

A comparative study of transgenic canola (*Brassica napus* L.) harboring either chimeric or native *Chit42* genes against phytopathogenic fungi

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Received: 15 June 2015 / Accepted: 30 December 2015
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Abstract Canola (*Brassica napus* L.), an agro-economically important crop in the world, is sensitive to many fungal pathogens. One strategy to combat fungal diseases is genetic engineering through transferring genes encoding the pathogenesis-related (PR) proteins such as chitinase which cause the chitin degradation of fungal cell wall. Chitinase *Chit42* from *Trichoderma atroviride* (PTCC5220) plays an important role in biocontrol and has high antifungal activity against a wide range of phytopathogenic fungi. This enzyme lacks a chitin binding domain (ChBD) which is involved in binding activity to insoluble chitin. In the present study, we investigated the effect of chitin binding domain fused to *Chit42* when compared with native *Chit42*. These genes were over-expressed under the CaMV35S promoter in *B. napus*, R line Hyola 308. Transformation of cotyledonary petioles was achieved by pBISM2 and pBIKE1 constructs containing chimeric and native *Chit42* genes respectively, via *Agrobacterium* method. The insertion of transgenes in T₀ generation was verified through polymerase chain reaction (PCR) and Southern blot analysis. Antifungal activity of expressed chitinase in transgenic plants was also investigated by bioassays. The transgenic canola expressing chimeric chitinase showed stronger inhibition against phytopathogenic fungi that indicates the role of chitin binding domain.

Electronic supplementary material The online version of this article (doi:10.1007/s13562-015-0347-1) contains supplementary material, which is available to authorized users.

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Keywords Transgenic canola · *Chit42* · Chimeric chitinase · Chitin binding domain · Antifungal activity

Abbreviations

DMAB Di-methyl amino benzaldehyde
CTAB Cetyl Trimethyl Ammonium Bromide
GLcNAc N-acetylglucosamine

Introduction

Canola provides oils for industrial lubricants, human consumption, animal feeds and also can be used as green fertilizer and composting crops (Dixon 2006; Seberry et al. 2009). The productivity of canola is limited by several abiotic and biotic stress factors (Grover and Pental 2003; Dutta et al. 2005; Ullah et al. 2012). Fungal diseases have served as a major cause of yield losses (Oerke et al. 1994). Agricultural operations, utilization of chemical compounds and using resistant varieties are three common procedures for controlling fungal diseases (Barone and Fruscianta 2007). Since the agrochemicals and conventional breeding approaches often have certain limitations, it is advisable to develop fungus-resistant plants through genetic engineering (Chang et al. 2002).

Chitinases are member of pathogenesis-related proteins which are often acid soluble, extracellular and protease resistant. Chitinases from *Trichoderma* sp. have been shown to have strong antifungal activities. They catalyze hydrolysis of Chitin that is a major component of many fungal cell walls, insect exoskeletons and crustacean shells and the second most abundant polysaccharide in nature (Collinge et al. 1993; Graham and Sticklen 1994; Neuhaus 1999).

Chitinase *Chit42* is an endo-chitinase of *T. atroviride* PTCC5220 which plays a key role in biocontrol activities

against phytopathogenic fungi (Limon et al. 2004; Harighi et al. 2006). *Chit42* lacks a chitin-binding domain (ChBD) (Kojima et al. 2005). Chitin-binding domain linked to the catalytic site of some chitinases via a linker region (Arakane et al. 2003; Limon et al. 2004). This domain is a tunnel-like structure which permits tight interaction with the polymeric substrate and facilitates chitinase binding, thus allowing efficient degradation of chitin (Van Aalten et al. 2001; Hardt and Laine 2004). *Serratia marcescens* is one of the most extensively studied chitinolytic bacteria producing multiple chitinases (Felse and Panda 1999; Zakariassen et al. 2009).

In current study to investigate the role of chitin binding domain from *S. marcescens* chitinase B on enzyme activity of *T. atroviride Chit42*, two constructs including *Chit42* and chimeric chitinase with fused ChBD at C-terminal site were transformed to canola plants via *Agrobacterium* method. The evaluation of the antifungal activity of the putative transgenic plants was carried out to study the effect of ChBD in the chimeric chitinase.

Materials and methods

Plant, fungal and bacterial materials

The seeds of canola (*B. napus* L.) R line Hyola 308, as a genes receptor, was provided by the Oilseed and Development Company, Tehran, Iran. *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Verticillium dahlia* and *Alternaria solani* were provided by Dr. H. Afshari-Azad from Iranian Research Institute of Plant Protection. The fungi were proliferated on PDA (Potato dextrose agar) medium and sub-cultured as needed.

Agrobacterium tumefaciens strain LBA4404 was used for plant transformation and *Escherichia coli* strain DH5 α (Cinnagen, Iran) was performed in all molecular biological experiments. The bacteria were grown in LB (Luria-Bertani) medium at appropriate temperatures (37 °C for *E. coli* and 28 °C for *A. tumefaciens*) along with shaking in 180 rpm.

Preparation of explants and the bacterial strain for transformation

Seeds were sterilized using 70 % ethanol for 2 min and 0.1 % HgCl₂ for 10 min. Then they were washed several times with sterilized water, plated on ½MS medium (Murashige and Skoog 1962) and incubated in the presence of light for 5 days. After germination, the cotyledonary petioles were cut and pre-cultured on CM solid medium (MS with 3.5 mg/L of Benzylaminopurine). After 2 days, the explants were subjected for transformation process. Single colonies of *A. tumefaciens* containing pBIKE1 (*chit42* gene) and pBISM2 (chimeric chitinase gene) were used to incubate in LB medium

including 50 mg/L of kanamycin and were grown overnight at 28 °C with constant shaking (180 rpm) to mid-log phase. The bacterial culture was transferred to fresh medium and cultivated until the optical density (OD 600) of 0.4 was achieved. Then the bacterial cells were collected by centrifugation and re-suspended in fresh ½MS medium for the subsequent inoculation step.

