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Agrobacterium-mediated transformation of cotton (*Gossypium hirsutum*) using a heterologous bean chitinase gene

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Abstract

Cotton (*Gossypium hirsutum* L., var. Coker 312) hypocotyl explants were transformed with three strains of *Agrobacterium tumefaciens*, LBA4404, EHA101 and C58, each harboring the recombinant binary vector pBI121 containing the *chi* gene insert and neomycin phosphotransferase (*nptII*) gene, as selectable marker. Inoculated tissue sections were placed onto cotton co-cultivation medium. Transformed calli were selected on MS medium containing 50 mg l^{-1} kanamycin and 200 mg l^{-1} cepotaxime. Putative calli were subsequently regenerated into cotton plantlets expressing both the kanamycin resistance gene and β-glucuronidase (*gus*) as a reporter gene. Polymerase chain reaction was used to confirm the integration of *chi* and *nptII* transgenes in the T_1 plants genome. Integration of *chi* gene into the genome of putative transgenic was further confirmed by Southern blot analysis. 'Western' immunoblot analysis of leaves isolated from T_0 transformants and progeny plants (T_1) revealed the presence of an immunoreactive band with MW of approximately 31 kDa in transgenic cotton lines using anti-chitinase-I polyclonal anti-serum. Untransformed control and one transgenic line did not show such an immunoreactive band. Chitinase specific activity in leaf tissues of transgenic lines was several folds greater than that of untransformed cotton. Crude leaf extracts from transgenic lines showed *in vitro* inhibitory activity against *Verticillium dahliae*. Transgenic plants currently growing in a greenhouse and will be bioassayed for improved resistance against *V. dahlia* the causal against of verticilliosis in cotton.

Abbreviations: B_5 vitamins – Gamborg et al. (1968) vitamins; CaMV – cauliflower mosaic virus; *chi* – chitinase; 2,4-D – 2,4-dichlorophenoxyacetic acid; GUS – β-glucuronidase; MS – murashige and shoog (1962); NAGA – *N*-acetyl glucosamine; *nptII* – neomycin phosphotransferase gene; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Cotton is one of the most important fiber crops with a raw material value of $5.5 billion per year worldwide. In recent years, genetic engineering of plants has resulted in the development of agricultural crops with desirable agronomic traits such as increased resistance to pests, pathogens, herbicides, and fiber-quality modification (John and Keller, 1996, Wilkins et al., 2000). Verticillium wilt, caused by *Verticillium dahliae*, is one of several biotic factors limiting yield under certain environmental conditions. Further, soil-borne fungal diseases are less amenable to chemical

control. *Gossypium* spp. are known to possess genes for resistance to fungal pathogens. Transfer of these resistance genes to *G. hirsutum* cultivars through conventional methods may take several years and it is rather difficult to breed disease-resistant cotton of commercial value from inter-specific hybrids (Borole et al., 2000). In recent years, the possibility of transforming plants with genes encoding pathogenesis-related proteins such as β-1,3-glucanase and chitinase, which have been implicated in plant disease resistance to fungal infection, has been explored.

Chitinases such as poly[1,4-*N*-acetyl-β-D-glucosaminid]glycan hydrolase (EC 3.2.1.14) are low molecular weight pathogenesis-related (PR) proteins that are often extracellular, acid soluble and protease resistant (Collinge et al., 1993, Graham and Sticklen, 1994; Neuhaus, 1999). They catalyze hydrolysis of β-1,4 linkages of *N*-acetyl-D-glucosamine chitin polymer of fungal mycelial walls into *N*-acetyl glucosamine oligomers. Both infectious agents and abiotic stress can cause induction of plant chitinases (Yun et al., 1997). Chitinases possess anti-fungal activity that causes *in vitro* lysis of hyphal tips as well as inhibition of spore germination in *Alternaria*, *Fusarium* and *Trichoderma* (Schlumbaum et al., 1986; Mauch et al., 1988; Sela-Buurlage et al., 1993; Schickler and Chet, 1997). Enhanced resistance to fungal diseases in plants has been attributed to induce chitinase activity during incompatible interactions (Boller et al., 1983; San et al., 1992). Several laboratories have been able to transfer plant- or microbial-derived chitinase genes into plants and develop transgenic crops with enhanced resistance to fungal diseases. These include transgenic tobacco and canola (Broglie et al., 1991; Terakawa et al., 1997), pickling cucumber (Raharjo et al., 1990), rice (Nishizawa et al., 1999; Kishimoto et al., 2002), grapevine (Yamamoto et al., 2000), peanut (Rohini and Sankara, 2000) and cotton (Emani et al., 2003).

Cotton is a major cash crop in north-eastern Iran in proximity to the Caspian sea region. Verticilliosis is a vascular fungal disease caused by *Verticillium dahliae* and is considered as a major wilt disease in cotton growing areas in Golestan Province. The objective of this study was to transform cotton var. Coker 312 with a heterologous bean chitinase gene using *Agrobacterium* system for enhancing resistance to verticilliosis. Constitutive expression and subsequent elevation of chitinase levels in transgenic cotton can increase resistance to root-invading vascular pathogens.

Materials and methods

Plasmid construction

DNA manipulations were carried out according to the standard procedures (Sambrook and Russell, 2001). A 1.0 kb bean chitinase gene from pCH18 (Broglie et al., 1991) was subcloned into the *Eco* RI site of *lac*Z operon in pGEM-7Zf(+) (Promega), as an intermediate cloning vector, followed by transformation of *E. coli* strain DH5α and screening for *lac*Z$^-$ transformants. The insert was digested with two different restriction enzymes, *Xba*I and *Sac*I, and subsequently ligated into the homologous sites in pBI121 (Clontech, Washington, DC) to yield a recombinant pBI121-BCH. The chitinase gene expression was under the constitutive CaMV35S promoter control (Figure 1). The plasmid was transformed into competent cells of *Agrobacterium* strains by freeze–thaw method (An, 1987).

