Enhanced Resistance to *Verticillium dahliae* in Transgenic Cotton Expressing an Endochitinase Gene from *Phaseolus vulgaris*

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Abstract: The cotton wilt disease caused by the fungus *Verticillium dahliae* reduces total yield and quality in cotton worldwide. Since traditional breeding has achieved only low levels of wilt resistance, we used a transgenic approach to produce wilt tolerant cotton. We produced fertile transgenic T_2 cotton lines by *Agrobacterium*-mediated transfer of an endochitinase gene from *Phaseolus*. Transgene integration and expression were confirmed by PCR amplification, Southern blot and Western blot analyses. Seedlings of homozygous T_2 plants with high levels of chitinase expression were evaluated for their tolerance to *Verticillium dahliae* by infecting them with a spore suspension in a greenhouse. The transgenic plants demonstrated higher degrees of tolerance to the fungus than non-transgenic plants, as measured by foliar disease symptoms, vascular discoloration and plant height. Transgenic plants were also taller than the control ones and are currently being grown in the greenhouse and will be crossed with Iranian cotton breeding lines in a future study.

Keywords: fungal disease; *Gossypium hirsutum*; induced resistance; transgenesis

Cotton is a major cash crop in north-eastern Iran in proximity to the Caspian Sea region and is cultivated on 150 000–200 000 ha. Verticilliosis is a vascular fungal disease caused by *Verticillium dahliae* and is considered as the major wilt disease in cotton-growing areas in Golestan province, Iran. Quantity and quality of produced cotton are affected by several biotic and abiotic factors during the growing season. Among the biotic factors, the vascular wilt diseases are of most concern due to the soil-born nature of their cause which reduces the efficiency of chemical fungicides drastically (McFadden *et al*. 2001). The soil-inhabiting fungus *V. dahliae* Kleb. was reported to cause substantial yield losses in cotton (Bolek *et al*. 2005). In addition, the increasing spread of aggressive strains of verticillium wilt in cotton-growing areas is another concern of the major growers because the fungus could easily be spread in soil or crop debris by adhering to vehicles and machinery, or by flood and irrigation water. *V. dahliae* infects the plant through the roots and then invades the vascular tissue, blocking water uptake and causing yellowing, leaf mottle and sometimes wilting and death. Infection causes not only the dropping of bolls and the reduction of yield by 20–60%, but also reduces the quality of cotton fibre (Wang *et al*. 2004). At present, selection and breeding of new lines with higher levels of resistance to verticillium wilt are among the major approaches to control this disease. However, due to the limited resistance sources in cotton, it is therefore relatively difficult to transfer resistance to cultivated cotton

and to select cultivars with a high level of resistance to verticillium wilt via conventional plant breeding. Also, the development of resistant plants through conventional plant breeding is time-consuming and laborious. Hence, the selection of new varieties with high levels of resistance to the disease using biotechnological approaches and development of new cotton cultivars resistant to verticillium wilt are the most effective and feasible approaches to combat the problem (Mert *et al*. 2005).

On the other hand, plants have developed various mechanisms to defend themselves against these fungi which include the production of low molecular weight secondary metabolites, proteins and peptides with antifungal activity (Wu & Bradford 2003). Among all induced responses, production of "Pathogenesis Related (PR) proteins" is the most important because they can lead to an increased resistance of the whole plant against a pathogenic attack (Sexton & Howlett 2006). Among antifungal PR proteins, chitinase is of great biotechnological interest for engineering plants to increase resistance to phytopathogenic fungi (Dempsey *et al*. 1998). The enzyme chitinase is capable of degrading chitin, a linear homopolymer of β-1,4-N-acetyl-D-glucosamine residues, which constitutes 3–60% of the cell wall of most fungi (Collinge *et al*. 1993). Boller *et al*. (1983) identified an endogenous chitinase activity in cotton which increased 4.7 fold after ethylene treatment. However, it was reported that the endogenous production of chitinase in cotton was not sufficient to control the disease. Chitinase genes have been used to engineer a variety of crops, such as canola (Brogue *et al*. 1991), wheat (Shin *et al*. 2008), grapevine (Yamamoto *et al*. 2000), and cotton (Emani *et al.* 2003; Tohidfar *et al*. 2005). Several chitinase genes, i.e. classes 1 and 2, have been identified in cotton (Chlan & Bourgeois 2001). Class I chitinases (Goshi; Chi1; 1, Goshi; Chi1; 2 and Goshi; Chi1; 3) can be induced by ethylene[15], whereas class 2 chitinases (Chi2; 1 and Chi2; 2) can be induced by salicylic acid (SA) (Zhang & Punja 1994; Hudspeth *et al*. 1996).

