ORIGINAL RESEARCH PAPER

Cloning and functional analysis of a novel chitinase gene *Trchi1* from *Trichothecium roseum*

Hongquan Xian · Jiarui Li · Liqing Zhang · Duochuan Li

Abstract Chitinases produced by mycoparasites play an important role in disease control in plants. To explore the functions of chitinases in *Trichothecium roseum*, we cloned a new chitinase gene named *Trchi1* from *T. roseum* by RT (reverse transcription)-PCR techniques. The *T. roseum* gene, *Trchi1*, contains an 1278-bp ORF that shares 76 % similarity with chitinase from *Bionectria ochroleuca* (ABV57861 3G6L_A). A plant expression vector, containing the *Trchi1* gene driven by the CaMV35S promoter, was constructed and transformed into tobacco via *Agrobacterium tumefaciens*. Southern blot analysis showed that *Trchi1* was integrated into the tobacco genome. Total chitinase activity in *Trchi1*-transgenic tobacco leaves was enhanced 2.2- to 5.8- times with respect to non-transgenic leaves. Transgenic tobacco plants transformed with the *Trchi1* gene had increased resistance to *Alternaria alternata* and *Colletotrichum nicotianae*.

Keywords Chitinase genes · Disease resistance · Transgenic tobacco · *Trichothecium roseum*

H. Xian · L. Zhang · D. Li
Institute of Plant Protection, Shandong Agricultural University, Taian 271018, China
e-mail: lidc20@sdau.edu.cn

H. Xian
College of Life Sciences, Qingdao Agricultural University, Qingdao 266109, China

J. Li (✉)
Department of Plant Pathology, Kansas State University, Manhattan, KS 66502, USA
e-mail: jili2@ksu.edu

L. Zhang
Taishan Medical College, Taian 271018, China

Introduction

Fungi that parasitize the mycelia of other fungi are termed mycoparasites. Mycoparasites of plant pathogenic fungi have received much attention due to their use as biocontrol reagents. *Trichothercium roseum* is a parasitic fungus that can parasitize *Sclerotinia sclerotiorum* and *Pestalotiopsis funerea* (Huang and Kokko 1993) and inhibit a variety of plant pathogenic fungi (Munshi et al. 2009; Jayaprakashvel et al. 2010; Shafat 2010).

Chitin is the second most abundant organic and renewable compound in nature after cellulose. Chitinases are chitin-degrading enzymes that have important biological functions and immense potential applications. In recent years, much research has focused on fungal chitinases, especially at the molecular level (Li 2006). Chitinases produced by mycoparasites can inhibit mycelial growth, spore germination, and germ tube elongation (Viterbo et al. 2001), degrading not

only the tips of mature hyphae, but also the chitin-glucan complex of the cell wall and sclerotia (Lorito et al. 1993). Extracellular chitinase produced by *Trichothecium roseum* has been reported to be involved in the process of re-parasitic and antagonistic interactions (Li et al. 2004).

Chitinase genes isolated from different fungi have been used to enhance the resistance of crops and trees to fungal pathogens. The mechanism of chitinase action involves chitin degradation and cell wall disruption, leading to fungal cell lysis (Dahiya et al. 2006). Bolar et al. (2001) transformed *ech42* encoding endochitinase and *nag70* encoding exochitinase from *Trichoderma atroviride* into apple. The transgenic apple plants were highly resistant to *Venturia inaequalis*, the causal reagent of apple scab. Kern et al. (2010) demonstrated that expression of a chitinase gene from *Metarhizium anisopliae* in tobacco plants conferred resistance against *Rhizoctonia solani*. Hassan et al. (2009) observed that a family 19 chitinase (Chit30) from *Streptomyces olivaceoviridis* ATCC 11238 expressed in transgenic pea affected the development of *Trichoderma harzianum* in vitro. Jia et al. (2010) found that overexpression of the chitinase gene *BbchiT1* from *Beauveria bassiana* resulted in enhanced resistance to *Cytospora chrysosperma* in *Populus tomentosa* Carr. Kumar et al. (2009) provided a powerful demonstration of the disease protection for *Rhizoctonia solani* using transgenic cotton plants that expressed an endochitinase gene from *Trichoderma virens*.

