IJST (2015) 39A3: 331-339
Iranian Journal of Science & Technology

http://ijsts.shirazu.ac.ir

Heterologous expression of *chit*36 gene from *Trichoderma atroviride* in canola reduces development of lesion caused by *Sclerotinia sclerotiorum*

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Abstract

An endochitinase gene (*chit*36) was isolated from the biocontrol fungus *Trichoderma atroviride*. Chit36 was overexpressed under the CaMV35S constitutive promoter in canola. Transformation of cotyledonary petioles was achieved via *Agrobacterium tumefaciens*. The insertion of transgene was verified by PCR and DNA dot blotting. RT-PCR analysis indicated that the transgenic canola was able to express *chit*36 gene. Also, we used a transgenic over-expression approach in order to investigate antifungal activity of expressed *chit*36 on *Sclerotinia sclerotiorum*. Antifungal activity was detected in crude protein extracts from transgenic canola using radial diffusion assay. Also, lesion sizes of transgenic canola by *S. sclerotiorum* were significantly retarded compared to non transgenic canola plant using detached leaf assay.

Keywords: Antifungal activity; canola; *chit*36; *Sclerotinia sclerotiorum*

1. Introduction

Canola is one of the most important sources of edible vegetable oil and protein-rich products in the world. Like many other crops, the production of this crop is challenged by phytopathogenic fungi. The conventional fungal pathogen control method which uses chemical fungicides causes environmental pollution. Therefore, it is desirable to develop fungal-resistant plants through the introduction of foreign fungal-resistant genes. Foreign genes can be transferred into genomes of plants without altering any other agro-economically important traits (Chang et al., 2002). Since chitin is a structural polymer in many fungal pathogens, among fungal resistant genes, the chitinases which are potential antifungal agents through their chitin degradation activity, are excellent candidates for controlling the development of fungal pathogens (Pasonen et al., 2008; Jayaraj and Punja, 2007; Gentile et al., 2007; Maximova et al., 2006; Xiao et al., 2007; Kalai et al., 2006).

The endochitinase gene from *Trichoderma* sp. was expressed in crop plants and the transgenic lines provided a significant reduction in disease severity, comparable to the protection afforded by the fungicide on non-transgenic plants (Deng et al., 2007; Gentile et al., 2007; Wu et al., 2006).

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Received: 1 February 2015 / Accepted: 16 June 2015

In the present study, we cloned the *chit*36 gene from *T. atroviride* and introduced it into canola by *Agrobacterium*–mediated transformation. The introduced gene was expressed in transgenic plants and was shown to inhibit the *Sclerotinia sclerotiorum* growth.

2. Materials and methods

2.1. Enzymes and chemicals

All chemicals, culture media, plant growth regulators and antibiotics were purchased from Merck (Germany), unless stated otherwise. Restriction enzymes and other DNA-modifying enzymes were obtained from Roche Biochemical and Fermentas.

2.2. Plant material

The canola (*Brassica napus* L.) R line Hyola 308, was kindly provided by Oilseed and Development Co. Tehran, Iran

2.3. Microorganisms and growth conditions

T. atroviride PTCC5220 was obtained from Persian Type Culture Collection. It was identified in NIGEB, Tehran, and Iran among 30 *Trichoderma* isolates and used for DNA isolation. The stock culture was stored on agar (1.5%) slants

of MY medium (2% malt extract, 0.2% yeast extract, 1% maltose). *Escherichia coli* DH5α (Cinagene, Iran) and *A. tumefaciens* LBA4404 (NIGEB) was used. Bacteria were grown in LB medium at appropriate temperatures (37°C for *E. coli* and 28°C for *A. tumefaciens*) with shaking (200 rpm). pUC19 vector (Farmacia) were used for routine cloning and pBI121 (Novagen) was used as a binary plant expression vector.

2.4. Genomic DNA PCR amplification

For the purpose of amplification of *chit*36 gene from *T. atroviride*, two tailed primers, Chit36f and Chit36r (Table 1), were designed based on sequence similarity of existing *chit*36 genes from database. To facilitate subsequent cloning of the PCR-derived fragments, *Bam*HI and *Eco*RI restriction sites (bolded) were added to the 5'-end of these primers, respectively (Table 1).