Plant material and transformation procedures

Seeds of *G. hirsutum* var. Coker 312 were provided by the Cotton Research Institute of Iran, Gorgan, Golestan province. Cotton hypocotyl pieces (0.5 cm^2 surface area) obtained from 7 to 10 day-old sterile seedlings were dipped in *Agrobacterium* suspension cells grown to the late log phase

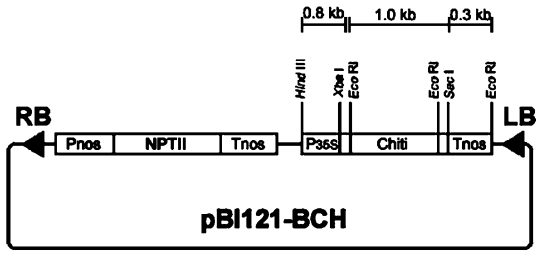

Figure 1. Chimeric gene map of the recombinant binary vector pBI121-BCH carrying the bean chitinase gene and *npt*II gene driven by CaMV35S promoter (P35S). LB, left border; RB, right border; *nptII*, neomycin phosphotransferase; *chi*, chitinase; Pnos, nopaline synthase promoter; Tnos, nopaline synthase terminator.

($OD_{600nm} = 0.6–0.8$). Explants were gently shaken in the bacterial suspension for 5 s to ensure contact of all hypocotyl edges with bacterial cells. The hypocotyl pieces were blot dried and placed on Whatman No. 1 filter paper and subsequently transferred to the co-cultivation MS (Murashige and Skoog, 1962) medium for 2 days at 26 ± 2 °C the dark (Firoozabady et al., 1987). After co-culture, hypocotyl pieces were transferred to callus induction medium (MS1) supplemented with MS salts, B_5 vitamin, 0.75 g l^{-1} $MgCl_2$, 30 g l^{-1} glucose, 0.46 μM kinetin, 0.45 μM of 2,4-D, pH 5.9, solidified with 0.2% phytagel supplemented with 50 mg l^{-1} kanamycin and 200 mg l^{-1} cefotaxime. Plates were incubated at 28 °C with a 16-h photoperiod (90 μmol m^{-2} s^{-1}). After 3–4 weeks, calli were excised from original explants and were transferred to fresh MS medium containing kanamycin. After another 2–3 weeks, calli were placed and maintained on embryo maturation medium (MS2), composed of MS salt supplemented with B_5 vitamin, 0.75 g l^{-1} $MgCl_2$, 1.9 g l^{-1} KNO_3 , 2.5 g l^{-1} phytagel, 30 g l^{-1} glucose, pH 5.8 and with 25 mg l^{-1} kanamycin for selection. Mature embryos were picked up and transferred to embryo germination medium (MS3), composed of MS salt supplemented with B_5 vitamins, 0.45 μM zeatin and 30 g l^{-1} sucrose under kanamycin selection (25 mg l^{-1}) (Kumar and Pental, 1998; Zhang et al., 2001). Germinated somatic embryos were placed in 500 ml jars containing MS salts supplemented with 100 mg l^{-1} *myo*-inositol, 0.5 mg l^{-1} thiamin·HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 3% sucrose and 0.15% (w/v) Gelrite, pH 5.7 (MS4). This medium was not supplemented with kanamycin in order to form roots on the plantlets (Zapata et al., 1998). Individual plantlets were transferred to pots containing a 1:1 (vol/vol) mixture of sterile soil and sand in plastic cups covered with polyethylene bags.

β-Glucuronidase (GUS) histochemical assay

Agrobacterium strain LBA 4404 harboring a binary vector pBI121 was used as the vector system for transformation. This vector contained *gus* gene as reporter gene driven by the CaMV35S promoter and the *nptII* gene driven by the CaMV35S promoter a selectable marker. *β*-Glucuronidase (GUS) gene expression was assayed in tissue T_1 transgenic cotton according to Jefferson et al. (1987).

Polymerase chain reaction analysis

Polymerase chain reaction (PCR) was carried out using specific primer pairs to amplify *nptII,* and *chi* transgenes from T_0 and T_1 transgenic cotton plants. Genomic DNA was extracted and purified from immature leaves following the protocol of Li et al. (2001). PCR was performed in a total volume of 25 μl reaction mixture consisting of 10× reaction buffer, 15 ng DNA template, 15 mM $MgCl_2$, 10 mM dNTPs, 60 ng of each primer and 0.5 unit *Taq* DNA polymerase (Cinagen Co., Tehran, Iran). PCR was carried out in a Touchgene (Model FTGO5TD) thermal cycler using the following conditions: initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for *nptII* and annealing at 62 °C for *chi* gene for 1 min, extension at 72 °C for 3 min and final extension at 72 °C for 5 min. The sequences of the primer pairs used in this assay are as follows: *nptII* 1: 5′-GAA CAA GAT GGA TTG CAC GC-3′*nptII* 2: 5′-GAA GAA CTC GTC AAG AAG GC-3′ and *chi*1: 5′-GAG TGG TGT GGA TGC TGT TG-3′ *chi*2: 5′-GCC ATA ACC GAC TCC AAG CA-3′.

Southern blot hybridization analysis

Ten microgram of cotton leaf DNA was completely digested with *Hin*dIII and *Eco*RI at 37 °C overnight in a total volume of 100 μl. Digested DNA fragments were separated on 0.8% agarose gels at 30 V for 8 h. DNAs were transferred onto nylon membranes (Hybond N^+, Amersham, UK) by capillary blotting. The *chi* gene fragment (1.0 kb) was labeled with DNA labeling Kit (Boehringer Mannheim) and used as probe in the filter hybridization. Detection was done using the DIG Detection Kit (Boehringer Mannheim).

