

# Evaluation of stability of *chitinase* gene in transgenic offspring of cotton (*Gossypium hirsutum*)

Masoud Tohidfar\*<sup>1</sup>, Hassan Rassouli<sup>2</sup>, Ali Haghazari<sup>2</sup>, Behzad Ghareyazie<sup>1</sup>, Javad Najafi<sup>2</sup>

<sup>1</sup>Agricultural Biotechnology Research Institute of Iran (ABRII) P.O. Box 31535-1897, Karaj, I.R. Iran <sup>2</sup>Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Zanjan, P.O. Box 313, Zanjan, I.R. Iran

## Abstract

Cotton cultivar Coker has been already transformed with recombinant pBI121-chi via *Agrobacterium tumefaciens*. The T-DNA region of pBI121-chi carries the *chitinase* (*chi*) gene from bean and is under the control of the CaMV35S promoter. T<sub>1</sub> and T<sub>2</sub> progenies of transgenic cotton containing the *chi* gene were used in this study. Polymerase chain reaction (PCR), Southern and Western blotting data confirmed integration and expression of the *chi* gene in the T<sub>1</sub> and T<sub>2</sub> progenies. The growth of *Verticillium dahliae* was significantly inhibited in an *in vitro* bioassay for which 100 µg of crude leaf protein extract derived from the T<sub>1</sub> plants was used. The 850-bp expected *chi* fragment was amplified for 77 transgenic plants from 128 T<sub>1</sub> and T<sub>2</sub> progenies, and 75 transgenic plants showed both *chi* and *nptII* bands. T<sub>0</sub> conduct bioassay, cotton seedlings were infected with the spore suspension (10<sup>6</sup> spores/ml), in a greenhouse. Fifty-five percent of the transgenic plants were able to restrict *V. dahliae* growth and symptoms. There were no distinguishable differences in the phenotypic appearance of transgenic plants compared to non-transgenics. These results showed that transgenic cotton expressing a bean chitinase exhibited enhanced resistance against *V. dahliae* in greenhouse and *in-vitro* assay as compared to the non-transgenic plants.

**Keywords:** Chitinase gene; Tolerance; Transgenic cotton; *Verticillium dahliae*

## INTRODUCTION

Cotton (*Gossypium hirsutum*) is a universally important crop. Cotton plants often suffer from many severe

diseases (Liu *et al.*, 2000). One of the most serious diseases of this crop plant is cotton wilt, which attacks seedlings and is caused by *Verticillium dahliae*. *Verticillium* wilt, caused by *Verticillium dahliae*, is a widespread disease that occurs in most cotton-producing areas. *V. dahliae* is a soil-borne pathogen that infects plants through the roots. Symptoms of infected cotton plants include stunting and wilting by some strains of *V. dahliae* and defoliation by other strains. The estimated loss of produce due to *V. dahliae* is 15-70%. Several classes of genes have been used in a genetic engineering approach to develop resistance in cotton to fungal pathogens. One group of genes, referred to as defence response genes encode proteins such as: β-1,3-glucanases, thaumatin-like proteins (tlps), ribosome-inactivating protein (RIPs), and chitinases. The defence response genes function in a variety of ways to inhibit fungal infection. Expression of these genes in transgenic plants has been shown to enhance fungal resistance (Muehlbauer and Bushnell, 2003). Although there are some tolerant cotton lines in Iran (e.g. Sahel), but their level of tolerance is not sufficient. It is therefore recommended to use genetic engineering approach to further enhance the resistance using genes like Chitinase (*chi*). As Iranian cotton varieties have low regeneration potential, most of the desirable genes are initially introduced into cultivar Coker which responds well to tissue culture. The resulting transgenic plants are then back crossed with other varieties.

The role of chitinase in plant defense against fungal attack has been very well documented (Lawrence and Novak, 2006; Adams, 2004). This enzyme is a glycanohydrolase which limits fungal growth by degrading poly [β-1,4-N-acetyl-b-D-glucozamine], i.e., chitin, the major structural polysaccharide of the fungal