Table 1. Oligonucleotides (primers) used in this study

Name	Oligonucleotides Sequence
Chi36f	5'- **TGGATCC**ATGACACGCCTTCTTGACG -3'
Chi36r	5'- **CGAATTC**TAACCAATGCGAGTAAGCA -3'
Chit36.2f	5'- **GCTCTAGA**ATGACACGCCTTCTTGACG -3'
Chit36.2r	5'- **GCGAGCTC**TAACCAATGCGAGTAAGCA -3'
M13f	5'- gCTAgTTATTgCTCAgCgg -3'
M13r	5'- gTAAAACgACggCCAgT -3'
35Sf	5'- GGCGAACAGTTCATACAGAGTCT-3'
Nosr	5'- CGCGATAATTTATCCTAGTTTGC-3'
virGf	5'- ATGATTGTACATCCTTCACG-3'
virGr	5'- TGCTGTTTTTATCAGTTGAG-3'

Fungal chromosomal DNA was prepared as described by Sun et al. (2002). Amplification of the DNA fragments encoding *chit*36 gene was performed using PCR. PCR reactions included 2.5 units of *Pfu* DNA polymerase, 1X buffer, 200 µM of each dNTPs, 2 µM $MgSO_4$ and 0.5 µM primers. PCR reaction conditions were 94°C for 30 sec, 42°C for 30 sec, and 72°C for 45 sec, for 28 cycles followed by a final extension of 10 min. PCR products were separated by agarose gel electrophoresis and products were purified by high pure PCR product purification kit (Roche) for cloning.

2.5. Sequence and computer analysis

Multiple sequence alignment was generated using Clustal W (*http://www.ebi-ac.uk/ClustalW*). DNA fragments were sequenced by a Commercial Service (Seqlab). The deduced amino acid sequence from *chit*36 gene was obtained by BLASTX Network Service (NCBI) and alignment of this amino acid sequence was done by clustal method with BLOSUM62 Service.

2.6. Cloning in plant expression vector

Full length DNA encoding the Chit36 (1035 bp) was amplified with specific primers (Chit36.2f and Chit36.2r) and recombinant pUC19 as template DNA using *pfu* polymerase and cloned into pBI121 at *Xba*I/*Sac*I restriction sites and confirmed by restriction enzyme and PCR patterns.

2.7. Preparation of the explants and the bacterial strain for transformation

Canola seeds were sterilized by being submerged in 70% ethanol for 5 min and then in 0.1% $HgCl_2$ for 8 min. They were then rinsed several times with sterilized water and plated on ½MS medium (Murashige and Skoog, 1962) under light for 5 days. After germination, the cotyledonary petioles were cut and placed on MS solid medium with 3.5mg/l benzylaminopurine (BAP) (CM) for pre-culture. After 2 days, the explants were used for transformation.

Single colonies of *A. tumefaciens* strain harboring recombinant pBI121 containing the *chit*36 gene were grown in LB medium supplemented with 50 mg/l kanamycin, and allowed to grow overnight at 27–28°C with constant shaking (200 rpm) to mid-log phase. The bacterial culture was then transferred to fresh medium and cultivated till OD_{600} = 0.4. The bacterial cells were collected by centrifugation and re-suspended in ½MS medium for use.

2.8. Transformation and selection procedure

Explants were immersed in the bacterial suspension for 1.5 min with constant shaking, placed onto the sterile filter paper to remove the excessive moisture, and then placed on the CM medium in Petri dishes for co-cultivation at 25°C for 2 days. The explants were washed with the sterile water containing 200 mg/l cephatoxim and then transferred to MS solid medium containing 3.5

mg/l BAP, 15 mg/l kanamycin, and 200 mg/l cephatoxim. After shoot initiation, the explants were transferred to MS solid medium with 25 mg/l kanamycin, and 200 mg/l cephatoxim. Regenerating shoots (about 3 cm in length) were transferred to MS solid medium with 2 mg/l 3-Indolebutyric acid (IBA), 25 mg/l kanamycin, and 200 mg/l cephatoxim for rooting and recovering complete plants. All the above media contained 3% (w/v) sucrose with pH 5.8, and all the explants, were cultured under 23–25°C and 16 h of day time with light intensity of 2000 Lux.

2.9. Molecular analysis of transgenic canola

Leaf material from canola was harvested, lyophilized and was ground into fine powder for extraction of genomic DNA by the method of Doyle and Doyle (1990). DNA fragment containing *chit*36 gene was amplified by PCR using the genomic DNA and the gene specific primers (Table 1).

2.10. Expression analysis

Specific mRNAs of the transgene were checked using reverse transcriptase (RT)-PCR. Total RNA was isolated from canola leaves using an RNA isolation kit (Roche). First strand cDNA was generated using the *chit*36 specific primer (chit36.2f). PCR amplification of the 1032 bp fragment of the above gene was achieved with specific primers (chit36.2f/chit36.2r) using the synthesized cDNA as template.