Western blot analysis

One gram of leaf tissue was flash frozen in liquid nitrogen and was powdered. Soluble proteins were extracted with one ml extraction buffer [4% (w/v) SDS, 5% 2-mercaptoethanol, 20% glycerol, 68 mM Tris–HCl (pH 6.8)]. Ten micrograms of

proteins from each sample was fractionated in 13% SDS-polyacrylamide gel as described by Laemmli (1970). 'Western' immunoblot analysis for chitinase was performed as described by Mohammadi and Karr (2002). After transferring the proteins onto a Hybond–C membrane (Bio-Rad) by a semi-dry trans-electroblotter (Sigma Co., UK), the membrane was probed with the anti-chit-I anti-serum (1:2000) (a gift from Dr. Els van Deventer, Zeneca Mogen International, Leiden, The Netherlands). The goat-anti-rabbit IgG alkaline phosphatase conjugate (1:2000) (Gibco) was used as secondary antibody. Total soluble protein was measured using the Bradford reagent (1976).

Enzyme assays

Young leaves from putative transgenic lines as well as untransformed cotton plants were flash frozen in liquid nitrogen and were ground to fine powder. The soluble proteins were then extracted in 50 mM sodium acetate buffer (pH 7.0). A colorimetric assay was performed using chitin (Sigma Chemical Company, St. Louis, USA) as the substrate following the method of Boller and Mauch (1988). The assay for each sample was performed three times.

The inhibitory effect of bean chitinase on the growth of V. dahliae in vitro

A non-defoliating strain (T_1) of *Verticillium dahliae* was used to study *in vitro* inhibitory activity of leaf extracts from transgenic cotton lines expressing chitinase gene. A single sclerotium of *V. dahliae* was placed in the center of Petri dishes (9 cm diam) containing potato dextrose agar (PDA). To allow initial mycelial growth, the plates were incubated at 27 °C. A mycelial plug (0.5 cm diameter) was placed in the center of a PDA plate surrounded by peripheral wells containing (a) 50 and (b) 100 µg leaf protein extracts prepared in 10 mM sodium acetate buffer (pH 5.0) from untransformed cotton plant, (c) 50 and (d) 100 µl of extraction buffer and (e) 100 and (f) 50 µg leaf protein extracts from transgenic cotton line #15. Plates were incubated at 27 °C for 7 days followed by examining mycelial morphology and measuring growth inhibition zone.

Results

Plant cell transformation and selection

Kanamycin-resistant microcalli (0.5 mm in diameter) emerged at the wound sites of hypocotyl segments co-cultivated with *Agrobacterium* strains 3 weeks after incubation on MS medium. Transformation frequency of embryos and plantlets were determined as the percentage of kanamycin-resistant embryos and plantlets based on leaf callus assay (Table 1). In this experiment, the transformation efficiency of *Agrobacterium* strain LBA4404 was higher than either EHA101 or C58 strain. Further, strain EHA101 was found to overgrow on some of the explants during the selection process thus interfering with callus growth on selection medium.

Plant regeneration

The transformed calli were placed and maintained on embryogenic medium (Zhang et al., 2001) under selection in which more the calli became embryogenesis, i.e. friable cream-colored granular calli that produced somatic embryos (Figure 2a, b). Germinated embryos with and without roots but containing a few leaves were then transferred to jars on MS4 medium. Root initiation was observed after 10–20 days. A total of 50 plants were obtained following transformation with strain LBA4404 whereas five and nine putative transformants were derived from strains EHA101 and C58, respectively. We observed various types of abnormal embryos and plantlets. At times, there were bare stems with no roots or cotyledon. Other embryos produced only roots but no cotyledons.

Table 1. Transformation frequency of cotton (var. Coker 312) embryos or plantlets regenerated from kanamycin-resistant calli

Bacterial strain	# of embryos or plantlets tested	# of transformants	Transformation freq. (%)[a]
LBA 4404	11	7.5	68
C58	9	2	22.2
EHA101	5	1	20

[a]Transformation frequency was determined as the percentage of embryos or plantlets that were kanamycin resistant on the basis of leaf callus assay.

