², while in transgenic lines it ranged from 12.22 ± 2.34 – 21.42 ± 5.67 mm², showing 47.9–70.31 % reduction in transgenic lines as compared to control plants. When these lines were compared for their endochitinase expression level and resistance to pathogen, a strong negative correlation between endochitinase activity and percentage of leaf area

Table 3. Inheritance of hygromycin resistance (*hph*) in T₁ of transgenic tobacco lines transformed by *Agrobacterium* LBA4404 harboring a pCAMBIA-ech42 in which *hph* is present as selectable marker gene.

Line	Resistant seedlings	Sensitive seedlings	χ^2 test (3:1)
Non-transformed	0	120	-
NT1	110	40	0.05
NT2	98	34	0.014
NT3	140	50	0.04
NT4	130	40	0.049
NT5	80	25	1.562
NT6	60	23	0.081

Fig. 11. (a) Endochitinase activity and average lesion diameter in control and transformed tomato lines LE1-LE8 inoculated with *Alternaria alternata*. Error bars indicate standard error of means. The value for the all transgenic line is significantly different from the control plant at $p < 0.01$. Mean separation was performed using the method of Tukey. (b) Left: non-transgenic and right: transgenic tobacco leaves inoculated with *A. alternata*.

Table 4. Fluorimetric assay for the expression level of *T. virens* endochitinase *ech42* in T_1 generation of transgenic tobacco lines.

T_1 plants of transgenic tobacco lines	Average endochitinase activity (pmol MU released min ⁻¹ μ g ⁻¹ of total protein) $\pm$ SE	Fold increase over non-transformed plant
control T_1	40.89 $\pm$ 0.51	1
10 T_1 # NT2	262.31 $\pm$ 0.03	6.6
10 T_1 # NT4	270.18 $\pm$ 0.39	6.7
10 T_1 # NT5	305.60 $\pm$ 0.24	7.47
10 T_1 # NT6	369.382 $\pm$ 0.9	9.01

MU-4 methyl umbelliferron

infected was observed ($R = -0.93$) in transgenic tomato lines.

Analysis of T_1 tobacco lines

At 25 mg l⁻¹ of hygromycin of tobacco the seedlings which remained green with well developed roots were classified as hygromycin resistant and easily distinguishable from pale seedlings with poor roots, which were classified as hygromycin sensitive. Table 3 shows the inheritance of hygromycin resistant trait in T_1 generation. Segregation analysis with the T_1 generation showed a Mendelian ratio of 3:1, which was found in all the lines. At $P < 0.05$, $n = 1$ all values fit into the hypothesis that the transgene segregated in Mendelian ratio of 3:1 and integration was at single

Fig. 12. Resistance of T_1 of transgenic tobacco lines NT2, NT4, NT5 and NT6 to *B. cinerea* infection. Each bar represents mean disease index $\pm$ SE. The value for the transgenic lines is significantly different from the control value at $P < 0.01$ (*).

site. Fluorimetric assay of T_1 generation indicated that endochitinase activity in T_1 tobacco plants is almost similar to their T_0 parental plant (Table 4). T_1 plants NT2 and NT4 lines showed average 6.5 times more activity as compared to non-transformed plants. T_1 plants of NT5 lines showed the average of 7.4 times and NT6 showed almost 9.1 times higher activity as compared to non-transformed plants. In case of bioassay with *Botrytis cinerea*, T_1 plants showed fewer disease lesions. A significant ($p < 0.01$) reduction in disease index value was observed in T_1 of all four transgenic lines of *N. tabacum* (Fig. 12). *In vivo* fungal infection assay of T_1 generation with *S. sclerotiorum* showed that there was significant reduction (38 % - 74 %) in percentage leaf area infected in T_1 generation of transgenic lines NT4, NT5 and NT6 except NT2 as compared to control plants at $p < 0.01$ level (Fig. 13).