\*Correspondence to: Masoud Tohidfar, Ph.D.  
Tel: +98 261 2703536; Fax: +98 261 2704539  
E-mail: gtohidfar@abrii.ac.ir

cell wall of *V. dahliae* (Adams, 2004). Basic chitinases isolated from bean (Schlumbaum *et al.*, 1986) and acidic ones isolated from cucumber (Zhang and Punja 1994) have shown fungicidal activity *in vitro*. Hence, chitinases hydrolyse the chitin in fungal hyphae and kill the fungus without causing damage to the plant cell. Several studies have demonstrated that enhanced chitinase levels in transgenic plants increase the resistance of plants to fungal pathogens (Nandakumar *et al.*, 2005; Rajasekaran *et al.*, 2005; Tohidfar *et al.*, 2005; Lorito *et al.*, 1998; Tabei *et al.*, 1998; Asao *et al.*, 1997; Broglie *et al.*, 1991; Toyoda, 1991). Molecular characterization and evaluation of transgenic cotton lines for resistance to *V. dahliae* and expressing chitinase gene was the objective of this study.

## MATERIALS AND METHODS

**Plant materials:** Seeds of first and second progenies (T<sub>1</sub> and T<sub>2</sub>) of transgenic T<sub>0</sub> plants (cultivar Coker) were transformed using plasmid pBI121-CHI (Tohidfar *et al.*, 2005). Along with seeds from non-transgenic control plants were used in this study. Seeds were sterilized in HgCl<sub>2</sub>, were rinsed in sterile water and were placed on the germination medium (MS; Murushige and Skoog, 1962) medium with 3% sucrose and 0.8% agar (w/v) as described by Tohidfar *et al.*, (2005). Plantlets were potted in soil for growth in the greenhouse. After 4 weeks, all plants and nontransformed control plants were inoculated by dipping the roots of separate groups of plants in spore suspensions of *V. dahliae* (10<sup>6</sup> spores/ml) for 20 min. After six weeks, symptoms were appeared and their changes in plant height chlorosis, leaf number and flowering date were noted every week (Mcffaden *et al.*, 2001).

**Polymerase chain reaction (PCR):** Genomic DNA of cotton plant was extracted from young leaves following the protocol of Haiwen *et al.* (2001), using the PCR Kit (Cinagen, Tehran, Iran) with following pairs of primers:

*nptII* F 5'-GAACAAGATGGATTGCACGC-3'  
*nptII* R 5'-GAAGAACTCGTCAAGAACGC-3'  
*chi* F 5'-GAGTGGTGTGGATGCTGTTG-3'  
*chi* R 5'-GCCATAACCGACTCCAAGCA-3'

The PCR profile included a 95°C for 5 min followed by 30 cycles for 30 s at 94°C, 1min at 60°C (*chi*) and 55°C (*nptII*) for annealing, 1min at 72°C for elonga-

tion and final extension at 72°C for 5 min. Expected PCR products size were about 785 bp and 870 bp for *nptII* and *chi* genes respectively.

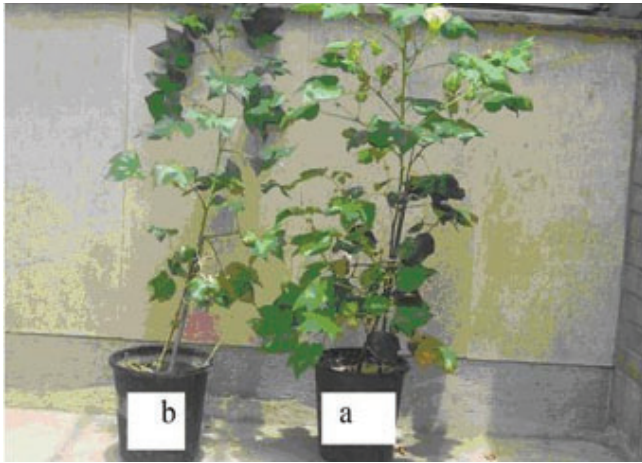
**Southern blot hybridization analysis:** Southern blot analysis of plant genomic DNA was carried out using standard protocols (Sambrook and Russell 2001) to confirm the transgenic status of plants showing a positive reaction in PCR analysis. Briefly, 20 µg of cotton genomic DNA was digested with *Xba*I and *Eco*RI, electrophorezed in 0.8% agarose gel and transferred onto Hybond- N<sup>+</sup> nylon membrane (Amersham, Buckinghamshire, UK). A 860 bp PCR product of the coding sequence of the *chi* gene was labeled with DIG DNA labeling kit (Boehring Mannheim, Germany) and used as probe.