Trichothecium roseum is an important mycoparasitic fungus with significant antifungal activity but studies on chitinases of *T. roseum* have been limited. Purified chitinase from *T. roseum* strongly inhibits the growth and development of chitin-containing fungi in vitro (Li et al. 2004). Expression of chitinase genes in plants improves their defense responses against various fungal pathogens. In particular, chitinases from mycoparasitic *Trichoderma* spp. were overexpressed in several agriculturally important plants such as lemon, cotton, apple, and carrot (Dana et al. 2006; Kumar et al. 2009). For example, the *chit42* gene of *T. harzianum* encoded a powerful endochitinase and was expressed constitutively in tobacco, and these transgenic plants showed broad resistance to fungal and bacterial pathogens, salinity, and heavy metals (Dana et al. 2006).

In this study, we cloned the chitinase gene *Trchi1* from *T. roseum* and transferred it into tobacco plants by the *Agrobacterium*-mediated transformation. Our results indicate that *Trchi1* may have efficacy for improving plant resistance to fungal pathogens.

Materials and methods

Bacterium and fungal strains, reagents, vectors, and plant materials

The M1 strain of *Trichothecium roseum* and plasmid pROKII (Sharma and Anjaiah 2000) were isolated and stored in our laboratory. *E. coli* DH5α strains, pMD 18-T Vector, RNA PCR Kit (AMV) Ver 3.0, TaKaRa *EX Taq, EcoR*I, and T4 DNA ligase were from TaKaRa Biotechnology Co., Ltd. (Dalian, China). *Xba*I and *Xma*I endonucleases were purchased from New England Biolabs (Ipswich, MA, USA). DIG High Prime DNA Labeling and Detection Starter Kit I was purchased from Roche Diagnostics GmbH (Mannheim, Germany). AMV Reverse Transcriptase was purchased from Promega (Madison, WI, USA). *Agrobacterium tumefaciens* strain LBA4404 and strains *A. alternata* and *C. nicotianae* were provided by Dr. Shao-Hui Chu, Dr. Yuancun Lian, and Dr. Guangmin Zhang, respectively, from the Institute of Plant Protection, Shandong Agricultural University, China. Tobacco (*Nicotiana tabacum* L.) cultivar NC89 plants were grown in the greenhouse at Shandong Agricultural University.

Cloning of the *Trchi1* gene

According to the amino acid sequences of chitinase genes from 16 different fungi (Supplementary Fig. 1), three conserved domains having amino acid sequences LSIGGWT, DG-D-DWE, and MFWEASA, respectively were selected for primer design. The degenerate primers Ps446, Ps561, and Pa were designed to amplify the chitinase gene from *T. roseum* (Supplementary Table 1).

Trichothecium roseum was cultured in SMCS medium at 25 °C for 3–4 days (Li et al. 2004). Total RNA was isolated from cultured *T. roseum* using Trizol reagent. First-strand cDNA synthesis was conducted using RNA PCR Kit (AMV) Ver. 3.0. Using cDNA first strand as a template, we performed nested PCR amplification using Ps446 and Ps561 as upstream
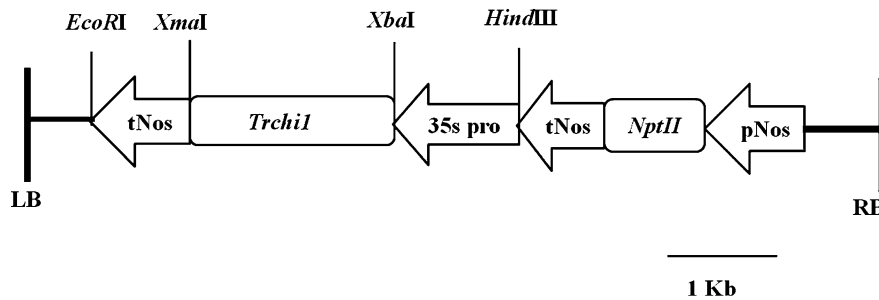
Fig. 1 T-DNA region of vector pROKII/ycTrchi1. *LB* left border, *RB* right border, *tNos* nos terminator, *Trchi1* 1,700-bp *Trchi1* coding sequence, *35 s pro* CaMV35 s promoter, *NptII* neomycin phosphotransferase II gene for kanamycin resistance, *pNos* nos promoter

primers and Pa as a downstream primer. The PCR amplification was carried out for 34 cycles. The initial denaturation reaction was done at 94 °C for 4 min. 34 cycles were performed with denaturation (94 °C, 30 s), annealing (50 °C, 45 s), and extension (72 °C, 1 min), followed by 72 °C for 10 min.