Transformation and selection procedure

Explants were submerged in a bacterial suspension for 5 min with constant shaking and then removed excessive moisture using sterile filter paper and put them on CM medium in Petri dishes for co-cultivation at 25 °C for 3 days.

After co-cultivation, due to inhibit the growth of *A. tumefaciens* attached to the explants, they were rinsed with sterile water including 200 mg/L cephatoxim and then transferred to MS medium containing 3.5 mg/L of BAP (Benzylaminopurine), 8 mg/L of kanamycin and 200 mg/L of cephatoxim. The explants were transferred to MS medium with 15 mg/L of kanamycin and 200 mg/L of cephatoxim, after shoot initiation. The regenerated shoots (about 3 cm in length) were separated from the explants and transferred to MS medium with 2 mg/L of 3-Indolebutyric acid (IBA) and 200 mg/L of cephatoxime for rooting and formation the complete plants. All the above media contained 3 % (w/v) of sucrose and 8 g/L of agar, pH 5.8. The explants were cultured at 23 ± 2 °C and under 16/8 h photoperiod with light intensity of 2000 Lux.

Molecular analysis of the transgenic canola

The leaves from the untransgenic and transgenic canola were collected, lyophilized and minced into a fine powder for extraction of genomic DNA (Doyle and Doyle 1991). PCR analysis was used for the first evidence of the transgene presence in the assumed transgenic plants. DNA fragment harboring of chimeric and native *Chit42* genes was amplified by PCR using the genomic DNA and (C42F4-5'-CGTTCCCGCAAGCAAGATCG-3'/NOSR-5'-CCAGTGAATTCCCGATCTAGTAAC-3') primers. PCR was carried out as explained: an initial denaturation at 94 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The resulting PCR products were detected by electrophoresis on 1 % (w/v) agarose gel.

Enzyme assay

Young leaves from putative transformants and untransformed canola plants were frozen in liquid nitrogen and were ground to make fine powder. The soluble proteins were also extracted in 50 mM sodium acetate buffer (pH 7.0). In this assay

chitinase activity was generally measured in the reaction mixture (total 500 ml) containing colloidal chitin as a substrate (3.8 mg) and the crud of enzymes from transgenic plants containing chitinases (200 µg/ml). The reaction was performed at 37 °C for 1 h and then centrifuged at 6000×g for 5 min. The supernatant was boiled along with potassium tetra borate buffer (100 µl) for 3 min. In following 3 ml of DMAB reagent [10 g of Di-methyl amino benzaldehyde in 100 ml of 10 N chloridric acid (12.5 % v/v) and glacial acetic acid (87.5 % v/v)] was added to the reaction, incubated at 37 °C for 20 min and the amount of GLcNAc produced in the reaction was calculated using the defined method by Zeilinger et al. (1999) according to the standard diagram of GLcNAc. One unit of enzyme activity was described as the amount of enzyme that catalyses the release of 1 µmol GLcNAc in 60 min at 37 °C. The assay for each sample was performed in three biological replicates. Total soluble protein concentration was determined according to Bradford (1976), using BSA (Bovine serum albumin) diagram as standard.

Southern blot analysis

Genomic DNA was extracted from fresh leaves of nontransgenic plants as the control and putative transgenic ones using the CTAB method (Doyle and Doyle 1991). PCR positive plants with high enzyme activity and untransformed control plants were analyzed by Southern blot assay to confirm the integration of the transgenes.

The extracted genomic DNA (20 µg) was digested using *Hind*III enzyme. The digested DNAs were seperated on 0.8 % (w/v) agarose gel, transferred onto a nylon membrane (Amersham Hybond N+; Amersham International Plc, Amersham, UK) and then hybridised to the Dig-dUTP labelled CaMV 35S probe. A partial internal fragment (631 bp in size) was achieved from PCR amplification of the CaMV35S fragment using CaMV35SF (5'-GGACTA ACTGCATCAAGAACACAG-3')/CaMV35SR (5'-GAAGGATAGTGGGATTGTGCGTC-3') primers and plasmid pBI121 containing the native and chimeric chitinase as template, subjected to DIG DNA labeling (Roche Applied Science GmbH, Mannheim, Germany) and used as a probe in hybridization experiments.

Bioassay of the transgenic canola plants

The antifungal activity of the extracted proteins from transgenic plants was tested using biological assays as mentioned in below. Canola leaf materials (0.5 g) were grinded to a powder in liquid nitrogen by a pestle and mortar. The soluble proteins were then extracted in 50 mM Tris-HCl (pH 8.8). The extracts were shaken for 1 h at 4 °C and subsequently centrifuged at 13,000 g for 35 min at 4 °C (Wang et al. 2006). The resulting supernatants were stored at 4 °C. The protein

content was determined against BSA according the Bradford assay (Bradford 1976).

Radial diffusion assay (Broglie et al. 1991)

For detection of chitinase antifungal activity of Chit42 and chimeric chitinase, fungal growth inhibitory assay was used. The zone of inhibition assay for antifungal activity was determined using 100 × 15 mm plates including 25 ml of PDA. After development of the mycelia, 5 mm holes were made around and at a distance of 1 cm away from the rim of the mycelia colony. Equal aliquots (40–45 µg) of crude proteins from transgenic plants contaning Chit42 and the chimeric chitinase were added to the holes. The plates were incubated at 28 °C for 24 h until mycelial growth had enveloped peripheral hole containing the negative control the extraction buffer of protein and the crude of proteins from untransformed plant. The fungal species included *S. sclerotiorum*, *R. solani*, *F. oxysporum* and *V. dahlia*. The assay for each sample was performed three replicates. Inhibition area was measured using Image Tools Software.