**Western blot analysis:** Extraction of proteins from fresh leaves of the PCR-positive plants and western blot analysis were performed according to Sambrook and Russell (2001). Ten µg of proteins extracted from leaves of PCR positive and control plants were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). After electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose filter. The rabbit anti-*chi*-I anti-serum (1:2,000 v/v) (a gift from Dr. Els van Deventer, Zeneca Mogen International, Leiden, The Netherlands) and alkaline phosphatase-conjugated goat antirabbit IgG (Promega, Madison, WI, USA; 1:5,000, v/v) was used as the primary and secondary antibody, respectively. Total soluble protein was measured using Bradford method (Bradford, 1976). BCIP/NBT (5-Bromo-4-chloro-3-indolyl Phosphate (BCIP)/Nitroblue Tetrazolium (NBT)) was used for visualization.

***In vitro* effect of transgenic plants on the growth of *V. dahliae*:** SS<sub>4</sub> isolate of *V. dahliae* was obtained from infected cotton fields in Gorgan, Iran. This isolate was used to produce a mycelial plug. The inhibitory activity of extracts from transgenic cotton was assessed *in vitro* following the method of Tohidfar *et al.*, (2005).

## RESULTS

***In vivo* effect of bean chitinase on the growth of *V. dahliae*:** All T<sub>1</sub> and T<sub>2</sub> transgenic plants survived



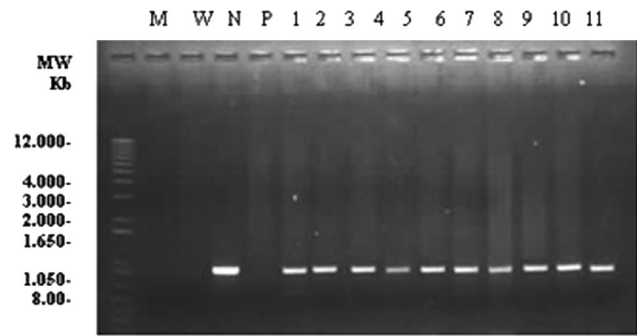
**Figure 1.** Comparison between a) transgenic b) non-transgenic cotton. Non-transgenic plant could not restrict *V. dahliae* growth and damage.

acclimatization and grew to maturity. Fifty-five percent of the examined transgenic plants could restrict *V. dahliae* growth and symptoms. There were no discernible differences in the phenotypic appearance, height, leaf number and flowering date of transgenic plants as compared to those of non-transgenic plants. However chlorosis (symptom of infection) were significantly less in transgenic plants compared to that of nontransgenic ones. Some of the transgenic plants developed into dwarves and could not restrict *V. dahliae* growth and symptoms (Fig. 1).

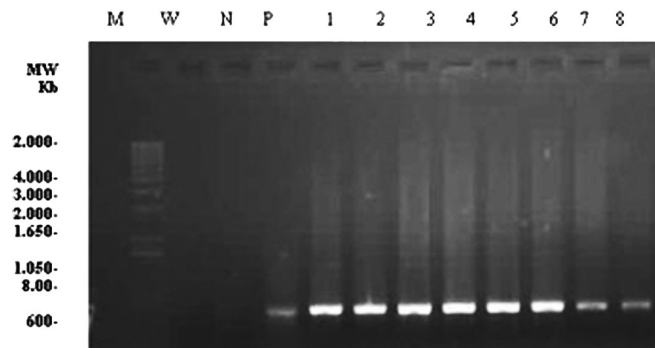
**PCR and Southern blot analysis:** PCR analysis was carried out initially to confirm the transgenic nature of the progeny plants. Bands of 870 bp (*chi*) and 785 bp (*nptII*) in length were amplified from nuclear DNA for 77 T<sub>1</sub> and T<sub>2</sub> transgenic plants (Table 1). Seventy five

**Table 1.** Stability of the chitinase (*chi*) gene in different transgenic events as judged by their transfer into the next generation. Table summarizes the results of PCR analysis of the T<sub>1</sub> progeny derived from 7 T<sub>0</sub> plants for the presence of the *chi* gene.

Line	<i>chi</i> +	<i>chi</i> -	Total
T <sub>1</sub> 11	30	14	44
T <sub>1</sub> 18	2	3	5
T <sub>2</sub> 20/1	15	14	29
T <sub>2</sub> 11/3	9	2	11
T <sub>1</sub> 28	4	1	5
T <sub>1</sub> 30	11	10	21
T <sub>1</sub> 35	6	6	12
Total	77	50	127



**Figure 2.** PCR analysis of DNA isolated from leaves of transgenic cotton using specific primer pairs for amplification of the 870 bp bean chitinase (*chi*) gene. M, 1.0 Kb plus DNA ladder (Gibco BRL); W, water (No DNA); P, positive control (pBI121-*chi*); N, negative control (non-transgenic cotton). Lanes 1-11 represent DNA from transgenic lines.