We previously reported on the production of a transgenic cotton line containing a chitinase gene from *Phaseolus vulgaris* under the control of CaMV 35S promoter using the *Agrobacterium* method (Tohidfar *et al.* 2005). The aim of this study was to investigate the expression of transgene and to evaluate the resistance of transgenic cotton plants expressing the chitinase gene against verticillium wilt under greenhouse conditions.

MATERIAL AND METHODS

Plant materials

Eighteen transgenic plants produced by *Agrobacterium*-mediated transformation using the plasmid pBI121-CHI as described previously (Tohidfar *et al*. 2005) were selfed for two generations. Three replicates of six transgenic T_2 cotton lines and three non-transgenic plants (as a control) were used in this study.

DNA extraction and PCR amplification

Genomic DNA was extracted and purified from young leaves following the protocol reported by Li *et al*. (2001). PCR was carried out using specific primer pairs to amplify the *chi* gene (850 bp) from transgenic cotton plants. Thermal cycler conditions had initial denaturation at 94°C for 2 min followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 60°C 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:
CHIF: 5'-GAG TGG TGT GGA TGC TGT TG-3'
CHIR: 5'-GCC ATA ACCGAC TCC AAG CA-3'.

Southern blot analysis

Ten micrograms of genomic DNA extracted from young leaves were completely digested with *Hind*III (for Line 11/4), *Eco*RI (for lines 11/57, 11/10, 11/9 and 11/44) and *Xba*I and fragments were separated on a 0.8% agarose gel for 12 h. DNAs were transferred onto nylon membranes (Hybond N+, Amersham, Little Chalfont, UK) by capillary blotting. The 850 bp PCR product of the coding sequence of the chitinase gene was labelled with DiG DNA Labelling and Detection Kit (Boehringer, Mannheim, Germany) and used as a probe. The hybridization temperature was optimized at 60°C and a mid-high stringency wash buffer (0.5× SSC and 0.1% SDS) was used.

Western blot

Total soluble protein was extracted from mature leaves of transgenic and control plants (Tohidfar *et al*. 2005). The protein was quantified using the

Bradford reagent (Bradford 1976). Ten micrograms of protein from each sample were fractionated on 13% SDS-polyacrylamide gel as described by Laemmli (1970). Western immunoblot analysis for *chi* gene was performed as described by Ghareyazie *et al*. (1997). After electrophoresis, protein was transferred onto nitrocellulose membranes in a BioRad Transblot apparatus (Bio-Rad, Richmond, USA) at 30V for 8 h. The membrane was then probed with an anti-chit-I anti-serum (1:2000) and the goat anti-rabbit IgG alkaline phosphatase conjugated antibody (1:2000) (Gibco, Eggenstein, Germany) was used as the secondary antibody.

Verticillium dahliae resistance assays on cotton seedlings

SS_4 isolate of *V. dahliae* was obtained from infected cotton fields in Gorgan, Iran. This isolate was used to produce a conidial suspension by growing on PDA plates for 4–7 days. Plates were flooded with sterile water and agitated with a glass rod. Approximately 0.5 ml of this conidial suspension was used to inoculate potato dextrose broth cultures in conical flasks that were grown under vigorous shaking (100 rpm) for 3 to 7 days at 24°C. The liquid cultures were filtered through cheesecloth, and the conidial concentration was determined using a haemocytometer. Conidial concentrations were adjusted to 10^6 conidia/ml with sterile water. Plants at the 6–8 leaf stage were inoculated by a stem puncture method (McFadden *et al*. 2001). Genotype response to *V. dahliae* inoculation was evaluated based on foliar disease symptoms, vascular discoloration and plant height. Four to eight weeks after inoculation, foliar symptoms were scored on a 1–5 scale as follows (McFadden *et al*. 2004):