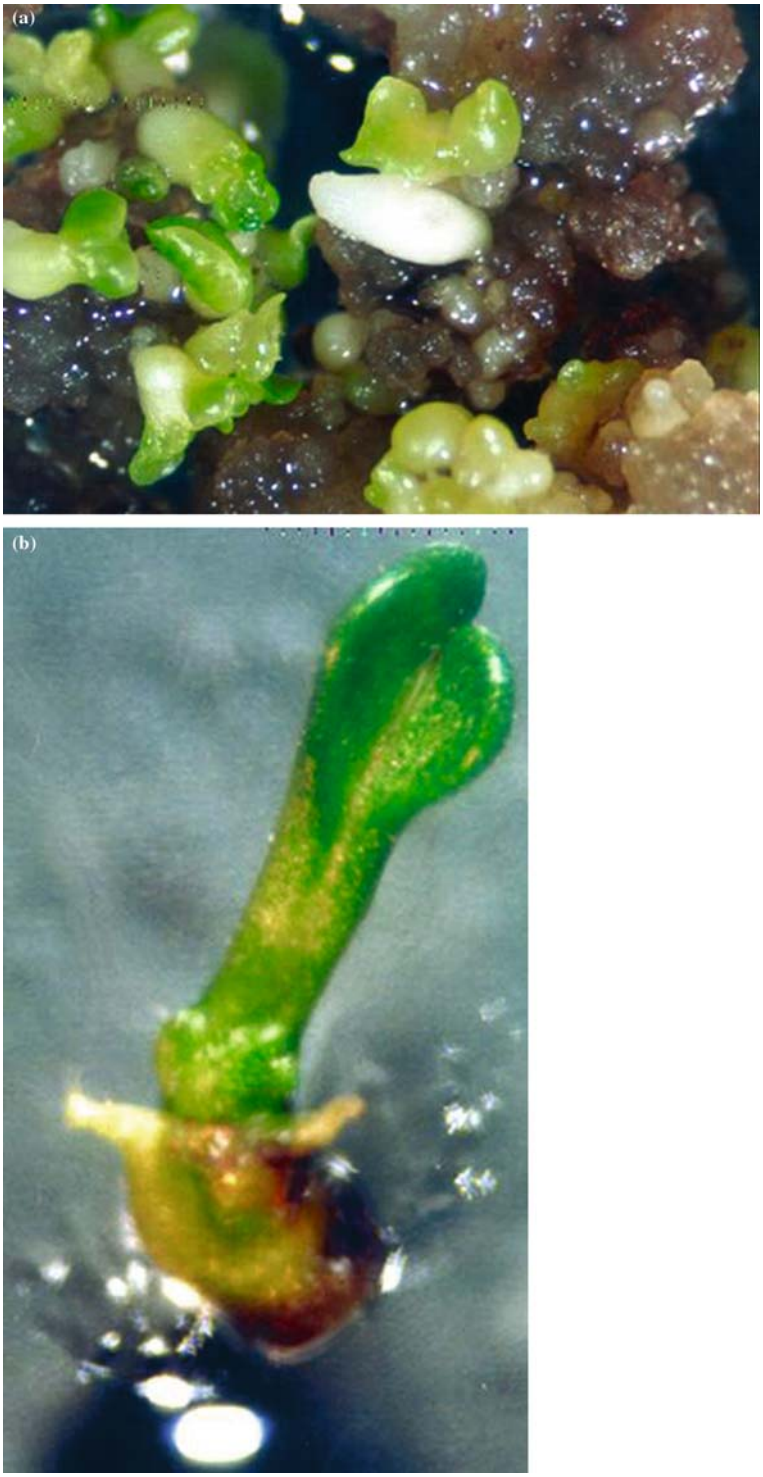

Figure 2. Cotton (var. Coker 312) calli on MS medium containing 50 mg l^{-1} kanamycin: (*a*) somatic embryos with different morphology; (*b*) germination of somatic embryo.

The frequency of abnormal plantlets in Coker 312 was around 3%. The 25 plants with well developed root systems and leaves were transferred to soil and left to flower and set seeds under the greenhouse conditions (Figure 3).

PCR analysis and GUS expression at T_0 and T_1 generations

A total of 23 randomly selected T_0 plants exhibiting some degree of kanamycin resistance were analyzed by PCR. Seventeen of these plants yielded a single DNA fragment of 785 bp for *nptII* gene (Figure 4). Stable expression of *gus* gene in young petals of T_1 transgenic plants was determined using the histochemical GUS assay (Figure 5). All 17 transformants showed positive results after amplification of the predicted 870 bp internal fragment of the chitinase gene. Untransformed T_0 cotton plants were negative for the *chi* gene (Figure 6a).

Transgenic cotton Lines of var. Coker 312 were successfully self-pollinated and T_1 seeds were produced. Those PCR products obtained in T_0 were also amplified in their T_1 progenies (Figure 6b).

Southern blot analysis

Genomic DNA isolated from leaves of PCR positive T_0 plants was subjected to Southern analysis to provide additional evidence of integration of the *chi* gene into the cotton genome. The results showed hybridization signals confirming that the transgene was successfully integrated in the plant genome. There was one band in the *Hin*dIII digested sample, and 3 bands for the sample digested with *Eco*RI as expected (Figure 7). No hybridization signal could be detected for the untransformed plant.

Expression of the bean chitinase gene in transgenic cotton at T_0 and T_1 generation

'Western' immunoblot analysis using the anti-chit-I polyclonal anti-serum revealed the presence of a

Figure 3. Transgenic cotton (var. Coker 312) expressing a heterologous bean chitinase gene 10 weeks old adult transgenic cotton bearing boll and flower.

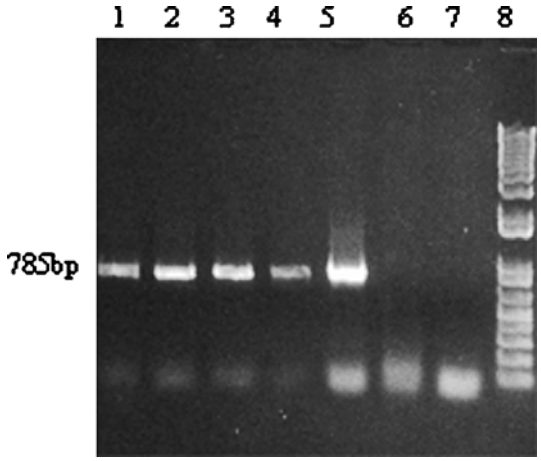

Figure 4. PCR analysis of DNA isolated from leaves of transformed cotton (var. Coker 312) using primer pairs specific for amplification of 785 bp *nptII* gene in agarose gel. Lanes 1–4, DNA from putative transgenic cotton lines; lane 5, DNA from pBI121-BCH; lane 6, DNA from untransformed cotton; lane 7, sterile water; M, 1.0 kb plus DNA ladder (Gibco BRL).

31 kDa protein band in eight transgenic cotton lines #4, 15, 16, 18, 20, 26, 28 and 29 (Figure 8a). Line #3 and untransformed control did not show any positive signal for bean chitinase protein. In addition to the expected 31 kDa protein, a 29 kDa band cross-reacting with the polyclonal anti-serum was also observed in all the transgenic lines tested. Transgenic cotton lines #15, 26 and 28 expressing a heterologous bean chitinase gene exhibited a stronger signal as compared to other lines. Western blot analysis of T_1 transgenic plants indicated the stable expression of *chit* gene in progenies of lines #20 and 18 (Figure 8b).

