Biotechnol Lett
DOI 10.1007/s10529-016-2058-7

ORIGINAL RESEARCH PAPER

Co-expression of chimeric chitinase and a polygalacturonase-inhibiting protein in transgenic canola *(Brassica napus)* confers enhanced resistance to *Sclerotinia sclerotiorum*

Mahboobeh Ziaei · Mostafa Motallebi · Mohammad Reza Zamani · Nasim Zarin Panjeh

Received: 31 August 2015 / Accepted: 2 February 2016

Abstract
Objectives Sclerotinia stem rot (SSR) caused by *Sclerotinia sclerotiorum* is one of the major fungal diseases of canola. To develop resistance against this fungal disease, the *chit42* from *Trichoderma atroviride* with chitin-binding domain and polygalacturonase-inhibiting protein 2 (PG1P2) of *Phaseolus vulgaris* were co-expressed in canola via *Agrobacterium*-mediated transformation.
Results Stable integration and expression of transgenes in T_0 and T_2 plants was confirmed by PCR, Southern blot and RT-PCR analyses. Chitinase activity and PGIP2 inhibition were detected by colorimetric and agarose diffusion assay in transgenic lines but not in untransformed plants. The crude proteins from single copy transformant leaves having high chitinase and PGIP2 activity (T16, T8 and T3), showed up to 44 % inhibition of *S. sclerotiorum* hyphal growth. The homozygous T_2 plants, showing inheritance in Mendelian fashion (3:1), were further evaluated under greenhouse conditions for resistance to *S. sclerotiorum.* Intact plants contaminated with mycelia showed resistance through delayed onset of the disease and restricted size and expansion of lesions as compared to wild type plants.
Conclusions Combined expression of chimeric *chit42* and *pgip2* in *Brassica napus* L. provide subsequent protection against SSR disease and can be helpful in increasing the canola production in Iran.

Keywords Antifungal activity · *Brassica napus* L. · Chimeric chitinase · Polygalacturonase-inhibiting protein · *Sclerotinia sclerotiorum* · Sclerotinia stem rot · Transgenic plants

Introduction

Canola (*Brassica napus* L.) is an important source of animal feed and industrially-used oil. The productivity of canola is limited by several abiotic and biotic stress factors. Fungal pathogens, such as *Sclerotinia sclerotiorum,* have served as a major cause of yield losses. *S. sclerotiorum* is destructive pathogen to oilseed rape which can cause the sclerotinia stem rot, rotting of leaves and pods, resulting in a substantial seed yield loss (Dong et al. 2008).

A1 **Electronic supplementary material** The online version of
A2 this article (doi:10.1007/s10529-016-2058-7) contains supple-
A3 mentary material, which is available to authorized users.

A4 M. Ziaei · M. Motallebi (✉) · M. R. Zamani ·
A5 N. Z. Panjeh
A6 National Institute of Genetic Engineering and
A7 Biotechnology (NIGEB), Tehran, Iran
A8 e-mail: motalebi@nigeb.ac.ir

A9 M. Ziaei
A10 e-mail: m_ziaei@nigeb.ac.ir

A11 M. R. Zamani
A12 e-mail: mrzamani97@yahoo.com

A13 N. Z. Panjeh
A14 e-mail: nassimzp@yahoo.com

Published online: 13 February 2016

Agricultural operations, utilization of chemical compounds and using resistant varieties are three common procedures for controlling fungal diseases (Barone and Frusciante 2007). Since the agrochemicals and conventional breeding approaches often have certain limitations, it is advisable to develop transgenic plants by inserting antifungal genes to confer resistance against fungal pathogens (Ceasar and Ignacimuthu 2012).