**Figure 3.** PCR analysis of DNA isolated from leaves of T<sub>1</sub> transgenic cotton using specific primer pairs for amplification of the 785 bp *nptII* gene. M, 1.0 kb plus DNA ladder (Gibco BRL); W, water (No DNA); P, positive control (pBI121 (*chi*)); N, negative (non transgenic plant) control. 1-8 represent DNA from T<sub>1</sub> lines.

plants showed both *chi* and *nptII* bands (Figs. 2 and 3). The  $\chi^2$  (chisquare test) test of T<sub>1</sub> plants (line 11) indicated that the inheritance of both *chi* and *nptII* genes follows the Mendelian ratio for a single copy gene (3:1). No amplification was obtained from nontransgenic plants (Table 2).

Southern blot analysis showed that the hybridization of probe to the undigested was exclusively at high molecular weight, indicating the integration of the gene into the cotton genome. When the DNA was digested with *EcoRI*, most of the hybridization was corresponded to an expected fragment of ~900 bp including the whole coding sequence of the *chi* gene. This result showed that there is at least one copy of the integrated *chi* gene in the genome (Fig. 4). When the DNA digested with *XbaI* was hybridized to the probe, only one band of different size was detected in each of

**Table 2.** The  $\chi^2$  (Chisquare test) for 44 T<sub>1</sub> plants derived from line T<sub>11</sub>. Accordingly the 3:1 mendelian ratio is confirmed.

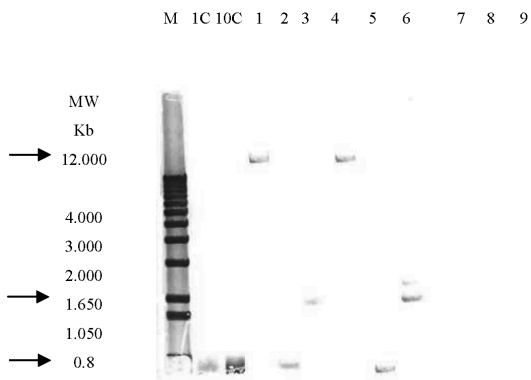
X	O <sub>i</sub>	E <sub>i</sub>	O <sub>i</sub> -E <sub>i</sub>	(O <sub>i</sub> -E <sub>i</sub> ) <sup>2</sup>	(O <sub>i</sub> -E <sub>i</sub> ) <sup>2</sup> /E <sub>i</sub>
3	30	33	-3	9	0.2727
1	14	11	3	9	0.818
Total	44	44	-	-	1.09ns

ns: non-significant

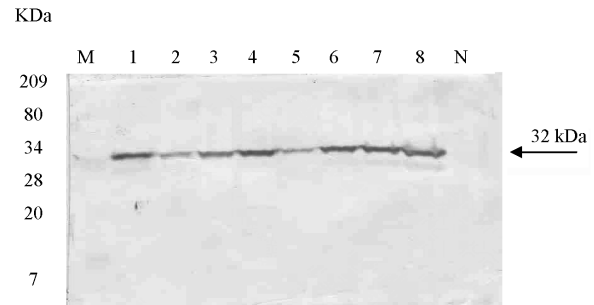
the different transgenic lines examined. Since there is only one *Xba*I site in the T-DNA, this result indicated the presence of only one single copy of the transgene in each of the tested events. No hybridization signal could be detected for the DNA extracted from untransformed plant.

**Western blot analysis:** Forty four transgenic plants were analyzed for *chi* expression using Western blot analysis. Extracts of thirty nine plants had one protein band of expected size (32 kDa) that reacted with a bean chitinase antibody (Fig. 5). The other 5 plants had no detectable chitinase. The level of chitinase expression varied among the transgenic plants. Untransformed control plants did not show any positive signal for the chitinase protein.