0 = no stunting nor leaf necrosis,
1 = no stunting with partial necrosis visible in leaf or cotyledon,
2 = mild stunting and/or partial necrosis of more than one leaf or cotyledon,
3 = moderate stunting and/or necrosis visible in more than a half of the foliar tissue of the plant,
4 = pronounced stunting and necrosis visible in each leaf,
5 = dead plant.

Scoring for vascular browning was made on a 1–5 scale as follows (McFadden *et al*. 2004):

0 = no vascular discoloration,
1 = discoloration restricted to the base of the stem only,
2 = discoloration of the "internode 0" (hypocotyl) region of the stem below the cotyledons,
3 = discoloration of the stem above the cotyledons,
4 = complete vascular discoloration of the stem,
5 = dead plant.

Vascular discoloration was visually evaluated by cutting the stems. Non-transgenic plant lines were inoculated with distilled water as negative controls. The plant height was measured at day 42 post inoculation.

RESULTS

PCR analysis

PCR analysis was carried out primarily to confirm the transgenic nature of T_2 plants. The 850 bp fragment was amplified from nuclear DNA for all transgenic plants tested. No amplification was observed for non-transgenic plants. The same amplification was observed when the plasmid pBI121-CHI was used as the template. Based on the progeny analysis by PCR amplification three out of the six T_2 lines studied were found homozygous.

Southern blot analysis

Figures 1–3 show Southern blot analysis of the transgenic T_2 lines. A 850 bp PCR product of the coding sequence of the *chi* gene was used as a probe. Hybridization to undigested DNA occurred exclusively at high molecular weight, indicating the integration of the gene into the cotton genome. When the DNA was digested with *Hind*III, an expected fragment of approximately 2.1 Kb that included the entire coding sequence of *chi* gene promoter and the terminator was obtained for transgenic line # 11/4. On the other hand, when the DNA was digested with *Eco*R1, an expected fragment of approximately 1.3 Kb that included the entire coding sequence of *chi* gene and the terminator was obtained for transgenic lines # 11/57, 11/10, 11/9 and 11/44 (Figure 1 and 3). These results showed that at least one intact copy of the *chi* gene was integrated into the cotton genome. Furthermore, once the DNA was digested with *Xba*I, only one band was detected for all the studied transgenic lines except for line # 11/57,

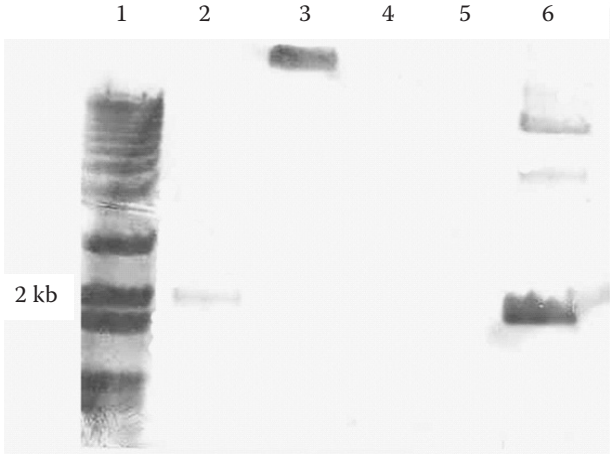

Figure 1. Southern blot analysis; *Hind*III digested DNA: lane 1 – 1.0 kb plus DNA ladder (Gibco BRL); lane 2 – digested DNA from transgenic line # 11/4; lane 3 – undigested DNA from transgenic line # 11/4; lane 4 – digested DNA from untransformed plant; lane 5 – undigested DNA from untransformed plant; lane 6 – digested plasmid pBI121-BCH