2.11. Dot blot analysis

Genomic DNA (15 µg) was denatured for 10 min in boiling water and chilled on ice. The denatured DNAs were spotted on a nylon membrane (Hybond N+, Amersham), and hybridized to Dig-dUTP labeled chit36 probe. A fragment (1032 bp in size) was obtained from PCR amplification of the *chit*36 gene using chit36.2f/chit36.2r primers and plasmid pBIMY3 containing *chit*36 as template and subjected to DIG DNA labeling (Roche) and used as a probe in hybridization experiments.

2.12. Bioassay of transgenic canola plants

Canola leaf material (3 g) was ground to a fine powder in liquid nitrogen using a mortar and pestle. Two volumes of 1 M NaCl in 20 mM NaOAc, pH 4.7 were added to the leaf material. The extracts were then shaken for 1 h at 4ºC. Extracts were subsequently centrifuged at 13,000g for 20 min at 4ºC. The pellets were discarded and the supernatants were used in the dialysis step. Samples were dialysed twice for 2 h at 4ºC against 20 mM NaOAc (pH 4.7). A 12,000 MW cutoff dialysis membrane was used. Extracts were subsequently centrifuged at 13,000g for 20 min at 4ºC and the supernatants were stored at -20ºC. The protein content was determined against BSA using Bradford assay (Bradford, 1976). The antifungal activity of crude extract of transgenic canola was tested against *S. sclerotiorum* using radial diffusion assay described by Broglie et al. (1991).

Also, susceptibility of transgenic and non transgenic canola to *S. sclerotiorum* was evaluated using a detached leaf assay described by Carstens *et al*. (2003).

3. Results

T. atroviride, showing high level of chitinolytic enzyme activity was used to amplify chitinase 36 (*chit*36) gene. Two specific primers (Chit36f and Chit36r) were designed based on *chit*36 genomic DNA sequences obtained from the GenBank database. Under experimental conditions, the genomic DNA was amplified using specific primers and *pfu* polymerase. A specific band, about 1 kb was amplified from *T. atroviride* chromosomal DNA (Fig. 1). The PCR product was analyzed by restriction pattern using *Pvu*II enzyme (Fig 1).

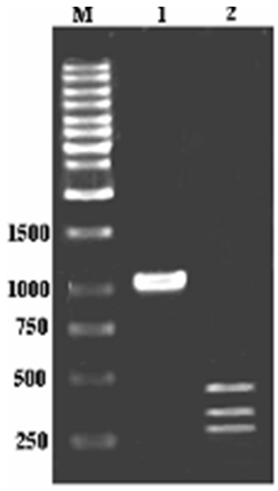

Fig. 1. Restriction pattern analysis of *chit*36 DNA: PCR amplification of *chit*36 DNA (line 1, approximately 1.0 Kb), digestion of PCR product using *Pvu*II (line 2), M-DNA size marker

Restriction pattern of *Pvu*II digested *chit*36 PCR product showed different patterns compared to expected pattern for known sequences available in the data base. However, since the PCR product was shown to have the expected size under stringent conditions of amplification the amplified DNA fragment was cloned into a cloning vector and designated as pUCMY1. The

new construct was confirmed by restriction and PCR patterns. Analysis of the sequence of the cloned fragment demonstrated that we have cloned PCR fragment with high homology to the previously reported chitinase sequences of different *Trichoderma* sp. (data not shown). The sequence was submitted to the NCBI data base under accession number EF432244. A homology search utilizing Clustal W, revealed high homology between *T. atroviride chit*36 cloned in present study and several chitinase genes of other fungi, including endochitinase (*chit*36) from *T. harzianum* (88.9%), endochitinase (*chit*36P1) from *T. atroviride* (88.9%), and chitinase (chit37) from T. *inhamatum* (80%). Analysis of the *chit*36 sequence from *T. atroviride* revealed an open reading frame, 1032 bp in length, encoding a protein of 344 amino acids (about 36 kDa) (Table 2).

Table 2. Nucleotide and deduced amino acid sequences of *T. atroviride* PTCC5220 *chit*36 gene. Primers are indicated by bold characters and stop codon by a period. *Pvu*II restriction sites are indicated by underline. Two new *Pvu*II sites (compared with the *chit*36P1 sequence from *T. harzianum*) were detected in this sequence. The altered nucleotides in new *Pvu*II sites are shown in bold.