The 3′-end flanking sequence of *Trchi1* was isolated by 3′-RACE PCR using RNA PCR Kit (AMV) Ver. 3.0. We used specific primers M8501pa1 and M8501pa2 as forward primers, and M13 Primer M4 as reverse primer.

The 5′-flanking sequence of *Trchi1* was cloned by TAIL-PCR (Liu et al. 1995). The first TAIL-PCR was performed by using 5MX8501Ps2, 5m7303ps2, and 5m7303ps1 as specific primers, and a random degenerate primer as forward primer. The annealing temperatures for specific amplifications were 60, 63, and 65 °C. The second TAIL-PCR amplification was performed using 5M8501RPs1, 5M8501RPs2, and 5M8501RPs3 as nested primers with annealing at 59, 63 and 60 °C over the three rounds of reactions.

Using *T. roseum* cDNA and genomic DNA as templates, psq8500 and paq8501 primers were used for PCR amplification. The PCR procedures were as follows: 94 °C for 4 min, 32 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min, and the final extension at 72 °C for 10 min.

For all above-mentioned PCR reactions, 2 μl cDNA, 2.5 μl of 10× Taq buffer, 2 μl of 10 mM dNTP, 0.5 μl (2.5 U) *EX Taq*, and 10 pmol of each pair of primers in 25 μl were used. The PCR products were separated by electrophoresis on 1 % agarose gels in TAE buffer and were observed by fluorescence with UV light after staining with ethidium bromide. PCR products were ligated to the cloning vector pMD18-T by T4 DNA ligase and transformed into *E. coli*

DH5α. Positive colonies were confirmed by PCR and sequencing.

Construction of plant expression vector

Using *T. roseum* cDNA as the template, ycm8501bs and ycm8501ba primers were used for the amplification of the full-length *Trchi1* gene, which was cloned into the pMD18-T vector using T4 DNA ligase, and the resulting vector was named pMD18-T/*ycTrchi1* (Supplementary Fig. 2c). The vectors pROKII and pMD18-T/ycTrchi1 were digested with *Xba*I and *Xma*I, and ligated using T4 DNA ligase (Supplementary Fig. 2d). The final plant expression vector was named pROKII/ycTrchi1 (Fig. 1), and was confirmed by PCR and enzyme digestion.

Genetic transformation of tobacco

The pROKII/ycTrchi1 was transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze–thaw method (Chen et al. 1994). Leaves of in vitro cultured tobacco NC89 plants were cut into 0.5 cm × 0.5 cm leaf discs and immersed into the *Agrobacterium* culture for 10 min. The leaf discs were then cultured on MS medium (Murashige and Skoog 1962) in the dark for 2–3 days, then cultured on MS supplemented with 3 mg 6-BA/l, 0.2 mg NAA/l, 100 mg kanamycin/l, and 500 mg carbenicillin/l and solidified with Gelrite. When regenerated shoots were approx. 2 cm, the adventitious buds were moved into MS medium for root formation. When the seedlings were 5–7 cm high, they were transferred to soil and moved into a greenhouse for further growth. T_1 transgenic seeds were harvested from different T_0 transgenic lines, and T_1 transgenic seedlings were used for molecular analysis and fungi bioassay.

Molecular identification of transgenic tobacco plants

Genomic DNA was isolated from T_1 transgenic tobacco plants according to the methods of Dellaporta et al. (1983). Using tobacco genomic DNA as the template, PCR was performed to confirm the presence of transgenes using specific primers psq8500 and paq8501. Southern hybridization was used to identify the integration pattern of transgenes. Transgenic tobacco genomic DNA was digested with *Hin*dIII. We used a 432-bp fragment of *Trchi1* amplified with specific primers ycm8501bs and 5M8501RPs4 as the template to synthesize probes labeled with digoxin. The procedures for Southern hybridization were referred to the DIG High Prime DNA Labeling and Detection Starter Kit I.