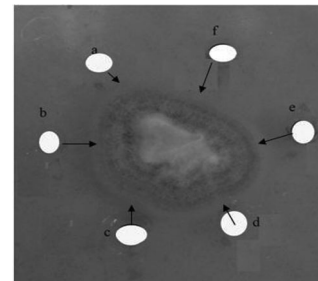
**In vitro effect of bean chitinase on the growth of *V. dahliae*:** Leaf tissue extracts from T<sub>1</sub> and T<sub>2</sub> plants



**Figure 4.** Southern blot analysis of DNA isolated from leaves of T<sub>1</sub> transgenic cotton. M, 1.0 Kb plus DNA ladder (Gibco BRL); 1C, reconstructed one copy equivalent of transgene in a haploid cotton genome; 10C, reconstructed ten copy equivalent of transgene in a haploid cotton genome; lanes 1 and 4, undigested DNA extracted from transgenic cotton. Lane 2 and 5 *Eco*RI digested DNA extracted from transgenic cotton, lanes 3 and 6 *Xba*I digested DNA extracted from transgenic cotton, lane 7 undigested DNA extracted from non-transformed cotton, Lane 8, *Eco*RI digested DNA extracted from non-transformed cotton and 9, *Xba*I digested DNA extracted from non-transformed cotton.



**Figure 5.** Western immunoblot analysis of chitinase (*chi*) gene expression in leaf extracts of T<sub>1</sub> transgenic cotton lines. M, pre-stained standard molecular weight marker in kilo Daltons (kDa); N, extract from non-transformed cotton; lanes 1-8, leaf extracts of transgenic lines#11.2, 11.4, 11.7, 11.9, 18.1, 18.6, 28.1 and 28.4, respectively. The arrowhead indicates the expected 32-kDa chitinase band. The level of chitinase expression varied among the individual T<sub>1</sub> transgenic plants. Untransformed control did not show any positive signal for chitinase protein.

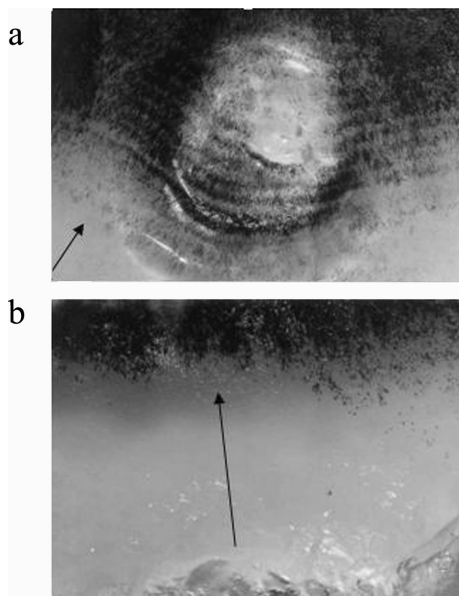


**Figure 6.** Inhibitory activity of leaf extracts from transgenic cotton line#18 expressing chitinase gene against *V. dahliae* on PDA. A mycelial plug (0.5 cm diameter) was placed at the center of PDA plate surrounded by peripheral wells containing (a) 50 and (b) 100 µg of leaf protein extracts prepared in 10 mM sodium acetate buffer (pH 5.0) derived from untransformed cotton plants, (c) 50 and (d) 100 µg of extraction buffer and (e) 50 and (f) 100 µg of leaf protein extracts from transformed cotton plant.

inhibited mycelial growth of *V. dahliae*, whilst the extracts from negative control did not have any inhibitory effect (Fig. 6). There was no significant difference between mycelial growth when different amounts of leaf extracts from transgenic plant were used at 50-100 µg range. Leaf tissue extracts from transgenic plants lysed the hyphal tip; hyphal morphology was distorted, and hyphae became gradually thinner and shorter, eventually turning dark and not sporulating (Fig. 7).

## DISCUSSION

In the last two decades efforts have been made on the transgenic expression of plant fungal chitinases in crop



**Figure 7.** A close-up picture showing the effect of leaf extracts from control or T<sub>1</sub> transgenic cotton on mycelial growth of *V. dahliae*. a) control, fungal mycelium has overgrown into the well on PDA and mycelia appear normal; whereas in b) mycelial growth has stopped before reaching the wells containing the extract from transgenic leaf tissue and have lysed.

plants, and significant improvements in the resistance to fungal diseases have been recorded (Nandakumar *et al.*, 2005; Rajasekaran *et al.*, 2005; Pappinen *et al.*, 2002; Lorito *et al.*, 1998; Tabei *et al.*, 1998; Asao *et al.*, 1997; Broglie *et al.*, 1991). Plant chitinases have been shown to inhibit fungal growth *in vitro* by degrading chitin polymers in fungal cell walls (Noel *et al.*, 2005; Nielsen *et al.*, 1994; Toyoda *et al.*, 1991; Hüttermann and Cwielong 1982). However, the genes which have been used in these studies encode basic chitinases. Acidic chitinases have also been shown to have antifungal activity *in vitro* (Nishizawa *et al.*, 1999; Raharjo *et al.*, 1996; Zhang and Punja, 1994; Schlumbaum *et al.*, 1986).