Disc diffusion assay (Nweze and Mukherjee 2010)

At first some fungal species such as *F. oxysporum*, *V. dahlia*, *A. solani* were grown on PDA plate at 28 °C for 5 days. The spores were washed with sterile water and then were collected and counted by Thoma Lam. Based on a modified method of Nweze, sterile paper disks (6 mm in diameter) were placed on the PDA medium. Then 1 µl from 2×10^7 cell/ml dilution of spore suspension of *F. oxysporum*, *V. dahlia* and *A. solani* was added to each disk. Following the same concentration of total proteins from leaf of transgenic canola containing chimeric and native *Chit42* were added to the disks. The protein from untransgenic plants and protein extracting buffer were served as control. Plates were incubated at 28 °C until spore germination and mycelia growth was seen in negative control disks. Finally, the inhibitory effect of Chit42 and chimeric chitinase enzymes on the growth of these fungi were compared relative to each other and to the controls. The assay for each sample was carried out in three replicates. Diameter of growth zones were measured using Image Tools Software.

Spore germination assay (Broekaert et al. 1997)

Spore suspension of *A. alternate*, *F. oxysporum* and *V.dahlia* (2×10^4 cell/ml) in half-strength PDB (Potato dextrose broth) containing the crude protein of transgenic canola with native or chimeric chitinase were incubated at 28 °C with shaking (150 rpm) for 48 h. Crude protein from untransgenic canola plant was used as a control. After 48 h incubation, the growth of the fungi was determined by measuring OD-values at 595 nm. The percentage of growth inhibition was calculated

as $100 \times$ the ratio of the A595 of the control minus the A595 of the sample over the A595 of the control. The experiments were conducted three times.

Light microscopy assay (Kronland and Stanghellini 1988 and Benhamou et al. 1993)

This assay used for studying the effect of Chit42 enzyme of transgenic canola plants on morphological changes and the cell wall of mycelium in *R. solani*. The crude protein (70 μ g) of transgenic canola was added to 2 days grown *R. solani* on a slide covered with a thin layer of PDA. Crude protein from untransgenic canola plant was used as a control. After 3, 6 and 24 h of incubation at 37 °C, the slide was examined for morphological changes and degradation of fungi cell wall under a light microscope.

Statistical analysis

The statistical differences were assessed based on the ANOVA (analysis of variance) using SPSS Software (Ver. 15, USA). Differences were considered significant at a probability level of $P < 0.05$. Mean values were compared using least significant different and Duncan tests.

Results and discussion

The main objective of this work was to obtain transgenic canola (*B. napus*) plants harboring a novel chitinase with chitin binding domain (chimeric chitinase) and native chitinase (*chit42*) genes and to evaluate the effect of this domain in antifungal activity. The ChBD from soil bacteria *S. marcescens* was selected to join to chitinase (*Chit42*) of *T. atroviride*.

Transformation and selection of transgenic plant

The constructs pBIKE1 containing *Chit42* gene and pBISM2 containing chimeric chitinase gene were used for over expression of these genes in canola. They are under the control of CaMV35S promoter and the nopaline synthase as terminator in the plant expression vector pBI121. Selectable marker gene in this vector is *nptII* (kanamycin resistance gene) (Supplementary Fig. 1).

These constructs were mobilized into *A. tumefaciens* and subsequently used for *B. napus*, R line Hyola 308 transformation. The explants were co-cultivated with *A. tumefaciens* and then transferred to selection medium containing kanamycin and cefotaxime. The independent transgenic canola lines were successfully rooted (Supplementary Fig. 2).

The success of *Agrobacterium*-mediated plant transformation is a biological progress which depends on various factors

such as; species genotype, strain of *Agrobacterium*, selectable marker, regeneration capacity of target cells and accessibility of the bacterium to the regenerable cells. Also, CaMV35S promoter used to ensure high levels of gene expression in all plant tissues. *Agrobacterium* mediated method for transformation and use of CaMV35S promoter have been reported by several researchers as impressive parameters (Cardoza and Stewart 2003; Kahrizi et al. 2007; Liu et al. 2010).

The experiment was performed with a total of 400 and 451 explants of canola using the pBIKE1 and pBISM2 constructs respectively. The numbers of regenerated shoots obtained at 8 mg/L kanamycin were 280 and 225 for these transgenes. Shoots were transferred to a medium containing 15 mg/L kamamycin and only 129 and 133 green shoots were obtained. Then, 65 and 59-rooted plantlets were transferred to pots (Table 1). PCR analysis confirmed the presence of the *Chit42* in 20 and chimeric chitinase in 21 kanamycin-resistant putative transgenic plants (Table 1). Successful introduction of transformation efficiency of *Chit42* and chimeric chitinase were calculated as 5 % and 4.65 % respectively (Table 1). The observed satisfactory transformation rates were similar to various reports in *B. napus* (Wang et al. 2005; Liu et al. 2011) and other *Brassica* species, *B. rapa* and *B. juncea* (Cho et al. 2001; Das et al. 2006). The transformants were phenotypically analyzed and compared with the controls and any abnormalities in the growth, size or reproduction did not show in transgenic lines.

Molecular analysis of regenerated plants

The ka namycin- resistant clones were subjected to PCR analysis for integration confirm of the transgenes chimeric chitinase and *Chit42*. The corresponding fragment, 700 bp of the *Chit42* gene, was amplified using specific primers (C42F4/NOSR) (Supplementary Fig. 3a). The lines containing chimeric chitinase were also analyzed by PCR using the same primers and the expected fragment (925 bp) was amplified from these transgenic lines (Supplementary Fig. 3b). The *Chit42* and chimeric chitinase specific primers did not amplify the corresponding fragments in untransformed plants.

A set of *virG* primers (*virGf/virGr*) was used to detect *Agrobacterium* contamination that might have escaped the selection. PCR detection under different conditions showed no bands using transgenic line DNA as template. A 738 bp band was found using *Agrobacterium* DNA as control (data not shown).