ATG ACA CGC CTT CTT GAC GCC AGC TTT CTG CTG CTG CCT GCT ATC GCA TCG ACG CTG TTT	60
M T R L L D A S F L L L P A I A S T L F	20
GGC ACC GCC TCT GCA CAG AAT GCG ACA TGC GCA CTG AAG GGA AAG CCG GCA GGC AAA GTC	120
G T A S A Q N A T C A L K G K P A G K V	40
TTG ATG GGA TAT TGG GAA AAT TGG GAT GGA GCA GCC AAC GGT GTT CAC CCT GGA TTT GGC	180
L M G Y W E N W D G A A N G V H P G F G	60
TGG ACA CCG ATC GAA AAC CCC ATC ATT AAA CAG AAT GGC TAC AAT GTG ATC AAC GCC GCC	240
W T P I E N P I I K Q N G Y N V I N A A	80
TTC CCC GTT ATT CTG TCA GAT GGC ACA GCA TTA TGG GAA AAC GAC ATG GCT CCT GAC ACT	300
F P V I L S D G T A L W E N D M A P D T	100
CAA GTC GCA ACT CC**A** GC**T** GAA ATG TGT GAG GCT AAA GCA GCT GGT GCC ACA ATT CTG CTG	360
Q V A T P A E M C E A K A A G A T I L L	120
TCA ATT GGA GGT GCT ACT GCT GGC ATA GAT CTC AGC TCC AGT GCA GTC GCT GAC AAG TTC	420
S I G G A T A G I D L S S S A V A D K F	140
ATC GCC ACC ATT GTA CCA ATC TTG AAG CAG TAC AAT TTT GAC GGC ATT GAT ATA GAC ATT	480
I A T I V P I L K Q Y N F D G I D I D I	160
GAG ACG GGG TTG ACC AAC AGC GGC AAT ATC AAC ACA CTT TCC ACA TCC CAG ACC AAT TTG	540
E T G L T N S G N I N T L S T S Q T N L	180
ATT CGC ATC ATT GAT GGT GTT CTT GCT CAG ATG CCT TCC AAC TTC GGC TTG ACT ATG GCA	600
I R I I D G V L A Q M P S N F G L T M A	200
CCT GAG ACA GCG TAC GTT ACA GGC GGT AGC ATC ACG TAT GGC TCT ATT TGG GGA GCG TAC	660
P E T A Y V T G G S I T Y G S I W G A Y	220
CTA CCT ATC ATC CAG AAA TAT GTT CAA AAC GGC CGG CTG TGG TGG TTA AAC ATG CAA TAT	720
L P I I Q K Y V Q N G R L W W L N M Q Y	240
TAC AAC GGC GAT ATG TAC GGT TGC TCT GGC GAC TCT TAC GCA GC**T** GGC ACC GTC CAA GGA	780
Y N G D M Y G C S G D S Y A A G T V Q G	260
TTC ATC GCT CAG ACT GAT TGC CTA AAT GCA GGA CTT ACC GTC CAA GGC ACC ACA ATC AAG	840
F I A Q T D C L N A G L T V Q G T T I K	280
GTT CCA TAC GAC ATG CAA GTA CCA GGC CTA CCT GCG CAA TCA GGA GCT GGA GGT GGC TAT	900
V P Y D M Q V P G L P A Q S G A G G G Y	300
ATG AAT CCA AGC TTG GTT GGA CAA GCA TGG GAT CAC TAC AAC GGT GCT CTG AAA GGC TTG	960
M N P S L V G Q A W D H Y N G A L K G L	320
ATG ACG TGG TCA ATC AAC TGG GAT GGA GCG GGT AAC TGG ACA TTT GGC GAC AAT TTG CTT	1020
M T W S I N W D G A G N W T F G D N L L	340
ACT CGC ATT GGT TAG	1035
T R I G .	344

For cloning of *chit*36 gene in plant binary expression vector pBI121 for transformation of canola, the fragment containing the *chit*36 coding region was cloned into *Xba*1/*Sac*1 sites of pBI121. Cloning of *chit*36 gene into this expression vector was confirmed by PCR and restriction patterns (Fig. 2).