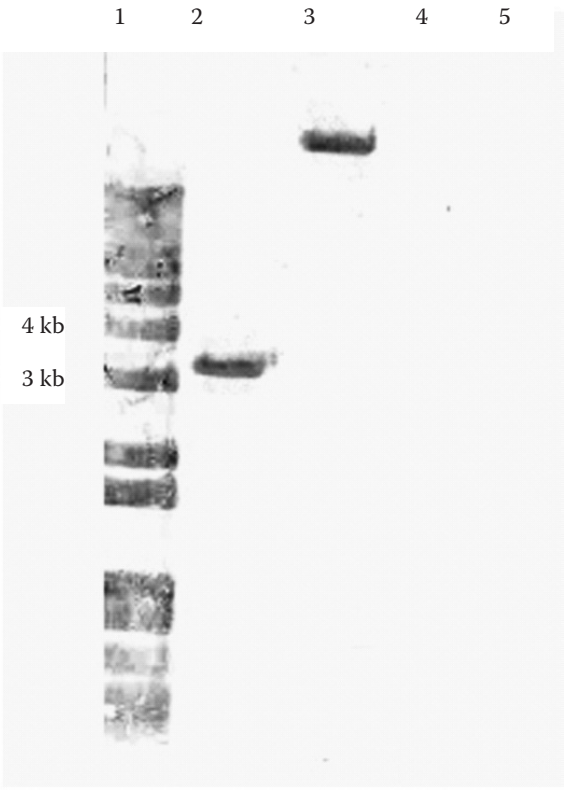

Figure 2. Southern blot analysis; *Xba*1digested DNA: lane 1 – 1.0 kb plus DNA ladder (Gibco BRL); lane 2 – digested DNA from transgenic line # 11/4; lane 3 – undigested DNA from transgenic line # 11/4; lane 4 – digested DNA from untransformed plants; lane 5 – undigested DNA from untransformed plants

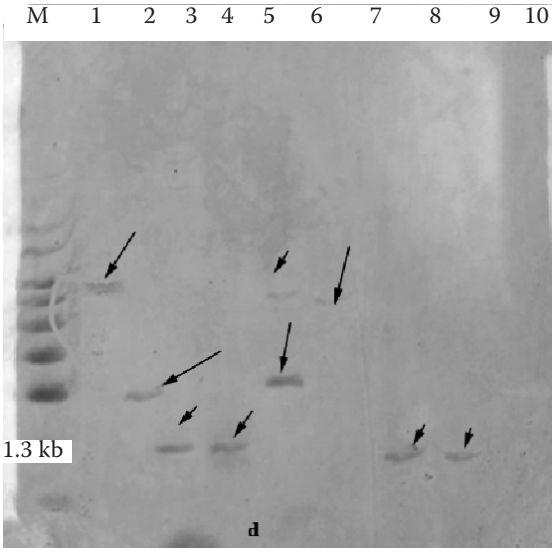

Figure 3. Southern blot analysis; *Xba*1 digested DNA: lane M, 1.0 kb DNA ladder (Fermentas); lane 1 – digested DNA from transgenic line # 11/10; lane 2 – digested DNA from transgenic line # 11/9; lane 5 – digested DNA from transgenic line # 11/57; lane 6 – digested DNA from transgenic line # 11/44; lane 9 – digested DNA from untransformed plant; *EcoR*1 digested DNA, lane 3 – digested DNA from transgenic line # 11/10; lane 4 – digested DNA from transgenic line # 11/9; lane 7 – digested DNA from transgenic line # 11/57; lane 8 – digested DNA from transgenic line # 11/44; lane 10 – digested DNA from untransformed plant