Enzyme activity assay

Chitinase activity from leaf tissues of the wild type and PCR positive transgenic plants, containing native and chimeric

Table 1 Transformation efficiency of tissue-cultured leaf explants of canola using the pBIKE1 and pBISM1 plasmid carrying the *Chit42* and chimeric chitinase genes respectively

Constructs	Number of explants	Number of regenerated shoots from explants (8 mg/l Kanamycin ^a)	Number of green shoots (15 mg/l kanamycin ^b)	Number of rooted plantlets and transferred to pots	Number of PCR positive plants ^c	Transformation efficiency ^d
pBIKE1 (<i>Chit42</i>)	400	280 (70 %)	129(46 %)	65	20(31 %)	5.00 %
pBISM1 (chimeric chitinase)	451	225(49 %)	133(59 %)	59	21(35 %)	4.65 %

^a The number (percentage) of regenerated shoots from explants incubated in the presence of 8 mg/l kanamycin over the number of explants

^b The number (percentage) of green shoots at 15 mg/l kanamycin over the number of regenerated shoots from explants at 8 mg/l Kanamycin^a

^c The number (percentage) of PCR positive plants over the number of explants transferred to pots

^d The number (percentage) of PCR positive plants over the number of explants

chitinase, was assayed in the presence of colloidal chitin. The specific enzyme activity of different transgenic plants varied from 0.72 ± 0.24 to 4.02 ± 0.39 U/ μ g for the lines containing (*Chit42*) and 0.79 ± 0.23 to 6.48 ± 0.52 U/ μ g for the lines harboring (chimeric chitinase). There was a significant difference between enzyme activities in transgenic lines (harboring chimeric and native *Chit42*) with control plant (wild type). Among all transgenic lines, T18, T7, T21, T13 and T2 harboring *Chit42* and T21, T9, T3, T2 and T1 with chimeric chitinase showed the highest specific enzyme activity. Furthermore, chimeric chitinase transformants (6.48 ± 0.52 to 2.46 ± 0.60 U/ μ g) in general demonstrated higher enzyme activity compared to transgenic lines harboring *Chit42* (4.02 ± 0.39 to 3.66 ± 0.15 U/ μ g) (Table 2). In previous study, the results revealed that the fusion of ChBD to chitinase improved the enzyme affinity to crystalline and colloidal chitin (Matroodi et al. 2013). The effect of ChBD on binding chitinase to chitin substrate was also described by Hashimoto et al. (2000) where they showed that the ChBD deletion from chitinase A1 greatly decreased the efficiency of chitin degradation. The enzyme activities of all T₀ transformants having Chimeric and native *Chit42* showed various levels due to their different copy number and localization of integrated heterologous transgenes (Yamamoto et al. 2000; Iyer et al. 2000; Matzke

et al. 2000). The transgenic lines harboring chimeric chitinase (T21, T9, T3, T2 and T1) and native *Chit42* (T18, T7, T21, T13 and T2) with high enzyme activity were selected for Southern blot analysis in order to define the number of integrated T-DNAs.

Southern blot analysis

Southern blot analysis was performed in PCR positive plants demonstrating high chitinase activity and the results showed that the transgenes were integrated into the transformed plants. Amplified CaMV35S promoter was labeled as probe. Genomic DNA from transformed lines containing chimeric chitinase (T21, T9, T3, T2 and T1) and *Chit42* (T21, T18, T13, T7 and T2) were digested with *Hind*III (Supplementary Fig. 1). Each hybridization band corresponded to one separate transgene copy, because the integrated genes were cut ones by *Hind*III enzyme while the other *Hind*III site was localized in the plant genome. Whereas each integration event occurs independently and by chance, transgenic plants exhibited unique hybridization profiles. The transgene copy number was appraised as one in T9, T3 and T1 lines with chimeric chitinase and T18 and T2 lines harboring *Chit42* genes. Two bands were detected in T2 (chimeric chitinase), T21 and T7 harboring *Chit42*. In the rest of the lines, T21 and T13

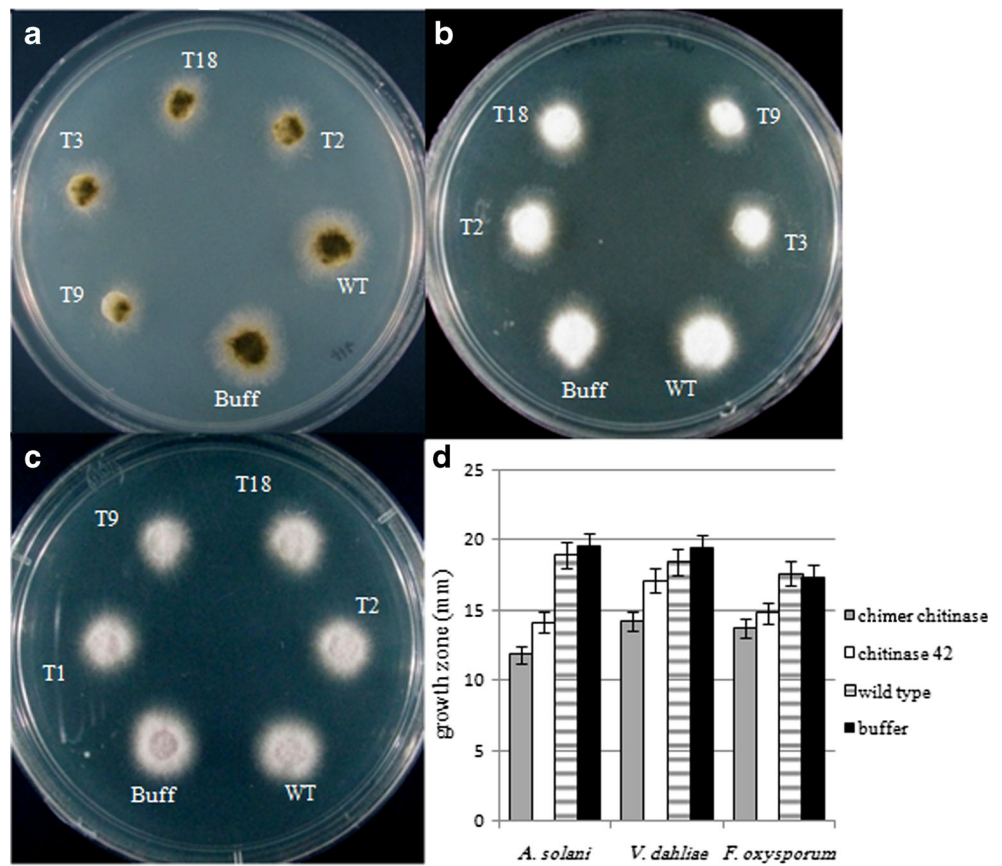
Table 2 Comparison of enzyme specific activity in leaf tissues of two transgenic canola lines and untransformed control plant