Analysis of T_0 plants for chitinase activity

Further, chitinase specific activity was different among transgenic lines assayed. As shown in Table 2, chitinase specific activity was several folds greater in six transgenic lines tested as compared to the untransformed control and line #3. However, no significant differences were observed between three of the transgenic cotton lines and the controls. Chitinase specific activity in transgenic cotton plants strongly correlated with the expression of signals observed in 'Western' immunoblotting.

The inhibitory effect of bean chitinase on the growth of V. dahliae in vitro

In vitro mycelial growth of *V. dahliae* was inhibited upon the use of leaf tissue extracts from transgenic cotton plants. This inhibitory effect was proportional to the concentration of chitinase extract used in plate bioassay (Figure 9). No inhibition

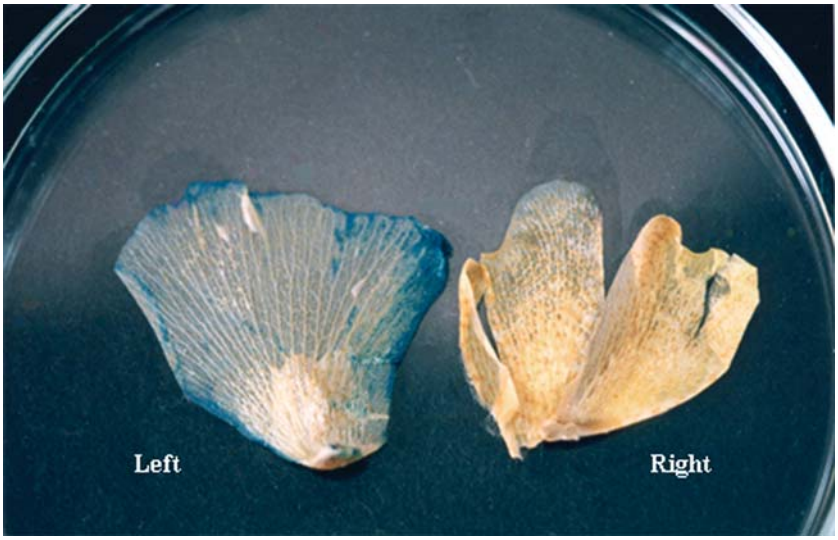

Figure 5. Histochemical detection of the stable analysis of *gus* gene expression in petal tissues of transgenic T_1 plants (control, right side) showing the characteristic deep blue coloration after overnight incubation in GUS buffer at 37 °C; no GUS activity was detected in untransformed control plant.

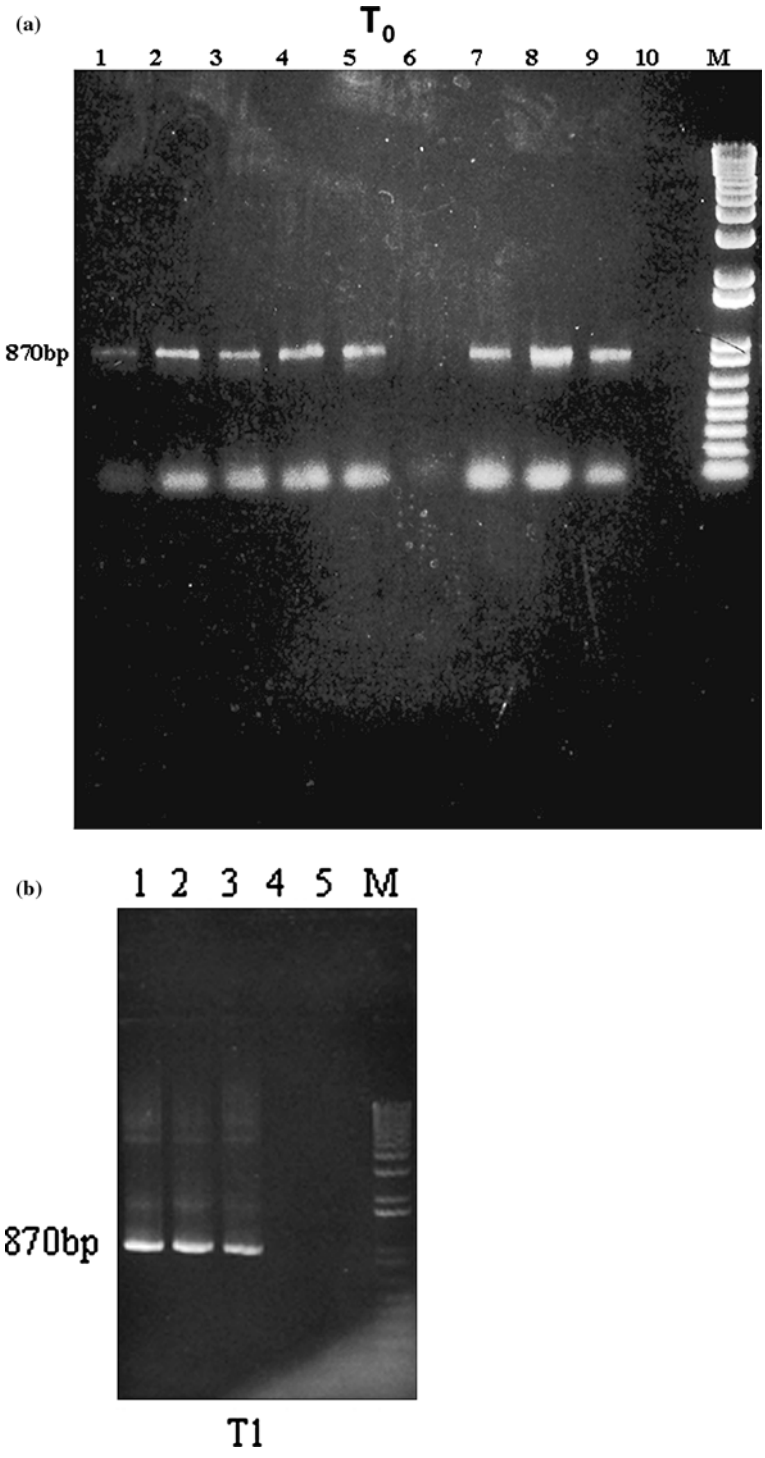

Figure 6. PCR analysis of DNA isolated from leaves of transgenic cotton (var. Coker 312) using specific primer pairs for amplification of 870 bp bean chitinase (*chi*) gene in agarose gel. (*a*) T_0 progeny. Lanes 1–5 and 7–9 represent DNA from transgenic lines; lane 6, DNA from untransformed cotton; lane 10, sterile water; M, 1.0 kb plus DNA ladder. (*b*) T_1 progeny selected from the T_0 transformant. Lanes 1 and 2 represent DNA from transgenic lines; lane 3, DNA from plasmid pBI121-BCH; lanes 4, DNA from untransformed cotton; lane 5, sterile water; M, 1.0 kb plus DNA ladder (Gibco BRL).