RT-PCR analysis of transgenic tobacco plants

Total RNAs from different transgenic tobacco plants were isolated using Trizol. For RT-PCR, 1 μg total RNA was reverse transcribed using AMV Reverse Transcriptase following the manufacturer's instructions. We performed RT-PCR using the gene-specific primers psq8500 and paq8501 (Supplementary Table 1). PCR cycling comprised an initial step at 94 °C for 5 min, followed by 31 cycles at 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 50 s.

Enzyme assay

Tansgenic tobacco leaves (1 g) were powdered in liquid N_2, transferred to a microcentrifuge tube, mixed with 3 ml 0.05 M phosphate buffer (pH 6.5) and centrifuged for 20 min at 4 °C at ∼10,000 *g*. The supernatant was used directly for chitinase activity measured as previously reported (McCreath and Gooday 1992) using 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside as substrate.

Fungi resistance of transgenic tobacco plants

A. alternata and *C. nicotianae* were placed in the center of a potato dextrose agar (PDA) plate and cultured at 25 °C to cover the entire plate. Two mature leaves per plant from transgenic plants were taken and non-transgenic plants at similar growth stage. Three small wounds were made between secondary veins of

the backside of half the leaf with a needle and the fungi were inoculated into the wound sites. Three wounds were also made in the other half of each leaf but were not inoculated to be used as controls. The leaves were sealed with plastic wrap and placed on 4 layers of wet gauze in a porcelain plate at 25 °C. After 9 days, we checked inoculated leaves for fungal growth.

Results and discussion

Cloning of *T. roseum* chitinase gene

A 656-bp DNA sequence of *T. roseum* chitinase gene was obtained using Ps561 and Pa primers. Starting from this sequence, by 3′-RACE PCR, a 481-bp sequence was obtained, with 108-bp overlapping with the original 656-bp sequence. After three rounds of TAIL-PCR using nested primers 5MX8501Ps2, 5m7303ps2, and 5m7303ps1, we achieved a 1,000-bp fragment with 286-bp overlapping with 656-bp sequence. For the second TAIL-PCR using specific primers 5M8501RPs1, 5M8501RPs2, 5M8501RPs3, we obtained a 650-bp fragment with 108-bp overlapping with the 1,000-bp sequence. These four fragments were assembled in silico. Finally, using cDNA and genomic DNA as templates, we amplified the full length *T. roseum* chitinase gene by PCR using specific primers psq8500 and paq8501 designed on the vitual sequence. A 1,700-bp and 1,818-bp fragments were obtained from cDNA and genomic DNA, respectively (Supplementary Fig. 2a, b), sequenced, and deposited in Genbank with accession numbers GU361768 and GU361767, respectively.

The sequence analysis of *Trchi1* gene and putative protein

The *Trchi1* gene contains a 1,278-bp ORF, which putatively encodes a 425 amino acid protein. The genomic sequence contains two introns of 62 and 56-bp.

The deduced amino acid sequences of *Trchi1* gene were compared with those of other chitinases using the BLASTx program. Phylogenic relationships between the homologous genes were analyzed by the DNA-MAN software (Supplementary Fig. 1). The results showed that *Trchi1* had the highest similarity (76 %) with a chitinase from *Bionectria ochroleuca* (Genbank
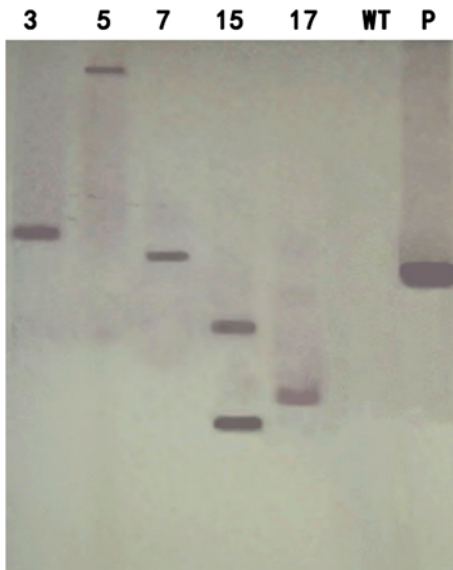
Fig. 2 Southern blot analysis. A 432-bp fragment of the *Trchi1* gene was used as the template to synthesize probes labeled with digoxin. 3, 5, 7, 15, and 17: T_1 transgenic lines, *WT* non-transgenic plants, *P* positive control, vector pROKII/ycTrchi1 (1 ng)
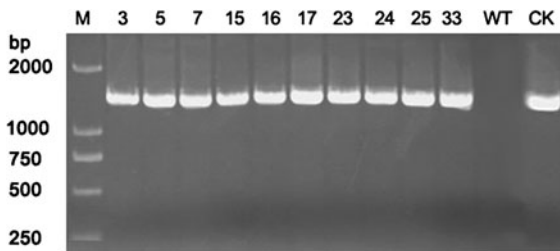