Transgenic line (<i>chit42</i>)	Specific activity ^a	Transgenic line (chimeric chitinase)	Specific activity ^a
T18	$4.02^b \pm 0.39$	T21	$6.48^b \pm 0.52$
T7	$3.6^b \pm 0.18$	T9	$6.3^b \pm 0.35$
T21	$3.3^b \pm 0.5$	T3	$4.8^b \pm 0.30$
T13	$3.18^b \pm 0.15$	T2	$4.44^b \pm 0.16$
T2	$2.46^b \pm 0.60$	T1	$3.66^b \pm 0.15$
untransformed canola	0.78 ± 0.19	untransformed canola	0.78 ± 0.19

^a One unit of specific activity is defined as the enzyme activity catalyzing the formation of one μ mol of N-acetylglucosamine h⁻¹ (μ g protein)⁻¹

^b Difference is significance at 1 % using Least Significant Difference test (LSD) and the standard deviations are indicated by \pm values

Fig. 1 Inhibitory activity of the chimeric chitinase and Chit42 toward **a** *A. solani*, **b** *V. dahlia*, **c** *F. oxysporum* and **d** Growth zone diameter of pathogenic fungal growth. *T18* and *T2* (*Chit42*); *T9*, *T3* and *T1* (chimeric chitinase); Buff: Extraction buffer of protein as negative control; *WT*: the crude of proteins from wild type or untransformed canola plant as negative control. Results represent the average and standard deviation of three experiments



containing chimeric and native *Chit42* respectively, three bands were indicated. The transgenic lines carried one to three

copies of these genes which are according to the results of Moloney et al. (1989), who reported single or multiple copy

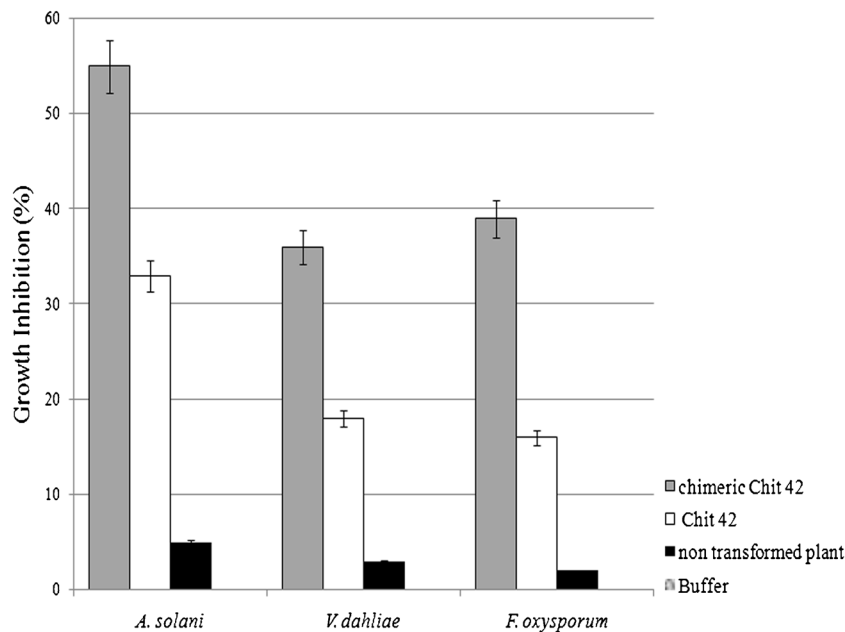


Fig. 2 The growth inhibition (%) of Chit42 and chimeric chitinase on spore germination. Growth of *A. solani*, *F. oxysporum* and *V. dahlia* in crude protein extracts from transgenic plant containing chimeric chitinase (*T9*, *T3* and *T1*) and *Chit42* (*T18* and *T2*) and non-transformed (*WT*) canola leaves. Absorbance of the reaction mixture (crude protein extract +

spore suspension + potato dextrose broth) after 48 h incubation was measured at 595 nm. Bars represent means and standard errors and those with different lower case letters are significantly different at ($p > 0.05$) by least significant difference (LSD) test. Results represent the average and standard deviation of three experiments