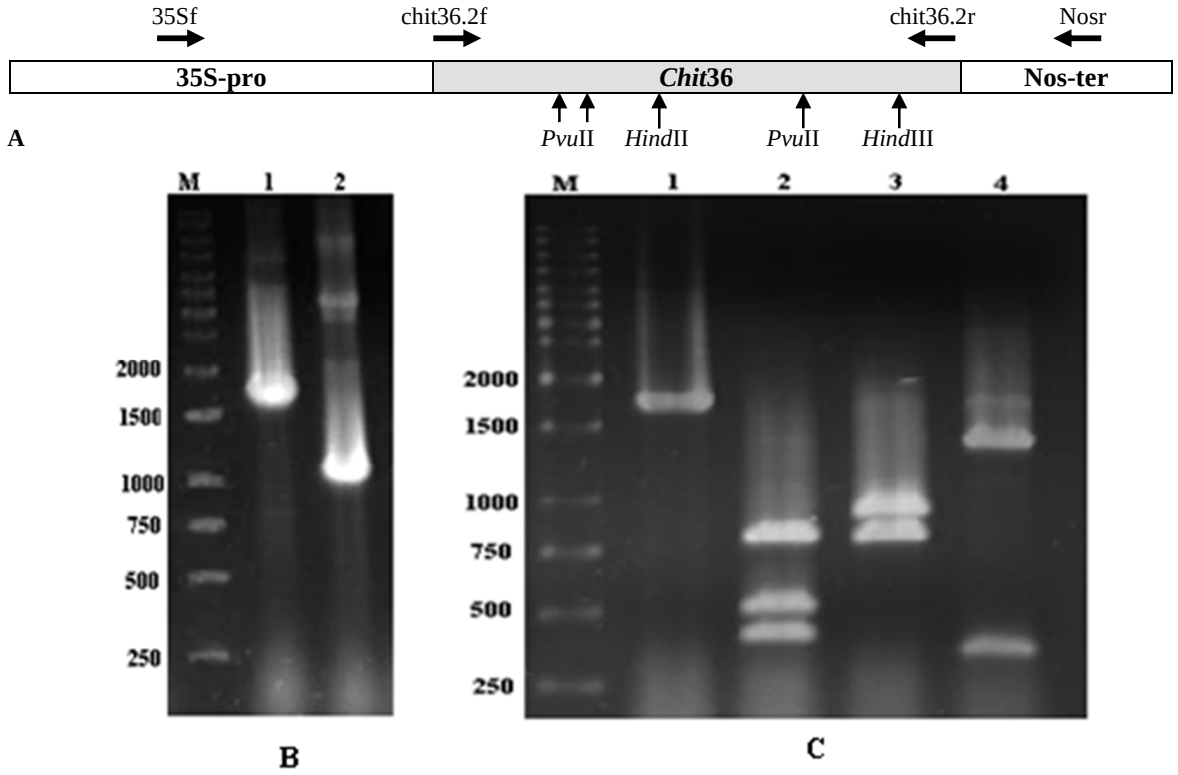

Fig. 2. A. Schematic representation of expected restriction pattern based on *chit*36 sequence of *T. atroviride* PTCC5220 and position of different primers used for PCR conformation. The position of restriction sites in *chit*36 DNA are indicated on the map. B. Confirmation of cloning *chit*36 gene in pBI121 by PCR using 35Sf/Nosr line 1, and Chit36.2f/Chit36.2r line 2. C. Confirmation of pBIMY3 containing *chit*36 by PBR (PCR based RFLP), line1. Amplification of DNA fragment containing *chit*36 coding sequence with its flanking regions by 35Sf/Nosr primers, lines 2, 3, and 4. Digestion of PCR product using *Pvu*II, *Hind*II, and *Hind*III respectively, M- DNA size marker

The open reading frame of *chit*36 gene in pBI121 (pBIMY3) which is between the CaMV35S promoter and Nos terminator was confirmed by DNA sequencing (data not shown).

The pBIMY3 was mobilized into *A. tumefaciens* and subsequently used for transformation of 5 days old cotyledonary petioles of canola. The shoots were regenerated from the cotyledonary petioles 1-2 weeks after planting. Independent transgenic canola lines were successfully rooted on kanamycin-containing selection media. The transgenic plants were hardened off in the greenhouse (Fig. 3) and shown to contain the *chit*36 transgene using PCR (Fig. 4). The gene specific PCR primers did not amplify a *chit*36 DNA fragment in the untransformed sample (Fig. 4).

A set of *vir*G primers (virGf/virGr) used for detection of any *Agrobacterium* contamination that might have escaped the selection. PCR detection under various conditions showed no detectable band using transgenic plants DNA as template. A 738 bp band was detected using *Agrobacterium* DNA as control (data not shown).

Also, restriction pattern analysis on PCR product of putative transgenic plant was performed using *Pvu*II restriction enzyme (PCR based RFLP, PBR) (Fig. 5). The results confirmed the presence of *chit*36 gene in transgenic plants.