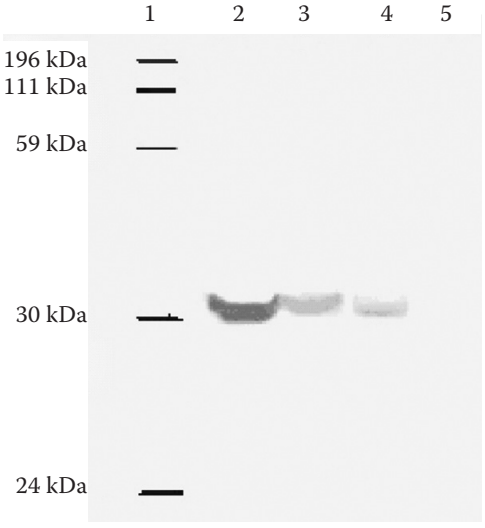

Figure 4. Western blot analysis of chitinase (*chi*) gene expression in a leaf extract of three different transgenic T_2 cotton lines; ten µg of total soluble protein was loaded in each well; lane 1 – molecular size markers in kDa; lane 2–4 – leaf extracts from transgenic lines; lane 5 – extracts from untransformed plant

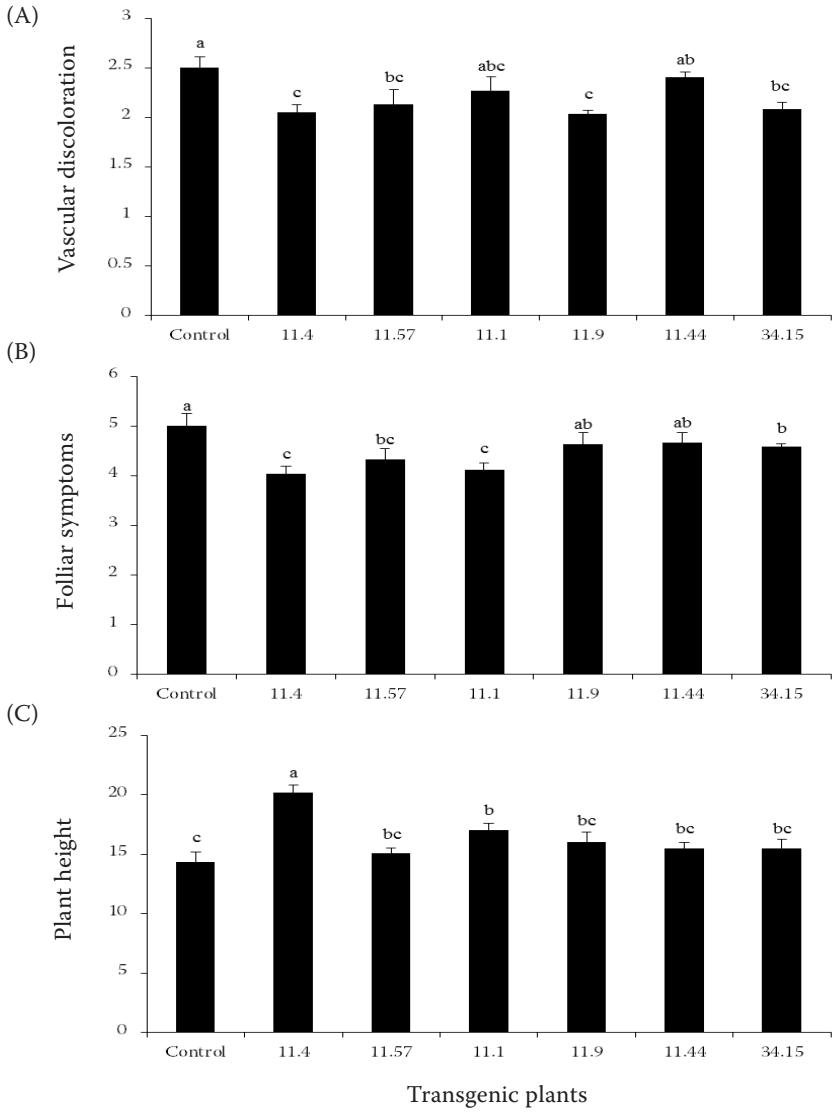

Figure 5. Responses of transgenic T_2 plants and non-transgenic cotton plant to *Verticillium dahliae* inoculation; A – vascular discoloration; B – foliar symptoms; C – plant height (in cm)

which resulted in the production of two bands (Figure 3). Since there was only one *Xba*I site in the T-DNA, this finding revealed the presence of only one single copy of the transgene in transgenic lines # 11/4, 11/9, 11/10 and 11/44 and two copies of the transgene in line # 11/57. No hybridization signal could be detected for the DNA extracted from non-transgenic plants.