Chitinases are low molecular weight pathogenesis-related (PR) proteins that are often extracellular, acid-soluble and protease resistant. These enzymes are expressed not only for regular growth and development but also in response to fungal diseases (Cletus et al. 2013). Chitinase *Chit42* is an endo-chitinase of *Trichoderma atroviride* which plays a key role in biocontrol activities against phytopathogenic fungi (Harighi et al. 2006). This enzyme catalyzes hydrolysis of chitin that is a major component of many fungi cell walls and the second most abundant polysaccharide in nature. Only a few of the fungal chitinases contain a chitin-binding domain (ChBD) which is linked to the catalytic site via a linker region. ChBDs exhibit a high specificity to chitin and its binding activity is reversible (Kojima et al. 2005). *Chit42* does not contain a chitin-binding domain (ChBD) and in this study a chimeric *chit42* was used by adding a chitin-binding domain from Chitinase B of *Serratia marcescens* (Matroodi et al. 2013).

During plant infection, most phytopathogenic fungi produce enzymes like endo-polygalacturonases (PGs) that can degrade the plant cell wall. PGs cleave the internal bonds of homogalacturonan, the constituent of the smooth region of pectin. The function of PGs can be hampered by polygalacturonase-inhibiting proteins (PGIPs), localized in the cell wall of many plants (Benedetti et al. 2011). PGIPs form specific complexes with PGs and reduce their activity and slowing down cell wall deconstruction (Casasoli et al. 2009). Several studies have shown that PGIP reduces the susceptibility to fungal attack in different transgenic plants (Janni et al. 2008; Ferrari et al. 2012). In this investigation, the *pgip2* gene from *Phaseolus vulgaris* (PvPGIP2), encoding one of the most efficient PG inhibitor was used (Casasoli et al. 2009).

Different types of single or combinational foreign genes are now being introduced into plants to enhance their resistance against fungal diseases. In this study, we introduced combined genes of chimeric *chit42* and *pgip2* derived from *T. atroviride* and *P. vulgaris*, respectively, into canola. Those transgenic plants were tested for their potential to enhanced protection against the fungus *S. sclerotiorum.*

Materials and methods

Plant material, microorganisms and growth conditions

Canola (*Brassica napus*) R line Hyola 308, was used as a receptor and was kindly provided by the Oilseed and Development Co., Tehran, Iran.

Sclerotinia sclerotiorum was kindly provided by H. Afshari-Azad, Iranian Research Institute of Plant Protection, Tehran, Iran. The fungal strain was grown on potato/dextrose/agar (PDA) medium (potatoes, infusion 200 g/l, dextrose 20 g/l and agar 15 g/l) at 25 °C and subcultured as needed.

Escherichia coli DH5α (Cinnagen, Iran) was used in all molecular biological experiments and *Agrobacterium tumefaciens* LBA4404 was used for plant transformation procedure. The bacteria were grown in lysogeny broth (37 °C for *E. coli* and 28 °C for *A. tumefaciens*) with shaking (180 rpm). Media were supplemented when required with ampicilin and kanamycin (100 and 50 μg/ml) respectively.

Agrobacterum strain and molecular construct

To produce a binary and double genes construct, the *Eco*RI DNA fragment (2585 bp) carrying the modified CaMV35S promoter/*chit*42 from *Trichoderma atroviride* (accession number; DQ132792.1) with a chitin-binding domain from *Serratia marcescens* (accession number; X15208)/NOS terminator was cutout from pJETMZ1 construct (Matroodi et al. 2013). This construct is made of pJET vector plus chimeric *chit*42 along with CaMV35S promoter and NOS terminator which containing *Eco*RI sites on both sides of it. Digested fragment of pJETMZ1 was inserted into the pBIAH23 construct (Mohammadzadeh et al. 2012) at the *Eco*RI site, located downstream of the NOS terminator. The pBIAH23 construct is harboring the *pgip2* gene of *Phaseolus vulgaris* cultivar Naz Red Bean (accession number; DQ105560) that was cloned between the CaMV35S promoter and NOS terminator in the T-DNA region of pBI121 binary vector. The new construct designated as pBIMZ2.