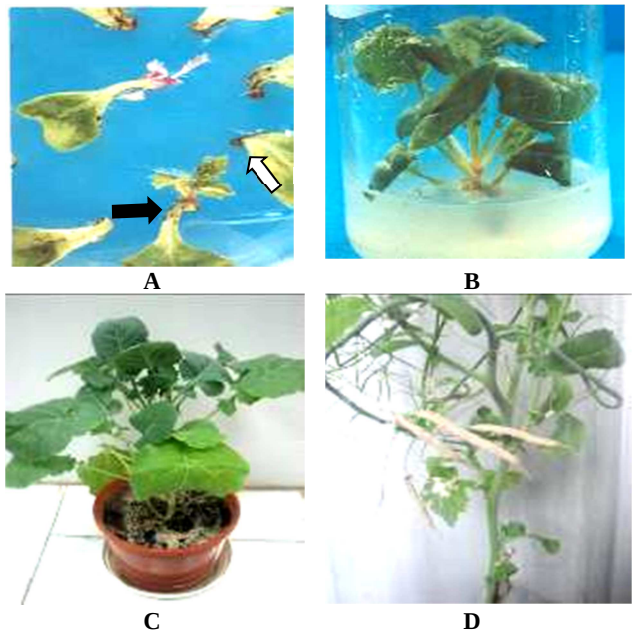

Fig. 3. Transformation and regeneration of transgenic canola plants. A, Shoot regeneration from cotyledonary petioles after transformation (1-2 weeks), chlorotic shoots (marked with white arrow) and green kanamycin-resistant shoot (marked with black arrow) formed on selection medium; B, Regenerated plantlet in growth medium; C, Regenerated plantlet in pot and acclimated to non-aseptic environment; D, Transgenic canola plant pods in the greenhouse

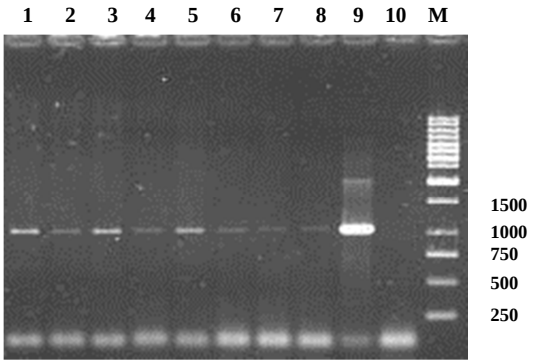

Fig. 4. PCR analysis of putative transgenic canola. A 1032 bp band was amplified using specific primers (chit36f/chit36r) and DNA of putative transgenic plants as template. Line1-8, putative transgenic canola; line 9, pBIMY3 plasmid DNA as positive control; line 10, non-transgenic canola DNA as negative control; M, 1 Kb DNA ladder

Dot blot analysis on PCR positive transgenic plants were performed and the results confirmed the integration of the exogenous gene into the genomes of transgenic canola plants (Fig. 6). No hybridization signal occurred in non-transgenic control plants.

Expression of the specific mRNA transgene (*chit*36) in the transformed canola lines was proven by means of RT-PCR. RNA was isolated from leaf tissues for generation of cDNA. The expected size (1032 bp) of the amplified cDNA fragment was detected in transformed lines (Fig. 7). Non-transformed plant was used as negative controls and no transcript was detected. To ensure that the amplified fragment was not due to the presence of trace amount of genomic DNA in the RNA solution, the RNA solution was used without reverse transcriptase for *chit*36 DNA amplification. No PCR band was detected from RNA sample, indicating this amplified band from transgenic lines is from mRNA of *chit*36.

In order to test the antifungal activity of expressed Chit36 from transgenic canola on actively growing culture of *S. sclerotiorum*, total protein extracts from leaves of transgenic and non-transgenic plants were tested using radial diffusion assay. Antifungal activity was detected in extracts from transgenic canola, whereas this activity was not found in non-transgenic canola extracts (Fig. 8). Culture medium of *T. atroviride* induced with colloidal chitin was used as positive control.