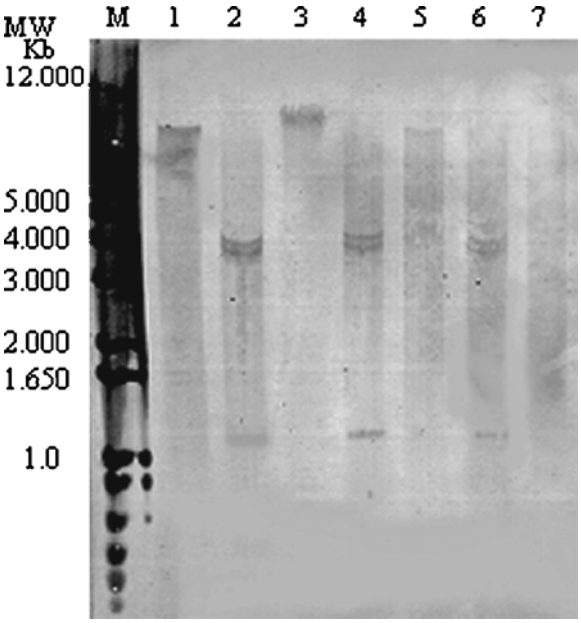

Figure 7. Southern blotting analysis of DNA isolated from leaves of T_0 transgenic cotton lines. M, 1.0 kb plus DNA ladder (Gibco BRL); lane 1, *Hin*dIII digested DNA from transgenic line #28; lane 2, *Eco*RI digested DNA from transgenic line #28; lane 3, *Hin*dIII digested DNA from transgenic line #18; lane 4, *Eco*RI digested DNA from transgenic line #18; lane 5, *Hin*dIII digested DNA from transgenic line #20; lane 6, *Eco*RI digested DNA from transgenic line #20; lane 7, *Hin*dIII digested DNA from untransformed plant.

zone was detected using boiled enzyme extract or extraction buffer alone. Close microscopic examination of *V. dahliae* revealed the adverse effects of tissue extract from transgenic cotton leaf upon fungal mycelial growth. Morphological change appeared following exposure of mycelium to the purified transgenic cotton chitinase. The hyphal tip showed marked swelling and subsequently lysis was also observed. Hyphal morphology was distorted, hyphae became thinner and shorter, turned dark and did not sporulate (Figure 10a, b).

Discussion

In this study, we were able to readily obtain transgenic cotton plants using the transformation–regeneration system as described earlier (Kumar and Pental, 1998; Zapata et al., 1998; Zhang et al., 2001). Keys to the transformation system were rapid callus initiation at the hypocotyl wound sites and the use of selectable kanamycin resistance marker. Rapid callus initiation (on MS medium containing 0.46 μM kinetin and 0.45 μM 2,4-D) was critical for the recovery of a high number of transformed microcalli at the periphery of the inoculated tissues. This could be due to a better recovery of transformed cells on this medium, and high competency of dividing cells for transformation with *A. tumefaciens* or both (Firoozabady and Galbraith, 1983, 1984). In our experiment using the hypocotyl as an explant for inoculation (Firoozabady et al., 1987), a few untransformed calli proliferated rapidly. These tissues were highly chimeric due to lack of complete contact between explants and kanamycin-containing medium. Excision of calli from explants was essential for promoting growth of calli and avoiding *Agrobacterium* contamination. Small size calli had a lower rate of survival. We believe that this situation is similar to the requirement for a critical minimum cell density reported for the growth of cells or protoplasts (Shneyour et al., 1984). Glucose was used as a sole carbon source for callus induction and embryo maturation since sucrose encouraged production of phenolics by cotton explants. The production of abnormal embryos and plantlets is most likely due to the length of time generally required for cotton tissue culture.

The *Agrobacterium* strain used had a major effect on transformation efficiency of cotton. It was found that use of strain LBA4404 yielded a higher degree of transformation efficiency in cotton as compared to other strains tested. This is in agreement with reports of Firoozabady et al. (1987), Umbeck et al. (1987) and Chen et al. (2002) on cotton transformation. But the results of other studies indicated that the super-virulent strains EHA101 and EHA105 were more suitable for stable transformation of cereals (Donaldson and Simmonds, 2000) and certain dicots (Rashid et al., 1996).

Majority of lines that gave positive results for PCR analysis were further confirmed by Southern hybridization. In the pBI121-BCH construct there is only one *Hin*dIII site in the 5′ of 35S promoter, while 3 *Eco*RI sites in pBI121-BCH. It seems that the lines #18, 20 and 28 were obtained from a dependent transformation event, because the bands obtained after *Eco*RI digestion were of the same size. It is estimated that there is only one copy of the transgene in T_0 plants.