Our study also shows the antifungal effect of chitinase in our transgenic progenies. There are some reports on the production of transgenic plants with improved resistance to fungal diseases using a chitinase gene (Grisson *et al.*, 1996; Lin *et al.*, 1995; Jach *et al.*, 1992; Broglie *et al.*, 1991). Several reports have also stressed the advantages of using chitinases for plant protection because these fungicidal enzymes are part of the plant defense system and not harmful to plants as the substrate chitin is not found in plants (Lorito *et al.*, 1998; Collinge *et al.*, 1993). Copy number of the transgene (*chi*) is one of the most important factors implicated in the stability of gene expression in

transgenic plants (Katleen, 2005). Our results showed the integration of a single copy of *chi* for line#11 and several other independent events (data not shown).

Significant differences in the level of expression of the chitinase gene among the transgenic T<sub>0</sub> cotton plants have been reported earlier (Tohidfar *et al.*, 2005). Our western blot analysis showed the differential expression of chitinase among the progenies. When the transgenic T<sub>1</sub> and T<sub>2</sub> cotton plants were challenged with *V. dahliae*, they developed less necrotic areas than nontransgenic plants, resulting in an overall improved resistance. A positive correlation was established between the level of chitinase expression and restriction of fungal growth and damage. It has also been suggested that glycosidic fragments released by degradation of chitin can serve as elicitors of additional plant defense responses (Kurosaki *et al.*, 1987). Five of the progeny plants had no detectable chitinase. This could be resulted from a gradual differential gene silencing process (Jerzy *et al.*, 2001).

## CONCLUSION

We also tested the chitinase transgenic cotton against *Verticillium dahliae*, we observed a low level of resistance against this pathogen. This may be due to the fact that the chitinase gene alone may not provide effective protection against the pathogen where chitin is either not a major constituent of the fungal cell wall or is not easily accessible to enzyme action. It may be possible to confer resistance to this pathogen by combining chitinase genes with other antifungal genes such as beta 1,3 glucanase. Regardless, our results with *Verticillium dahliae* provide the first convincing demonstration of the usefulness of a bean-derived endochitinase in conferring an effective resistance against a soil-born in cotton.

## References

- Adams DJ (2004). Fungal cell wall chitinase and glucanases. *Microbiology* 150: 2029-2035.
- Asao H, Nishizawa Y, Arai S, Sato, T Hirai M, Yoshida K, Shinmyo A, Hibi T (1997). Enhanced resistance against a fungal pathogen *Sphaerotheca humuli* in transgenic strawberry expressing a rice chitinase gene. *Plant Biotechnol.* 14: 145-149.
- Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248-254.