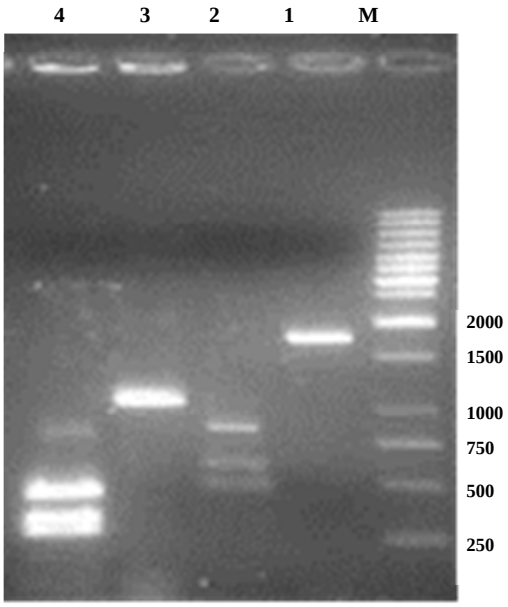

Fig. 5. Confirmation of transgenic plant by PBR. line 1, amplification of DNA fragment containing *chit*36 coding sequence with its flanking regions by CaMV35S/Nosr primers, lines 2, Digestion of PCR product (from line 1) using *Pvu*II; line 3, amplification of DNA fragment containing *chit*36 coding sequence by chit36f/chit36r primers, line 4, Digestion of PCR product (from line 3) using *Pvu*II; M, 1 Kb DNA ladder

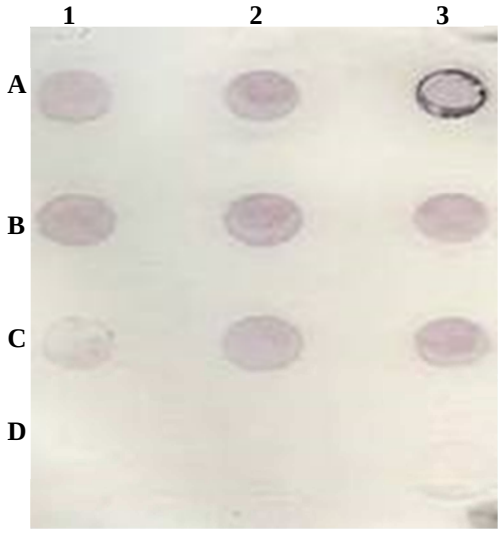

Fig. 6. Genomic DNA D dot blot analysis of putative transgenic canola with the total DNA loaded onto hybond N+ membrane hybridized with a digoxigenin- labeled *chit* 36 probe. A1, A2, B1, B2, B3, C1, C2 and C3, DNA from leaves of transgenic canola plants of T0 generation; A3, DNA from pBIMY3 plasmid as positive control; D3, DNA from non-transgenic plant as negative control

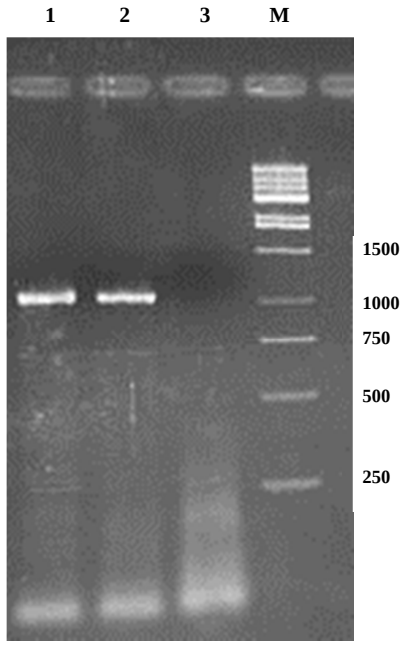

Fig. 7. RT-PCR analysis of transgenic canola expressing *chit*36 gene. Line 1 and 2, RT-PCR of transgenic canola plants; line 3, PCR amplification using RNA extraction (without revers transcriptase) as negative control (It ensures the amplified fragment in line 1 and 2 are not due to DNA contamination); M, 1 Kb DNA ladder

Also, to assess the effect of expressed *chit*36 in transgenic canola on *S. sclerotiorum* resistance, experiment was performed using greenhouse acclimated transgenic line in a detached leaf assay. Lesion expansion occurred on leaves and proceeded aggressively in untransformed control. Lesion sizes were significantly retarded in the transgenic canola line (Fig. 9). The transgenic lines were phenotypically analyzed and compared to untransformed controls and did not show any abnormalities with regards to growth, size or reproduction.

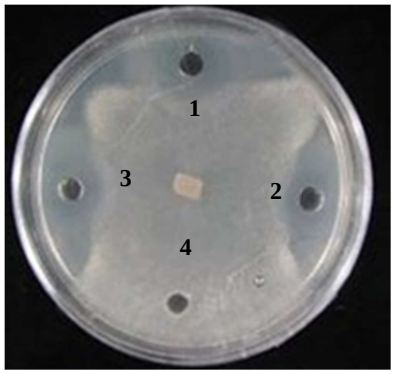

Fig. 8. Radial diffusion assay of crude protein extracts of transgenic canola against *S. sclerotiorum.* 1 and 2, protein extraction from transgenic canola; 3, culture medium of *T. atroviride* as positive control; 4, protein extraction from non transgenic canola as negative control