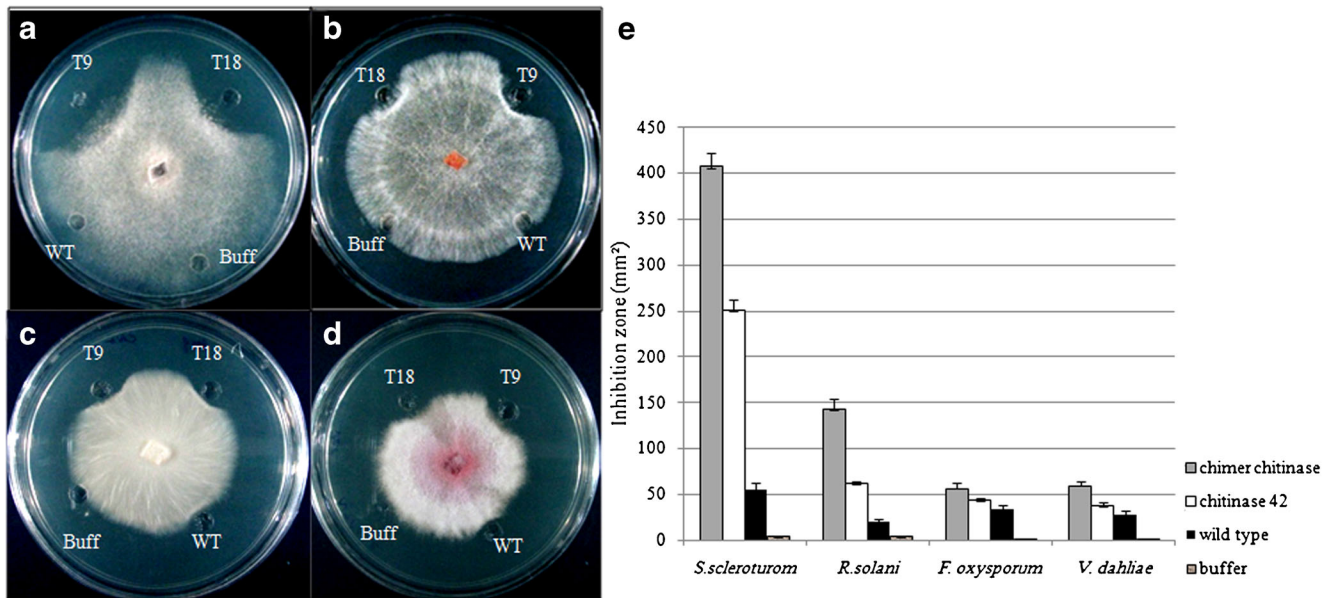


Fig. 3 Inhibitory activity of the chimeric chitinase and Chit42 toward **a** *S. sclerotiorum*, **b** *R. solani*, **c** *F. oxysporum* and **d** *V. dahliae*; **e** Inhibition zone diameter of pathogenic fungal growth. T18 (*Chit42*); T9 (chimeric chitinase); Extraction buffer of protein as negative control (*Buff*); the crude of proteins from wild type or untransformed canola plant as

negative control (*WT*). In diagram the bars represent means and standard errors for chimeric Chit42 (T9, T3 and T1) and Chit42 (T18 and T2) and those with different lower case letters are significantly different at ($p > 0.05$) by least significant difference (LSD) test. Results represent the average and standard deviation of three experiments

insertion into the canola genome. The size of hybridization bands from all tested transgenic plants were more than 2.5 kb for chimeric chitinase and 2.4 kb for *Chit42*. The

HindIII digested pBIKE1 plasmid was used as positive control. No transgene insertion was detected in untransformed plant (Supplementary Fig. 4).

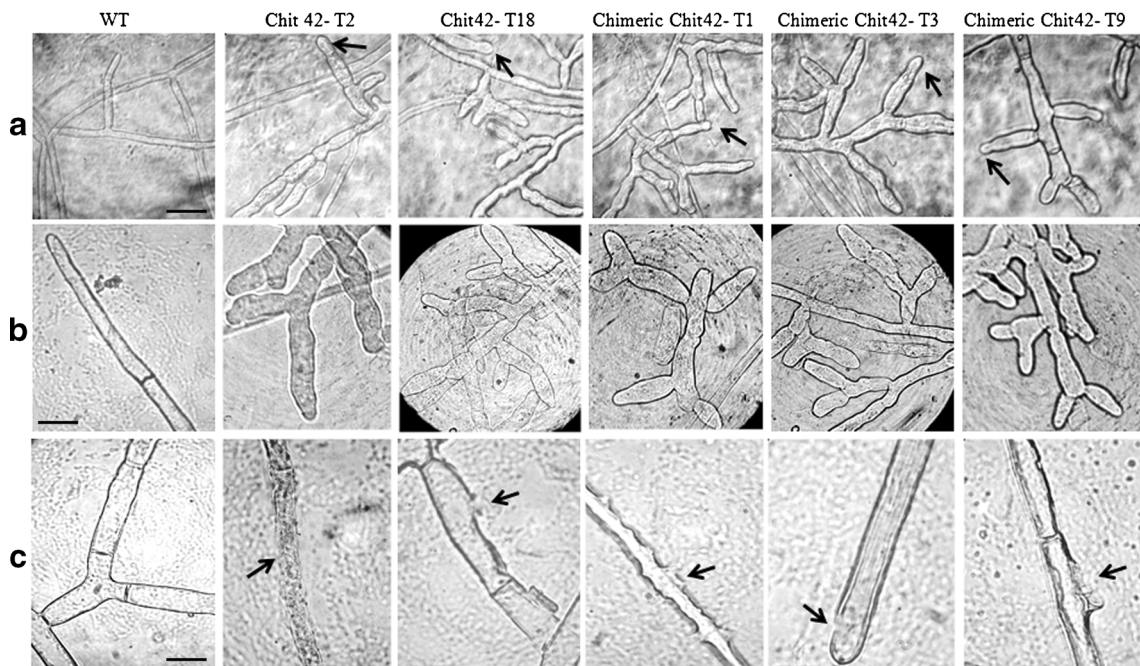


Fig. 4 Morphological changes induced in *Rhizoctonia solani* hyphae after exposure to the chitinase enzyme. **a** Mycelium of *R. solani*, 3 h after addition of proteins containing chimeric chitinase and native Chit42. The apical zone of young, emerging branches shows pronounced swelling (arrows). **b** Mycelium of *R. solani*, 6 h after addition of chitinase enzyme. Emerging branches are abnormally

shaped and markedly swollen. **c** Mycelium of *R. solani*, 24 h after addition of chitinase. Emerging degradation of *R. solani* cell wall shows pronounced swelling (arrows). All showed events including single copy of transferred gene with high chitinase activity. WT; Control hyphae exposed to crude proteins of untransformed plant showing a regularly mycelium. Scale bar = 20 μ m

Antifungal activity

In order to study antifungal activity of transgenes, the crude proteins were extracted from single copy transformant leaves containing chimeric (T9, T3 and T1) and native *Chit42* (T18 and T2) genes. The inhibitory effect was evaluated in vitro against five major phytopathogenic fungi. According to disk diffusion assay results, hyphal growth of *F. oxysporum*, *V. dahlia* and *A. solani* substantially decreased using total proteins of all transgenic lines. Moreover, germinated spores in the presence of chimeric chitinase were lower than lines with *Chit42* in all cases. No inhibition was observed in the case of control plant (Fig. 1).