DISCUSSION

Chitinases are capable of degrading chitin -a linear polymer of β -1, 4-N-acetyl-o-glucosamine residues, which constitutes 3-60 % of the cell wall of many phytopathogenic fungi (Collinge *et al.*, 1993). In the present study, transgenic plants of tobacco and tomato were found to be absolutely normal in their phenotype. There are reports that expression of *T. harzianum* endochitinase resulted in abnormal phenotype of plants (Bolar *et al.*, 2000). However, in the present study, none of the transgenic plants of tobacco and tomato, expressing endochitinase showed morphological

Fig. 13. Resistance of T_1 of transgenic tobacco lines to *S. sclerotiorum* infection. Each bar represents percentage leaf area infection on single leaf. The numbers above each set of bar represent mean of percentage lesion area $\pm$ SE. The value for the transgenic lines is significantly different from the control value at $P < 0.01$ (*).

abnormalities. This result is in agreement with the earlier reports (Lorito *et al.*, 1998; Mora and Earle, 2001; Emani *et al.*, 2003), where endochitinase expression did not adversely affect the plant phenotype.

Results of RT-PCR confirmed that the gene was being transcribed in the transgenic plant. In a separate experiment, only the total RNA sample without reverse transcription was subjected to analysis by PCR using the same primers. As expected, no product was obtained, confirming that there was no DNA contamination in the RNA (data not shown) and therefore the PCR product observed by RT-PCR were solely due to the presence of the foreign gene transcript.

Ren *et al.* (2000) reported that the endochitinase from bacterium *Pseudomonas luminiscense* can hydrolyse the substrate 4 MU- β -(glcNAc)₃ to 100 %, while exochitinase can hydrolyse only 13 %. With the help of fluorimetric zymography, it is possible to detect endochitinase activity in the crude plant extract. The results of fluorimetric assay, obtained in the present study were compared to control plants to eliminate any error, which could have resulted from the presence of exochitinase/endochitinase in the sample. The 42 kDa enzyme was identified by fluorimetric zymogram, where endochitinase hydrolysed the substrate, resulting in the release of MU and strong fluorescence was observed where the enzyme was present.

In the present study, transgenic lines of tobacco and tomato showed different levels of endochitinase expression, which varied from 4–11 times, as compared to control plants. Difference in expression level was also found in different tissues such as in root, stem and leaf of the same plant. In the present study, *ech42* gene is driven by CaMV 35S promoter, which is a strong and constitutive promoter, which is in agreement of the earlier report by Emani *et al.* (2003). In plant tissue, expression of gene, which is under 35S promoter is subjected to several regulatory elements and may reflect differential levels of expression in different organs of the same plant (Benefy and Chua, 1990). It has also been reported that 35S promoter can confer different developmental and tissue specific expression pattern (Benefy *et al.*, 1989; Sunilkumar *et al.*, 2002).

Southern blot hybridization using genomic DNA of transgenic tomato plants showed single copy integration in all highly expressing lines. Result of Southern blot hybridization in the present study is in agreement with the previous report by Emani *et al.* (2003), where all the highly expressing lines showed single copy

integration of transgene. There are reports that integration of two or more copies of transgene may sometimes lead to gene silencing (Tang *et al.*, 2006).