Western blot analysis

The accumulation of chitinase from *Phaseolus vulgaris* in transformed cotton was examined by Western blot analysis using the anti-chit-I polyclonal antiserum. A specific positive band of 32 kDa, which was consistent with the molecular weight of *Phaseolus vulgaris* chitinase, was detected in extracts from homozygous transgenic lines. In contrast, no band was detected in extracts from untransformed plants (Figure 4). Transgenic cotton line 11/4 expressing a heterologous chitinase gene from *Phaseolus vulgaris* exhibited a higher signal as compared to those of the other lines.

Evaluation of resistance to *V. dahliae*

All six T_2 homozygous lines evaluated in the greenhouse were significantly more resistant to *V. dahliae* than the control plants ($P < 0.05$). Three transgenic lines expressed similar levels of resist-

Figure 6. Comparison of a transgenic cotton plant expressing a bean chitinase gene with the non-transgenic control plant; A – disease symptoms (leaf coloration) after inoculation with 10^6 conidia ml/l suspension of *V. dahliae*; B and C – vascular browning in cotton plants inoculated with 10^6 conidia ml/l suspension, B – horizontal and C – vertical cutting of the stem; a – untransformed plant inoculated with distilled water; b – transformed plant inoculated with 10^6 conidia ml/l suspension; c – untransformed plant inoculated with 10^6 conidia ml/l suspension

ance. The foliar symptoms observed in the transformed lines ranged from 4.4 to 4.6, compared to 5.0 for the control, while their vascular discoloration ranged from 2.05 to 2.6, compared to 2.8 for the control (Figure 5A and B). The plant height measured in the transformed lines was in the range of 15.05 to 20.0 cm, compared to 13.04 cm for the control (Figure 5C).

The plant height in the transgenic plants was significantly higher than that of the control ($P < 0.05$). The disease was assessed in the transgenic lines based on two criteria; the plant height and the foliar and vascular symptoms. Data pooled from foliar and vascular symptoms were scored over a range of disease responses from resistant to susceptible. For transgenic lines that showed clear-cut resistance or susceptibility, there was no difference between the two scoring methods. Line 11/4 was the most resistant line as judged by the distribution of vascular browning scores, foliar symptoms, and plant height (Figure 6).

DISSCUSSION

Verticillium dahliae is a serious disease of cotton and has resulted in significant economic losses around the world. Available resistance to *Verticillium dahliae* in cotton is inherited in a quantitative manner. Three loci (CM12, STS1, 3147-2) have significant effects on resistance to verticillium wilt. Two loci are located on LG-1 and one on LG-2 and both linkage groups are located on chromosome 11 (Bolek *et al.* 2005). In previous studies, the involvement of chitinase in the cotton defence mechanism against fungal diseases was revealed using different genotypes with different levels of resistance (McFadden *et al.* 2001; Emani *et al.* 2003; Li *et al.* 2003). Boller *et al.* (1983) demonstrated that there was an increase in chitinase activity in cotton after ethylene treatment. Chlan and Bourgeois (2001) showed that buffer extracts of ethylene-treated cotton tissues contained a chitinase protein that had a chitinolytic activity

and cross-reacted with its specific antibody. This protein was present in the treated extracts, but not detectable in control tissue extracts. Consistently with these results, Emani *et al.* (2003) also showed that the expression of the defence response gene endochitinase in transgenic cotton led to resistance against *V. dahliae* in a greenhouse.

The role of chitinase in plant defence against a fungal attack has been documented very well (Leah *et al.* 1991; Lawrence & Novak 2006). This enzyme is a glycanohydrolase which limits the fungal growth by degrading poly [β-1,4-N-acetyl-β-D-glucosamine], i.e. chitin, the major structural polysaccharide of the fungal cell wall including the cell wall of *V. dahliae* (Adams 2004). Hence, chitinases hydrolyse the chitin in fungal hyphae and restrict the fungal growth without causing damage to the plant cell.