Fig. 3 RT-PCR identification of transgenic tobacco plants M: DL2000 marker; 3, 5, 7, 15-17, 23-25, 33: T_1 transgenic lines, *WT* non-transgenic tobacco, *CK* positive control, vector pROKII/yctrchi1 (1 ng)

accession No. 3G6L_A). Surprisingly, *Trchi1* had low similarity with chitinases from mycoparasites, indicating that *Trchi1* may be a new chitinase gene. Sequence analysis using Scanprosite software (http://www.expasy.ch/tools/scanprosite/) showed that the deduced *Trchi1* protein belongs to family 18, which was confirmed by the specific sequence FDGIDIDWE (position 165–173) for this family. *Trchi1* also contains a highly conserved region DGXDXDXE, which is the substrate-binding domain. Sequence analysis of *Trchi1* by SignalP-NN software (http://www.cbs.dtu.dk/services/SignalP) indicated that the putative amino acid sequence of *Trchi1* contains a signal peptide
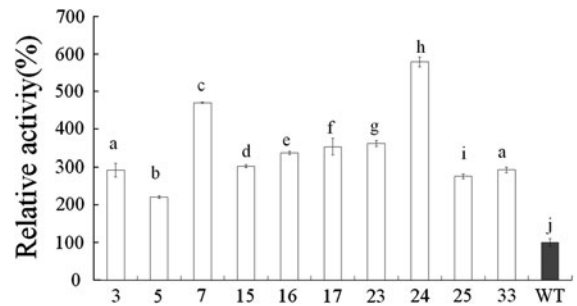

Fig. 4 Chitinase activity of wild-type and transgenic tobacco plants. The data were analyzed by Data Processing System v7.05, and comparison between different transgenic lines and the wild type (Relative activity = 100 %) were performed with SSR Duncan multiple range test. 3, 5, 7, 15–17, 23–25, 33: T_1 transgenic lines, *WT* wild type

formed by 20 amino acids, suggesting that *Trchi1* encodes a secreted protein.

Molecular identification of T_1 transgenic tobacco plants

We obtained transgenic tobacco plants by *Agrobacterium*-mediated transformation with the binary vector pROK and most of them grew normally and set seeds. PCR amplification of genomic DNA from putative transgenic plants showed that the expected 1.5-kb *Trchi1* amplicon could be obtained from 86 % of the plants (data not shown). Southern hybridization showed that in all PCR-positive transgenic tobacco plants, the *Trchi1* gene was integrated into the tobacco genome. In addition, most of the transgenic plants had one copy of the transgene (Fig. 2).

Trchi1 expression analysis in T_1 transgenic tobacco plants

We isolated RNA from 10 different positive transgenic tobacco plants, as confirmed by Southern blot analysis. RT-PCR analysis showed that the expected 1,500 bp band was obtained from all of them, whereas it was not amplified from the non-transgenic plants, demonstrating that the *Trchi1* gene was transcribed in the transgenic lines (Fig. 3). All 10 transgenic plants displayed much higher chitinase activity than the control plants, with a 2.2- to 5.8-fold increase (Fig. 4), indicating that the active Trchi1 protein was produced.