The inhibition rate was also calculated using spore germination assay. Significant differences were found between transgenic and negative controls (wild type plant and protein extraction buffer). These results showed that the restriction of hyphal growth in *A. solani*, *F. oxysporum* and *V. dahlia* fungi using crude enzymes containing chimeric chitinase with inhibition rate of 55 %, 39 % and 36 % was stronger, when compared to those with *Chit42* with inhibition rate of 33 %, 16 % and 18 % respectively (Fig. 2).

As shown in Fig. 3, the extracted total proteins (45 µg) from transgenic lines exhibited antifungal activity against *R. solani*, *S. sclerotiorum*, *F. oxysporum* and *V. dahlia* based on radial diffusion assay. According to this result chitinase enzyme exhibited stronger inhibition against *S. sclerotiorum* and *R. solani* (Fig. 3a, b and e) than *F. oxysporum* and *V. dahlia* fungi (Fig. 3c, d and e). This may be due to the intrinsic variability of chitin in fungal cell wall, which exists in several forms (Van de Velde and Kiekens 2004; Aranaz et al. 2009). In addition, the antifungal activity of chimeric chitinase was significantly higher than *Chit42* (Fig. 3e). This finding seems to result from the subsite structure in the binding cleft of this enzyme (Sasaki et al. 2002). The same results have also been reported by Matroodi et al. (2013). Protein extraction buffer and crude proteins from wild type plants, as negative control, showed no or negligible inhibition against four fungal pathogens tested.

Microscopic observations revealed that the morphology and the cell wall of *R. solani* were changed and degraded after exposure to crude protein extracts from transgenic plants. Hyphal samples growing in PDA medium deposited on microscopic slides were studied at 3, 6 and 24 h using light microscopy (Fig. 4). Observation of control sample exposed to crude proteins of untransformed plant showed the presence of a dense and regular mycelium. They were described by their hyphal natural diameter and regular shape (WT in Fig. 4). Morphological changes, mainly localized at the hyphal tips, were recognizable within 3 h after exposure of hyphal samples to the crude proteins of transgenic plants. The apical zone of most hyphae displayed distinct swelling (Fig. 4a, arrows). The transgenic plants containing either of enzymes

are able to degrade the cell wall of *R. solani* mycelium (Fig. 4). After 6 h exposure, the *R. solani* hyphae were shown abnormal growth with noticeable swelling, branching and distortion (Fig. 4b). Then after 24 h enzyme exposure, hyphae was noticeably reduced in size and the degradation of cell wall was occurred (Fig. 4c, arrows). Similar to our findings, a number of other studies have implicated the chitinase enzyme to be responsible for degradation of the cell wall of phytopathogenic fungi (Harighi et al. 2006).

In conclusion, the addition of a ChBD increases enzyme activities and antifungal properties of chitinase, so the transgenic canola expressing chimeric chitinase showed stronger inhibition against phytopathogenic fungi. Transformation of genes with enhanced antifungal activity would allow us to develop fungal resistant crop plants and is likely to reduce dependence on chemical fungicides. Meanwhile, this work demonstrates that, the expression of these genes had no deleterious phenotypic effects on the transgenic plants.

Acknowledgments This research was financially supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB) of I. R. Iran.

Compliance with ethical standards

Conflict of interest The authors declare that they do not have any conflict of interest.

References

- Arakane Y, Zhu Q, Matsumiya M, Muthukrishnan S, Kramer KJ (2003) Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem Mol Biol* 33:631–648
- Aranaz I, Mengibar M, Harris R, Panos I, Miralles B, Acosta N, Galed G, Heras A (2009) Functional characterization of chitin and chitosan. *Curr Chem Biol* 3:203–230
- Barone A, Frusciante L (2007) Molecular marker-assisted selection for resistance to pathogens in tomato, Marker-Assisted Selection, Current status and future perspectives in crops, livestock, forestry and fish. pp 153–164
- Benhamou N, Broglie K, Broglie R (1993) Antifungal effect of bean endochitinase on *Rhizoctonia solani*: ultrastructural changes and cytochemical aspects of chitin breakdown. *Can J Microbiol* 39: 318–328
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Broekaert WF, Cammue BPA, De Bolle MFC, K T, GW d S, RW O (1997) Antimicrobial peptides from plants. *Crit Rev Plant Sci* 16: 297–323
- Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254: 1194–1197
- Cardoza V, Stewart CN (2003) Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyls segment explants. *Plant Cell Rep* 21:599–604