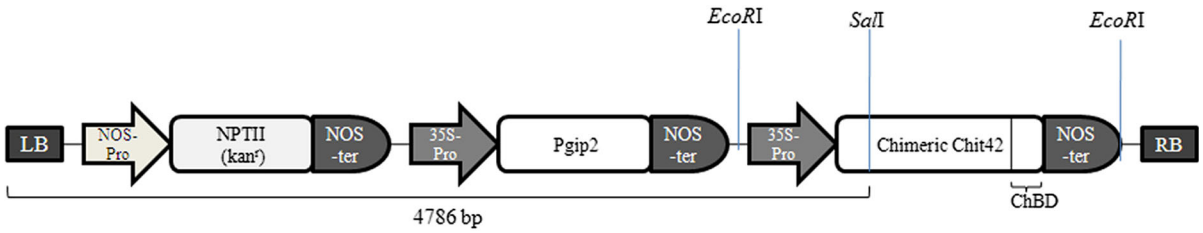

Fig. 1 Schematic representation of T-DNA region in binary vector pBIMZ2 harboring chimeric *chit42*, *pgip2* and neomycin phosphotransferase (*npt*II) genes with expected restriction patterns. Chimeric *chit42* and *pgip2* genes are driven by CaMV35S promoter and NOS terminator and *npt*II by NOS promoter and terminator. LB and RB: *left* and *right* border sequences of the T-DNA region, respectively. CaMV35S promoter: 35S promoter of cauliflower mosaic virus, NOS-terminator: terminator of nopaline synthase gene

The pBIMZ2 harbors neomycin phosphotransferase II (*npt*II) gene as a selectable marker which is kanamycin-resistant gene for plant selection, the chimeric *chit*42 and *pgip2* genes, encoding chitin-hydrolyzing enzyme and polygalacturonase inhibiting protein respectively, within the T-DNA region (Fig. 1). This construct was confirmed by PCR patterns using different combinations of gene and vector specific primers and was also mobilized into *A. tumefaciens* by freeze–thaw method (Sambrook and Ruussell). Therefore, these genes can be integrated together when transferred into plant cells.

Preparation of explants and the bacterial strain for transformation

Seeds were sterilized in 70 % (v/v) ethanol for 2 min and then in 0.1 % (v/v) sodium hypochlorite for 10 min. They were then rinsed with sterilized water several times and plated on ½MS (Murashige and Skoog) medium with 3 % (w/v) sucrose and 8 g agar/l in a growth room at 25 ± 2 °C, 16-h photoperiod with cool white fluorescent light of 2000 lux for 5 days. After germination, the cotyledonary petioles were cut and pre-cultured on CM solid medium (MS with 3.5 mg benzylaminopurine/l—BAP). After 2 days, the explants were used for transformation. Single colonies of *A. tumefaciens* strain harboring pBIMZ2 containing the chimeric chitinase and *pgip2* genes was used to incubate LB medium supplemented with 50 mg kanamycin/l and allowed to grow overnight at 28 °C with constant shaking (180 rpm) to mid-growth phase. The bacterial culture was then transferred to fresh medium and cultivated until an OD_{600} of 0.4 was obtained. The bacterial cells were collected by centrifugation ($\sim 5000 \times g$ for 5 min) and re-suspended in fresh ½MS medium for the subsequent inoculation step.

Canola transformation and selection procedure

The explants were immersed in a bacterial suspension for 5 min with constant shaking, then placed onto the sterile filter paper to remove excessive moisture and placed on CM medium in Petri dishes for co-cultivation at 25 °C for 3 days in the dark. After co-cultivation, the explants were washed with sterile water containing 200 mg cephatoxim/l to inhibit the growth of *A. tumefaciens* attached to the explants and then transferred to MS solid medium containing 3.5 mg BAP/l, 8 mg kanamycin/l and 200 mg cephatoxim/l. After shoot initiation, the explants were transferred to MS solid medium with 15 mg kanamycin/l and 200 mg cephatoxim/l. The regenerated shoots (about 3 cm in length) were excised from the explants and transferred to MS solid medium with 2 mg 3-indolebutyric acid (IBA)/l and 200 mg cephatoxime/l for rooting and recovering the complete plants. All the above media contained 3 % (w/v) sucrose, 8 g agar/l and the pH of the media was adjusted to 5.6–5.8 prior to the addition of agar. Plant transformation was carried out as described by Moloney et al. (1989) with some modifications.