Fig. 5 Resistance to two pathogenic fungi of tobacco plants overexpressing the *Trthi1* gene A3, A7, and A17 are T_1 transgenic lines #3, #7, and #17, respectively, which were inoculated with *Alternaria alternata*. NA non-transgenic plants were inoculated with *A. alternata*. C3, C7, and C17 represented T_1 transgenic lines #3, #7, and #17, respectively, which were inoculated with *Colletotrichum nicotianae*. NC non-transgenic plants inoculated with *C. nicotianae*
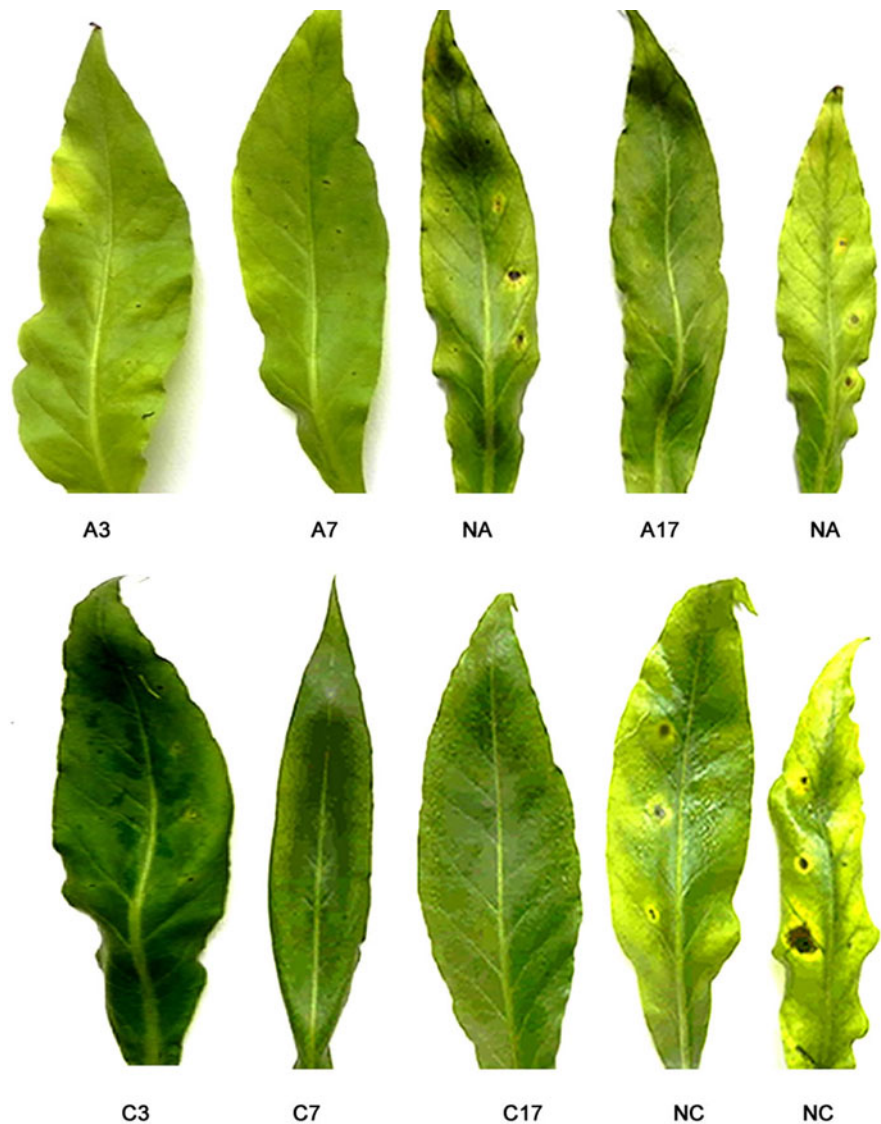
Resistance of transgenic T_1 tobacco plants to *A. alternata* and *C. nicotianae*

A. alternata and *C. nicotianae* were applied to the in vitro leaves of transgenic T_1 tobacco plants. Nine days after inoculation owith *A. alternata*, only 2.8 % (1 out of 36) of transgenic plants showed significant lesions, whereas all the negative control plants produced significant lesions (Fig. 5). Similarly, 9 days after *C. nicotianae* inoculation, only 7.1 % (3 out of 42) of transgenic plants showed significant lesions, whereas all the non-transgenic plants had significant lesions.

These experiments indicate that overexpression of the *Trchi1* gene greatly enhanced tobacco resistance against *A. alternata* and *C. nicotianae* Table 1.