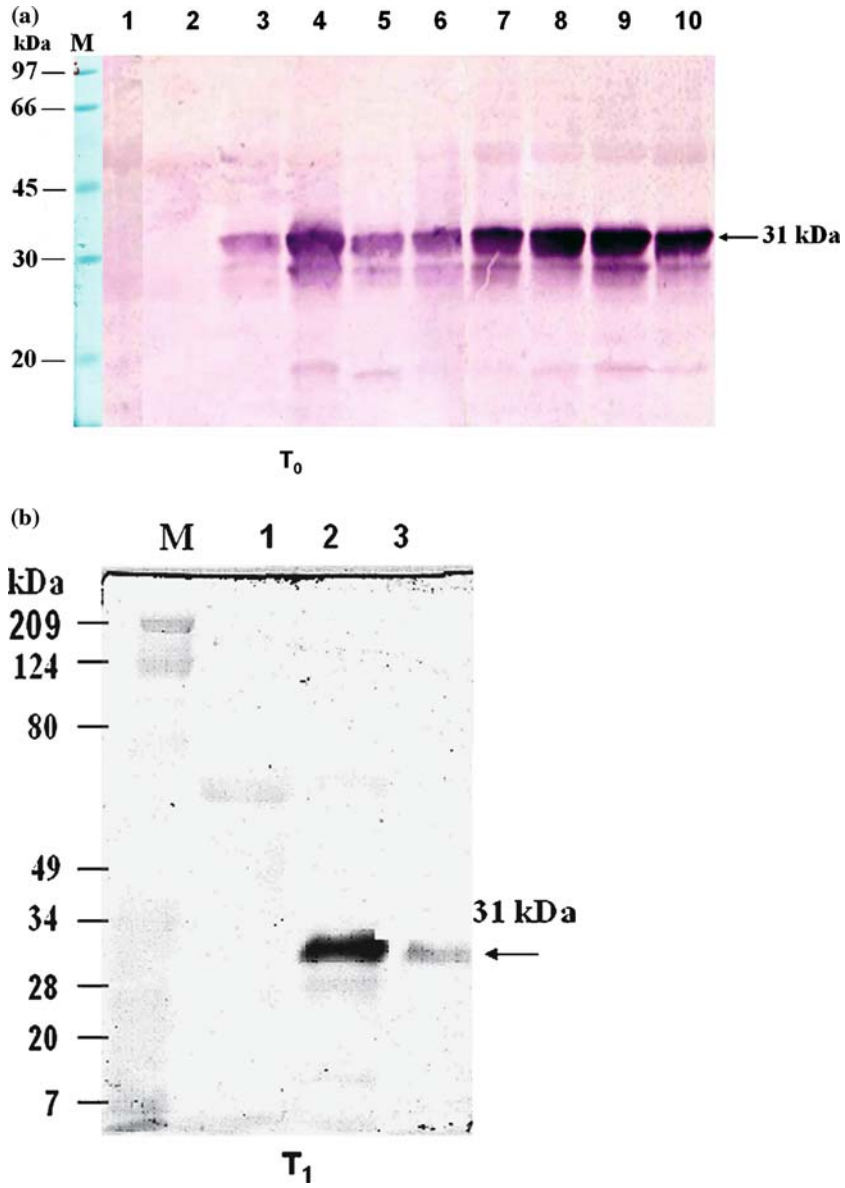

Figure 8. Western immunoblot analysis of chitinase (*chi*) gene expression in leaf extract of transgenic cotton lines. (*a*) T_0 progeny. Lane 1, extract from untransformed cotton; lanes 2–10, leaf extracts from transgenic lines #3, 4, 15, 16, 18, 20, 26, 28 and 29, respectively. M, molecular size markers in kDa. (*b*) T_1 progeny. M, prestained standards markers in kDa; Lane 1, extract from untransformed cotton; lane 2, leaf extracts from transgenic line #20; Lane 3, leaf extracts from transgenic line #18.

Bean chitinase gene expression level in T_1 transgenic cottons was variable as revealed by 'Western' immunoblotting and specific activity assay as well. Transgenic lines #15 and 28 exhibited stronger expression of *chi* gene than others. This may be the result of high copy number of integrated heterologous chitinase gene in these lines (Yamamoto et al., 2000). The lower MW immunoreactive band of about 29 kDa is presumably the proteolytic degradation product or modified form of the bean chitinase expressed in transgenic cotton plants as demonstrated in previous studies (Lin et al., 1995; Terakawa et al., 1997; Nishizawa et al., 1999). Transcript expression, translation and degradation, etc., can affect variations in *chi*

Table 2. Chitinase specific activity in leaf tissues of transgenic cotton lines (T_0) (var. Coker 312) relative to the untransformed control plant

Transgenic line (T_0)	Specific activity (μmol of *N*-acetyl-glucosamine min^{-1} (mg protein)$^{-1}$)[a]
T3	0.62
T34	1.56
T4	3.31
T20	5.29[b]
T16	6.13[b]
T29	6.30[b]
T26	7.10[b]
T15	13.86[b]
T28	19.56[b]
T33	36.96[b]
Control	0.05

[a]One unit of activity is defined as the enzyme activity catalyzing the formation of one μmol of *N*-acetyl-glucosamine min^{-1} (mg protein)$^{-1}$.
[b]Difference was significant at 1% using mean comparison test (LSD).

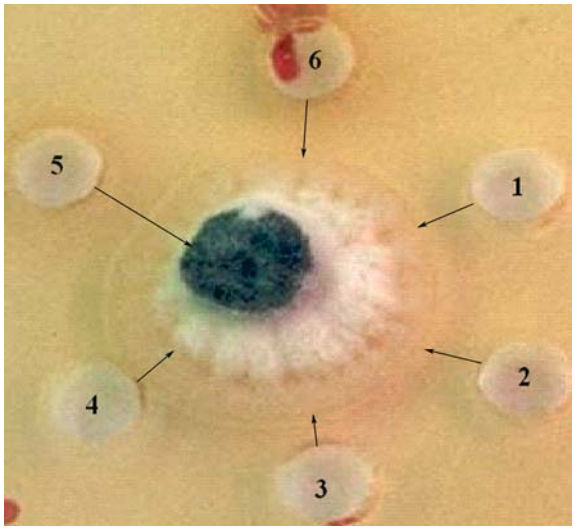

Figure 9. Inhibitory activity of leaf extract from transgenic cotton line#15 expressing bean chitinase (*chi*) gene against *V. dahliae* on PDA (potato dextrose agar). Samples were loaded into each individual well at the periphery and fungal mycelial plug was placed in the center of plate. Samples were as follows: (1) 50 and (2) 100 μg protein extract from leaf tissues of untransformed cotton prepared in 50 mM sodium acetate (pH 7.0) as an extraction buffer, (3) 50 and (4) 100 μl extraction buffer; (5) 100 and (6) 50 μg protein extract from leaf tissues of transgenic cotton.