We have observed good level of resistance in transgenic lines of tobacco and tomato to the fungal infection in both *in vivo* and *in vitro* assay. The pathogens used in this study are not typical pathogens of tobacco, but cause necrotic lesions on the leaves. *B. cinerea* is a fungal pathogen that infects a wide range of hosts (Choquer *et al.*, 2007), affecting different organs including leaves, stems, flowers and fruits (Hoeberichts *et al.*, 2003; Lorbiecke and Sauter, 1998). Early blight is a major fungal disease in tomato and is caused by *Alternaria solani*. The pathogens used in the present study have been widely used in the disease resistance assay (Terakawa *et al.*, 1997; Chakrabarti *et al.*, 2003; Jang *et al.* 2006). Transgenic plants with endochitinase gene from *T. harzianum* have shown enhanced resistance to *B. cinerea* and *A. alternata* (Lorito *et al.*, 1998). Transgenic broccoli plants also showed moderate level of disease resistance (Mora and Earle, 2001), Transgenic apple showed reduced disease severity from 0–99.7 % (number of lesions) or 0–90 % (percentage leaf area infected) (Bolar *et al.*, 2000). Emani *et al.* (2003) have also reported significant resistance in transgenic tobacco and cotton plants transformed with *ech42* gene from *T. virens*. Endochitinase degrades the fungal chitin by hydrolyzing the glycosidic bond between chitin monomers and in another mode of function, the chitin residues released by the action of chitinase act as the elicitor molecules and induce the plant defense mechanism, which include increased lignifications, phytoalexin synthesis, generation of reactive oxygen species and expression of PR-proteins. The results obtained in the bioassay suggest that the resistance of transgenic lines to fungal infection was due to the expression of *T. virens* endochitinase gene in the leaf of transgenic plants, as they showed enhanced resistance to fungal pathogens compared to control plants. All the bioassays were done in the control condition or in fact in those conditions, which are favourable for fungal growth. In bioassay, actively growing mycelia were used which provide the stringent conditions for screening of resistance or susceptibility of transgenic lines towards fungal pathogen. The method used can be said to be superior to the other bioassay methods where spore suspension has been used. The disease developed slightly slower in the transgenic lines (present study) as compared to control plants. This delay in symptom development could potentially allow plants carrying *ech42* in their genome to recover or adapt to changes in

field conditions that could potentially reduce disease spread. Since the method closely mimics the condition very similar to the soil by providing a constant pressure of pathogen infection, the plants surviving the challenge may equally perform in the field conditions.

NT3 line, which was expressing more endochitinase showed less resistance as compared to other transgenic tobacco lines. The behavior of NT3 line is interesting. The probable reason behind this is the integration of the gene in areas, where some of the native defense response genes may have been affected, resulting in the neutralization of enhanced expression of endochitinase enzyme (Chakrabarti *et al.*, 2003). It is necessary to do further work to find out why NT3 line is having less tolerance to phytopathogens.

From the inheritance and segregation ratio studies of tobacco it is concluded that (i) the hygromycin resistant gene is transferred to the T₁ generation. (ii) normal single gene Mendelian inheritance of *hph* gene has occurred in the progeny of transformed tobacco plants. Our report of normal Mendelian inheritance of antibiotic resistance gene is an addition to the growing literature such as petunia transformed with pMON120 type vectors (see Horsch *et al.*, 1983), in tomato transformed with pBin19 carrying the *nptII* gene (Cortina and Culianez-Macia, 2004), in peanut transformed with pBI121 carrying *nptII* gene (Cheng *et al.*, 1997), in rice transformed with *hpt*, *bar*, *gna* and *gusA* genes (Gahakwa *et al.*, 2000) and in barley plants transformed with *bar* gene (Bregitzer and Tonks, 2003). Transgene inherited in Mendelian ratio (present study) showed that there is single copy insertion in transgenic plants of tobacco. Alongwith *hph* gene, *ech42* gene and *gusA* gene were detected using PCR in T₁ plants of tobacco, which indicate that they are tightly linked with *hph* locus. The same level of endochitinase activity was measured in T₁ generation as in T₀ generation. The importance of monitoring the same level of endochitinase in different generations is to ensure the stability of expression of transgene over generations. Differences in expression level over generations have been reported in several plant species (Caligari *et al.*, 1993; James *et al.*, 2002; Vain *et al.*, 2002). The results indicate that transgenic tobacco plants obtained in this study are stable in T₁ generation. The significantly lower percentage of leaf area showing necrotic symptoms in transgenic leaves of the T₁ tobacco lines indicated that the expression of endochitinase in transgenic plants gave protection towards fungal infection. In the present study resistance of T₁ generation is more pronounced to *B. cinerea* than *S. sclerotiorum*. The possible reason behind

this is that some of the T₁ generation plants used in this study are probably homozygous and have enhanced endochitinase activity resulting in more inhibition of fungal infection in the T₁. The results of the present studies using transgenic plants of tobacco and tomato show that endochitinase gene (*ech42*) is a promising candidate gene for developing tolerance to phytopathogens in plants.

ACKNOWLEDGMENTS

MS is grateful to the CSIR, New Delhi for a research fellowship.

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