One approach to enhancing plant resistance to fungal diseases is to provide the host plant with an exogenous transgene, just as we have done in this study with the overexpression of *T. roseum Trchi 1* in tobacco. This strategy has been applied successfully to crops such as tobacco, potato, and apple (Li 2006); however, the chitinase genes are cloned mainly from plants, and only a few are originated from mycoparasites. In this study, overexpression of the *Trchi1*

Table 1 Analysis of fungal resistance of transgenic plants

	Alternaria alternata		Colletotrichum nicotianae	
	Transgenic	Non-transgenic	Transgenic	Non-transgenic
Total plants	36	18	42	18
Plants with lesions	1	18	3	18
Percentage of plants with lesions (%)	2.8	100	7.1	100

Twelve plants from T_1 transgenic lines (3, 7, and 17) were used for inoculation of *A. alternata* and 14 plants from each transgenic line were used for *C. nicotianae* inoculation, respectively

gene cloned from *T. roseum* significantly improved tobacco resistance on two severe fungal pathogens, *A. alternata* and *C. nicotianae*, as estimated by artificial inoculum in lab conditions. If confirmed in field conditions, this new source of resistance could broaden the resources for long-term and durable resistance to fungi in crops.

References

Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. Transgenic Res 10(6):533–543

Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques 16:664–670

Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782

Dana MM, Pintor-Toro JA, Cubero B (2006) Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress reagents. Plant Physiol 142:722–730

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini-preparation: version II. Plant Mol Biol Rep 4:19

Hassan F, Meens J, Jacobsen HJ (2009) A family 19 chitinase (Chit30) from *Streptomyces olivaceoviridis* ATCC 11238 expressed in transgenic pea affects the development of *T. harzianum* in vitro. J Biotechnol 143:302–308

Huang HC, Kokko EG (1993) *Trichothecium roseum*, a myco-parasite of *Sclerotinia sclerotiorum*. Can J Bot 71:1631–1638

Jayaprakashvel M, Selvakumar M, Srinivasan K, Ramesh S, Mathivanan N (2010) Control of sheath blight disease in rice by thermostable secondary metabolites of *Trichothecium roseum* MML003. Eur J Plant Pathol 126:229–239

Jia Z, Sun Y, Yuan L, Tian Q (2010) The chitinase gene (*BbchiT1*) from *Beauveria bassiana* enhances resistance to *Cytospora chrysosperma* in *Populus tomentosa Carr*. Biotechnol Lett 32:1325–1332

Kern MF, Maraschin Sde F, Vom Endt D, Schrank A, Vainstein MH, Pasquali G (2010) Expression of a chitinase gene from *Metarhizium anisopliae* in tobacco plants confers resistance against *Rhizoctonia solani*. Appl Biochem Biotechnol 160:1933–1946

Kumar V, Parkhi V, Kenerley CM (2009) Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from *Trichoderma virens* in response to interaction with *Rhizoctonia solani*. Planta 230:277–291

Li DC (2006) Review of fungal chitinases. Mycopathologia 161:345–360

Li DC, Zhang SH, Liu KQ, Lu J (2004) Purification and partial characterization of a chitinase from the mycoparasitic fungus *Trichothecium roseum*. J Gen Appl Microbiol 50(1):35–39

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8(3):457–463

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL, Di Pietro A (1993) Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathology 83(3):302–307

McCreath KJ, Gooday GW (1992) A rapid and sensitive microassay for determination of chitinolytic activity. J Microbiol Methods 14(4):229–237

Munshi NA, Tanki TN, Zargar MA, Sahaf KA, Raja TA (2009) Evaluation of some local microfungi for antagonism against mulberry root rot pathogen *Fusarium oxysporum* Schechlt. Appl Biol Res 11(1):6–11

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Shafat AR (2010) Management of powdery mildew, *Phyllactinia corylea* (Pers.) Karst of Mulberry (*Morus* sp.) using chosen biocontrol agents. J Biopest 3(2):483–486

Sharma KK, Anjaiah V (2000) An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens*-mediated genetic transformation. Plant Sci 159(1): 7–19

Viterbo A, Haran S, Friesem D, Ramot O, Chet I (2001) Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol Lett 200(2):169–174