mRNA accumulation and translation among transgenic lines of cotton. Transgenic line #3 showed no positive immunoreactive signal in 'Western' blotting despite the integration of transgene in its genome as confirmed by PCR. This might be the result of a positional effect and/or gene silencing which is one of the models for explaining differences in transgene expression (Meyer, 1985). Expression of the chitinase gene in the T_1 plants indicates the stable expression of the transgene.

Transgenic cotton lines showed varying degrees of chitinase specific activity are consistent with the results from other reports in which stable integration and expression of *chi* gene in canola and tobacco (Broglie et al., 1991), tobacco (Terakawa et al., 1997), grapevine (Yamamoto et al., 2000), peanut (Rohini and Sankara, 2000), pickling cucumber (Raharjo et al., 1990) and rice (Lin et al., 1995) led to significantly greater levels of chitinase in these transgenic plants and enhanced resistance to important fungal diseases caused by *R. solani*, *Sclerotinia sclerotiorum*, *Uncinula necator*, *Cercospora arachidicola*, *Botrytis cinerea and Magnaporthe grisea,* respectively. Broglie et al. (1991) have shown enhanced resistance to root rot fungal disease caused by *R. solani* in transgenic canola and tobacco plants constitutively expressing bean chitinase gene. Further, a 44-fold increase in chitinase activity in transgenic plants resulted in 40% decline in seedling mortality.

The anti-fungal property of bean chitinase gene product extracted from transgenic cotton suggest that chitinase plays a role in disease resistance of cotton. Such a chitinase gene may be highly useful for developing transgenic disease-resistant plants.

Work is currently underway to analyze transgene expression and inheritance pattern in subsequent generations of transgenic T_0 plants and their resistance to verticilliosis.

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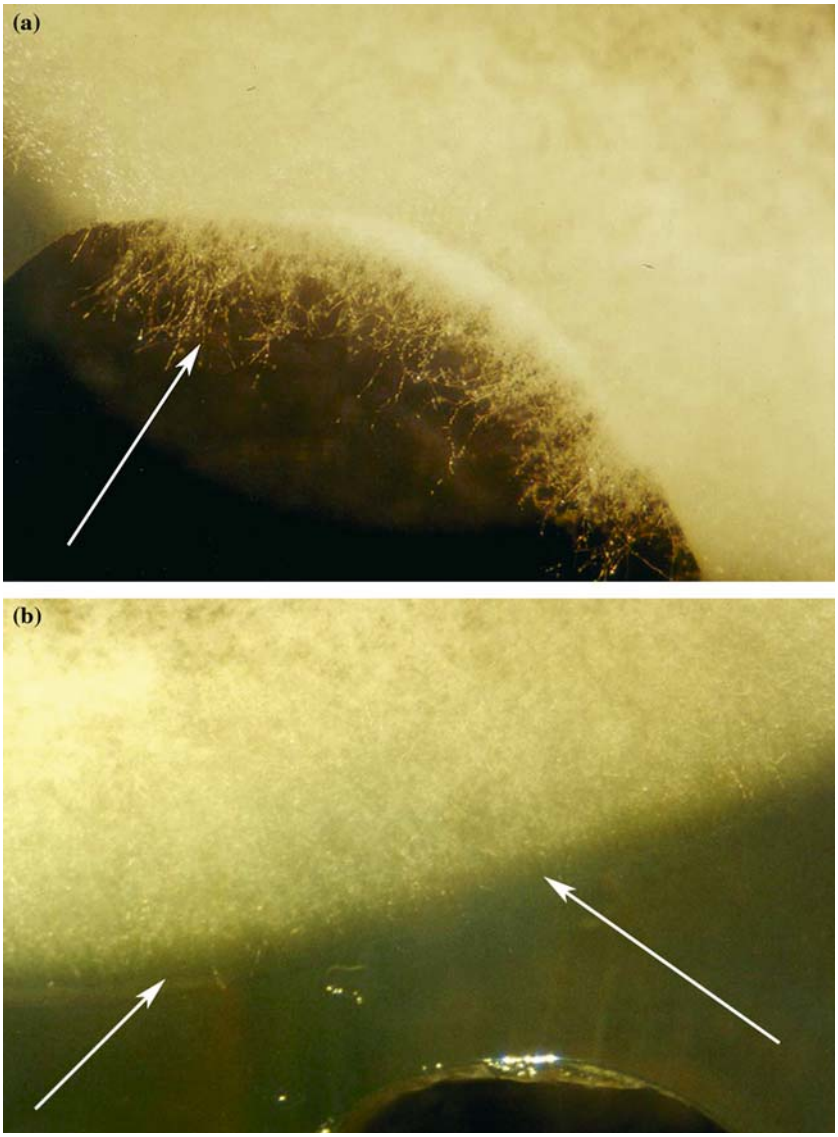

Figure 10. A close-up picture showing the effect of leaf extract from control or transgenic cotton line #15 on mycelial growth of *V. dahliae* as described in Figure 9. (*a*) In control, fungal mycelium has overgrown into the well in PDA and mycelia appear normal, whereas (*b*) mycelial growth has stopped before well containing extract from transgenic leaf tissue and mycelia are lysed.

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