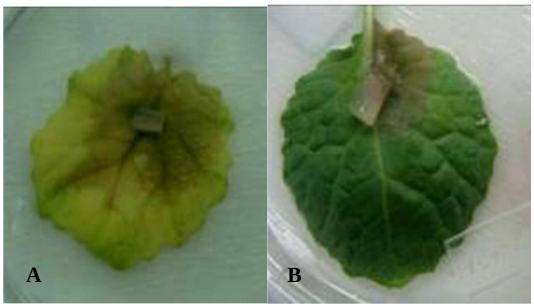

Fig. 9. *S. sclerotiorum* lesion development on detached leaves of transgenic (A) and non-transgenic (B) canola. Photograph was taken 48 hours after inoculation showing confined lesion growth in transgenic plants as compared to wild type

4. Discussion

The chitinase enzymes are fungicidal (Jayaraj and Punja, 2007; Xiao *et al*., 2007) and nontoxic to plants, animals and higher vertebrates (Lorito et al., 1996). However there are almost no reports demonstrating chitinase genes originating from plants or bacteria conferring transgenic plants resistance to several fungi indicating that these enzymes have a narrow spectrum of antifungal activity (Joosten et al., 1995; Lorito et al., 1998). The unsuccessful use of plant chitinases for plant protection probably comes from the fact that the genes code for enzymes usually affect only the hyphal tip and lack the ability to efficiently degrade spores or hard chitin structures (Joosten et al., 1995; Mauch et al., 1988). Whereas fungal chitinases are able to lyse not only the hyphal tip but also hard chitin wall of mature hyphae and other fungal preservation structures (Lorito et al., 1996; Lorito et al., 1998). Therefore, in this study, *T. atroviride*, was used for amplification of *chit*36 gene. DNA sequence information obtained from this isolate confirmed that the cloned PCR fragment shows high homology to the previously reported chitinase sequences of *T. harzianum* (Viterbo et al., 2001), *T. atroviride* (Viterbo and Chet, 2002), and *T. inhamatum* (Viterbo et al., 2002). Also, the *chit*36 DNA sequence cloned in this study contains no intron. The same results have been reported from other related fungi (Viterbo et al., 2001; Viterbo and Chet, 2002; Viterbo et al., 2002). The difference between *Pvu*II restriction pattern of the reported *chit*36P1 from *T. harzianum* (Viterbo et al., 2001) showing only one *Pvu*II site and that of *chit*36 in present study which is shown to have three *Pvu*II sites, demonstrated two new *Pvu*II sites (first and third *Pvu*II sites in Fig 3) which cause different *Pvu*II restriction pattern.

Transgenic plants expressing chitinase genes have shown enhanced fungal disease resistance in different species. Expression of endochitinase 42 from *Trichoderma* in tobacco and potato produced complete or nearly complete resistance to *Rhizoctonia solani* (Lorito et al. 1998). In recent years chitinase genes from *Trichoderma* sp. have been used to enhance fungal resistance in different transgenic plants (Deng et al., 2007; Gentile et al., 2007; Wu et al., 2006).

Stem rot is one of the most important fungal diseases in canola which is caused by *S. sclerotiorum.* It can cause serious losses in canola, especially in warm and humid areas which often occur in canola field in Iran. In our study, under experimental conditions, the transgenic canola plant transformed with *chit*36 gene from *T. atroviride* showed some resistance to stem rot disease. All the transgenic plants expressing chitinase activity suffered from less disease incidence than the control.

This work demonstrates a successful application of a single endochitinase gene from *T. atroviride* for controlling plant disease. Meanwhile, the expression of this gene had no deleterious phenotypic effect on the transgenic plants. The enhanced resistance of transgenic plants observed in detached leaf assay is consistent with the inhibitory effect proteins extracted from transgenic plants on the growth of *S. sclerotiorum in vitro* in radial diffusion assay. No inhibition was detected with protein extracts from non-transgenic plants. In the present study, it was demonstrated that the heterologous expressed Chit36 in canola inhibited the growth of *S. sclerotiorum*, which is an important first step in disease control strategies.

Acknowledgment

This research was financially supported by the National Institute for Genetic Engineering and Biotechnology (NIGEB).

Nomenclature

BAP	6-Benzyl-amino-purine
BSA	Bovine Serum Albumin
IBA	3-Indolbutyric acid
NCBI	National Center for Biotechnology Information
PBR	PCR-based RFLP
RT-PCR	Reverse transcription- polymerase chain reaction

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