- Chang M, Culley D, Choi J, Hadwiger L (2002) *Agrobacterium* mediated co transformation of a pea β -1,3 glucanase and chitinase genes in potato (*Solanum tuberosum* L. c. v. Russet Burbank) using a single selectable marker. *Plant Sci* 163:83–89
- Cho HS, Cao J, Ren JP, Earle ED (2001) Control of Lepidopteran insect pests in transgenic Chinese cabbage (*Brassica rapa* ssp *ekinensis*) transformed with a synthetic *Bacillus thuringiensis* cry1C gene. *Plant Cell Rep* 20:1–7
- Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U (1993) Plant Chitinases. *Plant J* 3:31–40
- Das B, Goswami L, Ray S, Ghosh S, Bhattacharyya S, Das S, Majumder AL (2006) *Agrobacterium*-mediated transformation of *Brassica juncea* with a cyanobacterial (*Synechocystis* PCC6803) delta-6 desaturase gene leads to production of gamma-linolenic acid. *Plant Cell Tissue Organ Cult* 86:219–231
- Dixon GR (2006) Vegetable Brassicas and related crucifers- volume 14 of crop production science in horticulture. CAB International, Glasgow
- Doyle JJ, Doyle JL (1991) Isolation of plant DNA from fresh tissue plant. *Plant Mol Biol* 9:340–342
- Dutta I, Majumder P, Saha P, Ray K, Das S (2005) Constitutive and phloem specific expression of *Allium sativum* leaf agglutinin (ASAL) to engineer aphid (*Lipaphis erysimi*) resistance in transgenic Indian mustard (*Brassica juncea*). *Plant Sci* 169:996–1007
- False PA, Panda T (1999) Regulation and cloning of microbial chitinase genes. *Appl Microbiol Biotechnol* 51:141–151
- Graham LS, Sticklen MB (1994) Plant Chitinases. *Can J Bot* 72: 1057–1083
- Grover A, Pental D (2003) Breeding objectives and requirements for producing transgenics for major field crops of India. *Curr Sci* 84: 310–320
- Hardt M, Laine RA (2004) Mutation of active site residues in the chitin-binding domain ChBDChiA1 from chitinase A1 of *Bacillus circulans* alters substrate specificity: use of a green fluorescent protein binding assay. *Arch Biochem Biophys* 426:286–297
- Harighi MJ, Motallebi M, Zamani MR (2006) Antifungal activity of heterologous expressed chitinase 42 (*Chit42*) from *Trichoderma atroviride* PTCC5220. *Iran J Biotech* 4:95–103
- Hashimoto M, Ikegami T, Seino S, Ohuchi N, Fukada H, Sugiyama J, Shirakawa M, Watanabe T (2000) Expression and characterization of the chitin-binding domain of chitinase A1 from *Bacillus circulans* WL-12. *J Bacteriol* 182:3045–3054
- Iyer LM, Kumpatla SP, Chandrasekharan MB, Hall TC (2000) Transgene silencing in monocots. *Plant Mol Biol* 43:323–346
- Kahrizi D, Salmanian AH, Afshari A, Moieni A, Mousavi A (2007) Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (K12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. *Plant Cell Rep* 26:95–104
- Kojima M, Yoshikawa T, Ueda M, Nonomura T, Matsuda Y, Toyoda H, Miyatake K, Arai M, Fukamizo T (2005) Family 19 chitinase from *Aeromonas* sp. No. 10S-24: role of chitin-binding domain in the enzymatic activity. *J Biochem* 137:235–242
- Kronland WC, Stanghellini ME (1988) Clean slide technique for the observation of anastomosis and nuclear condition of *Rhizoctonia solani*. *Phytopathology* 78: 820–822
- Limon MC, Chacon MR, Mejias R, Delgado-Jarana J, Rincon AM, Codon AC, Benitez T (2004) Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain. *Appl Microbiol Biot* 64:675–685
- Liu T, Liu L, Jiang X, Hou J, Fu K, Zhou F, Chen J (2010) *Agrobacterium*-mediated transformation as a useful tool for the molecular genetics study of the phytopathogen *Curvularia lunata*. *Eur J Plant Pathol* 126:363–371
- Liu H, Guo X, Naeem MS, Liu D, Xu L, Zhang W, Tang G, Zhou W (2011) Transgenic *Brassica napus* L. lines carrying a two gene construct demonstrate enhanced resistance against *Plutella xylostella* and *Sclerotinia sclerotiorum*. *Plant Cell Tissue Organ Cult* 106:143–151
- Matroodi S, Motallebi M, MR Z, Moradyar M (2013) Designing a new chitinase with more chitin binding and antifungal activity. *World J Microbiol Biotechnol* 29:1517–1523
- Matzke MA, Mette MF, Matzke AJM (2000) Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanism in plant and vertebrates. *Plant Mol Biol* 43:401–415
- Moloney MM, Walker JM, Sharma KK (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep* 8:238–242
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Neuhaus JM (1999) Plant chitinases (PR-3, PR-4, PR-8, PR11). In: Datta SK, Muthukrishnan S (eds) Pathogenesis-related proteins in plants. CRC Press, Florida, pp. 77–105
- Nweze E, Mukherjee P (2010) Agar-based disk diffusion assay for susceptibility testing of dermatophytes. *J Clin Microbiol* 10:3750–3752
- Oerke E, Dehne H, Schonbeck F, Weber A (1994) Crop production and crop protection-estimated losses in major food and cash crops. Elsevier Science, Amsterdam
- Sasaki C, Yokoyama A, Itoh Y, Hashimoto M, Watanabe T, Fukamizo T (2002) Comparative study of the reaction mechanism of family 18 chitinases from plants and microbes. *J Biochem* 131:557–564
- Seberry DE, Mailer RJ, Parker PA (2009) Quality of Australian canola. AOF (Australian Oilseeds Federation) Publishing physicsWeb.http://www.australianoilseeds.com/data/assets/pdf_file/0004/6592/2009_Book.pdf. Accessed 2009
- Ullah F, Banu A, Nosheen A (2012) Effects of plant growth regulators on growth and oil quality of canola (*Brassica napus* L.) under drought stress. *Pak J Bot* 44:1873–1880
- Van Aalten DMF, Komander D, Synstad B, Gaseidnes S, Peter MG, Eijsink VGH (2001) Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Pnas* 98:8979–8984
- Van de Velde K, Kiekens P (2004) Structure analysis and degree of substitution of chitin, chitosan and dibutylchitin by FT-IR spectroscopy and solid state ¹³C NMR. *Carbohydr Polym* 58:409–416
- Wang J, Chen Z, Du J, Sun Y, Liang A (2005) Novel insect resistance in *Brassica napus* developed by transformation of chitinase and scorpiion toxin genes. *Plant Cell Rep* 24:549–555
- Wang J, Zheng L, Wu J, Tan R (2006) Involvement of nitric oxide in oxidative burst, phenylalanine ammonia-lyase activation and Taxol production induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures. *Nitric Oxide* 15:351–358
- Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuta N (2000) Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. *Plant Cell Rep* 19:639–646
- Zakariassen H, Aam BB, Horn SJ, Varum KM, Sorlie M, Eijsink VGH (2009) Aromatic residues in the catalytic center of chitinase A from *Serratia marcescens* affects processivity, enzyme activity, and biomass-converting efficiency. *J Biol Chem* 284:10610–10617
- Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP (1999) Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genet Biol* 26:131–140