In this study it was shown that the expression of chitinase gene enhanced the resistance of cotton against *V. dahliae* infection under greenhouse conditions. The presence of only one band in *Hind*III and *Eco*RI digested sample confirmed that at least one copy of the intact *chi* gene and its promoter were stably integrated into the cotton genome. Moreover, since there was only one *Xba*I site in the T-DNA, the presence of one and two bands in the *Xba*I digested samples revealed the presence of only one single copy of the transgene in transgenic lines # 11/4, 11/9, 11/10 and 11/44 and two copies of the transgene in line # 11/57, respectively. This reduces the chance of silencing since the cytosine methylation silencing has been shown to be triggered by the presence of multiple copies of a transgene (Li *et al.* 2002; Butaye *et al.* 2005). Stability of gene expression was assured by high levels of chitinase expression in T_2 plants. The level of bean chitinase expression in T_2 plants varied as revealed by Western blotting. Strong expression of the gene in some of the transgenic lines could be related to their position in the genome. A few transgenic lines, i.e. 10/11 and 38/9, showed no positive immunoreactive signal in Western blotting despite the confirmed integration of the transgene in their genome (data not shown). This might be attributed to a positional effect and/or gene silencing which is one of the models used for explaining differences in transgene expression (Meyer 1985).

There are a few reports on the production of cotton transgenic plants with improved resistance to fungal diseases using a chitinase gene (Brogue *et al.* 1991; Grisson *et al.* 1996; Emani *et al.* 2003). Chitinase genes of different origins have also been transferred into rice (Nishizawa *et al.* 1999), grapevine (Yamamoto *et al.* 2000), Italian ryegrass (Takahashi *et al.* 2004) and wheat (Shin *et al.* 2008) in order to control *Magnaporthe grisea*, *Uncinula necator*, *Puccinia coronata* and *Fusarium graminearum*, respectively. Moderate to strong resistances were reported in those studies. To the best of our knowledge, a few reports exist on the transformation of cotton using antifungal genes (Emani *et al.* 2003; Rajasekaran *et al.* 2005). However, to date there has been no published report on the overexpression of a bean chitinase gene in cotton. Furthermore, complete resistance against any fungal disease by transferring a single gene encoding a defence protein has not been reported either.

In the present study, homozygous lines expressing levels of improved resistance were identified. All the transgenic T_2 lines except two lines, i.e. # 10/11 and 38/9, showed improved resistance. This was further confirmed by the Western blot findings where no chitinase expression was observed for lines # 10/11 and 38/9. McFadden *et al.* (2001) reported that T_3 cotton plants expressing chitinase protein in comparison with non-transgenic ones showed a lower reduction in their height after 6 weeks from inoculation due to verticillium infection. The rate of reduction in control and transformed plants was 30% and 10%, respectively. The difference between the results obtained in this study and those of McFadden *et al.* (2001) may be explained by differences in their integration position that could have affected the expression levels. When the transgenic T_2 plants were challenged with *V. dahliae* in the present study, they developed fewer necrotic areas than non-transgenic plants, resulting in an overall improved level of resistance. It could be assumed that this improved resistance was due to the activity of the chitinase enzyme encoded by the bean chitinase gene, leading to the hydrolysis of chitin. It has also been suggested that glycosidic fragments released by degradation of chitin can serve as elicitors of additional plant defence responses (Kurosaki *et al.* 1987).

Chitinase genes used in the previous studies encoded basic chitinase proteins (Zhang & Punja 1994; Nishizawa *et al.* 1999; Takahashi *et al.* 2004; Tohidfar *et al.* 2005; Shin *et al.* 2008). Acidic chitinases have been shown to have an-

tifungal activity *in vitro* (Zhang & Punja 1994; Tohidfar *et al*. 2005) similar to that of basic chitinases (Schlumbaum *et al*. 1986). This study was a pioneer report on the *in vivo* effective application of an acidic chitinase. The transgenic plants tested in this study clearly demonstrated an improved level of resistance to fungi in comparison with the control.

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