- Brogliè K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Brogliè R (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254: 1194-1197.
- Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993). Plant chitinases. *Plant J.* 3: 31-40.
- Haiwen L, Hemphill J, Wang JT, Gould J (2001). A Rapid and High Yielding DNA Miniprep for Cotton (*Gossypium* spp.). *Plant Mol Biol Rep.* 19: 183-183.
- Grisson R, Grezes-Besset B, Schneider M, Lucante N, Olsen L, Leguay JJ, Toppan A (1996). Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. *Nat Biotechnol.* 14: 643-646.
- Jach G, Logeman S, Wolf G, Oppenheim A, Chet I, Schell J, Logeman J (1992). Expression of a bacterial chitinase leads to improved resistance of transgenic tobacco plants against fungal infection. *Bioprocess* 1: 33-40.
- Hüttermann A, Cwielong P (1982). The cell wall of *Fomes annosus* (*Heterobasidion annosum*) as target for biochemical control. I. Direct lysis of the cell wall by lytic enzymes from *Trichoderma harzianum*. *Eur J Pathol.* 12: 238-245.
- Jerzy P, Whitham S (2001). Gene silencing and DNA methylation processes. *Plant Biol.* 4: 123-129.
- Katleen MJ, Butaye PA, Cammue SL, Delaure M FC (2005). Approaches to minimize variation of transgene expression in plants. *Mol Breed.* 16: 79-91.
- Kurosaki F, Tashiro N, Nishi A (1987). Secretion of chitinase from cultured cells treated with fungal mycelium walls. *Mol Plant Pathol.* 31: 211-216.
- Lawrence SD, Novak NG (2006). Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. *Biotechnol Lett.* 28: 598-599.
- Lin W, Anuratha CS, Datia K, Potrykus I, Muthukrishnan S, Datia SK (1995). Genetic engineering of rice for resistance to sheath blight. *Biotechnology* 13: 686-691.
- Liu J Y, Zhao G R, Li J (2000). Molecular engineering on quality improvement of cotton fiber. *Acta Bot Sin.* 42: 991-995.
- Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc Natl Acad Sci USA.* 95: 7860-7865.
- McFadden H, Beasley D, Brubaker C (2004). Assessment of *Gossypium sturtianum* and *G. australe* as potential sources of Fusarium wilt resistance to cotton. *Euphytica* 138: 61-72.
- Muehlbauer GJ, Bushnell WR (2003). Transgenic approaches to resistance. In: Leonard KJ, Bushnell WR, eds. *Fusarium head Blight of wheat and barley*. St Paul, MN: American Phytopathological Society Press.
- Nandakumar R, Babu S, Radjacommaro R, Raguchander T, Samiyappan R (2005). *Pseudomonas fluorescens* mediated antifungal activity against *R. solani*, sheath blight pathogen. *Phytopathol Mediterr.* 41: 109-119.
- Nielsen KK, Jorgensen P, Mikkelsen JD (1994). Antifungal activity of sugarbeet chitinase against *Cercospora beticola*: an autoradiographic study on cell wall degradation. *Plant Pathol.* 43: 979-986.
- Nishizawa Y, Nishio Z, Nakazono K, Soma M, Nakajima E, Ugaki M, Hibi T (1999). Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic japonica rice by constitutive expression of rice chitinase. *Plant Cell Report.* 99: 383-390.
- Noël A, Lévassieur C, Le VQ, Seguin A (2005). Enhanced resistance to fungal pathogens in forest trees by genetic transformation of black spruce and hybrid poplar with a *Trichoderma harzianum* endochitinase gene. *Physiol Mol Plant Pathol.* 67: 92-99.
- Pappinen, A, Degefu Y, Syrjälä L, Keinonen K, von Weissenberg K (2002). Transgenic silver birch (*Betula pendula*) expressing sugarbeet chitinase 4 shows enhanced resistance to *Pyrenopeziza betulicola*. *Plant Cell Rep.* 20: 1046-1051.
- Raharjo SHT, Hernandez MO, Zhang YY, Punja ZK (1996). Transformation of pickling cucumber with chitinase encoding genes using *Agrobacterium tumefaciens*. *Plant Cell Rep.* 15: 591-596.
- Rajasekaran K, Cary JW, Jaynes JM, Cleveland TE (2005). Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (*Gossypium hirsutum* L.) plants. *Plant Biotechnol J.* 3: 545-554.
- Sambrook J, Russell DW (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY. PP. 6.39-6.64.
- Schlumberg A, Mauch F, Vögeli U, Boller T (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature* 324: 365-367.
- Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998). Transgenic cucumber plants harbouring a rice chitinase gene exhibit enhanced resistance to Gray mold (*Botrytis cinerea*). *Plant Cell Rep.* 17: 159-164.
- Tohidfar M, Mohammadi M, Ghareyazie B (2005). *Agrobacterium-mediated* transformation of cotton (*Gossypium hirsutum*) using a heterologous bean chitinase gene. *Plant Cell Tissue Organ Cult.* 83: 83-96.
- Toyoda H, Matsuda Y, Yamaga T, Ikeda S, Morita M, Tamai T, Ouchi S (1991). Suppression of the powdery mildew pathogen by chitinase microinjected into barley coleoptile epidermal cells. *Plant Cell Rep.* 10: 217-220.
- Zhang YY, Punja ZK (1994). Induction and characterization of chitinase isoforms in cucumber (*Cucumis sativus* L.) effect of elicitors, wounding and pathogen inoculation. *Plant Sci.* 99: 141-150.