Molecular analysis of the transgenic canola

The leaf material (50 mg) from the transgenic and wild type canola was harvested, lyophilized and grinded into a fine powder for extraction of genomic DNA (Doyle and Doyle 1991). PCR amplification was used for initial evidence of the transgene presence in the putative transgenic plants. DNA fragment containing the

chimeric *chit42* and *pgip2* genes was amplified by multiplex PCR using the genomic DNA and specific primers (Supplementary Table 1), simultaneously. PCR was carried out as follows: 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 separated by electrophoresis on 1 % (w/v) agarose gel.

PCR-positive plants and wild type control plants were analyzed by Southern blot analysis to confirm the integration of the introduced genes. Genomic DNA (20 µg) was digested with *Sal*I (Fig. 1). The digested genomic DNAs were fractioned on 0.8 % (w/v) agarose gels, transferred onto a nylon membrane (Amersham Hybond N+) and hybridised to the Dig-dUTP labeled CaMV35S probe. A partial internal fragment (631 bp in size) was obtained from PCR amplification of the CaMV35S fragment using CaMV35S F/CaMV35S R primers (Supplementary Table 1) and plasmid pBI121 (accession number: AF485783.1) containing the chimeric *chit*42 and *pgip*2 as template and subjected to DIG DNA labeling (Roche Applied Science) and used as a probe in hybridization experiments.

Chitinase activity assay

Activity of chimeric chitinase enzyme checked by colorimetric method. Young leaves from putative transgenic lines as well as untransformed canola plants were frozen in liquid N_2 and were ground to fine powder. The soluble proteins were then 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 crude of enzymes from transgenic plants containing chitinase (200 µg/ml). The reaction was performed at 37 °C for 1 h and then centrifuged at $\sim 5000 \times g$ for 5 min. The supernatant was boiled with 100 µl potassium tetraborate buffer for 3 min. Then 3 ml of DMAB reagent [10 g di-methyl aminobenzaldehyde in 100 ml glacial acetic acid and 10 M HCl] was added to the reaction, incubated at 37 °C for 20 min and the amount of *N*-acetylglucosamine (GLcNAc) produced in the supernatant was determined by the method described by Zeilinger et al. (1999) using GLcNAc as a standard. One unit of enzyme activity was defined 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 three times as biological replicates. Total soluble protein concentration was measured by the Bradford method using bovine serum albumin (BSA) as a standard.

PGIP assay

To provide appropriate conditions for endopolygalacturonase enzymes of *S. sclerotiorum* from pectic zymogram medium [containing 2.64 g $(NH_4)_2SO4$, 0.34 g KH_2PO_4, 0.14 g $MgSO_4 \cdot 7H_2O$, 10 g citrus pectin, 1 l distilled water. pH adjusted to 4.5 (Sweetingham et al. 1986)] was used. A piece of mycelium of fungi was grown in a shake-flask in this medium. After 6 days growth at 26 °C, a liquid culture filtrate was obtained using Whatman filter paper No.1 and stored at −20 °C until used for enzyme assay or protein measurement.

Frozen leaves of the transgenic and PCR-positive plants were subjected to protein extraction. Tissue was homogenized in liquid N_2 and resuspended in 20 mM sodium-acetate buffer, pH 4.6 containing 1 M NaCl. Homogenates were incubated with gentle shaking for 1 h at 4 °C, centrifuged for 10 min at $10{,}000 \times g$ and supernatants were transferred to fresh tubes. The protein content was determined against BSA according to the Bradford method. The crude protein extracts were assayed for inhibitory activity against endo-polygalacturonases (PG) produced by *S. sclerotiorum*. The inhibitory effect of the Pgip2 against the fungal PG activity was measured using an agarose diffusion assay (Taylor and Secor 1988). PGs and/or crude plant protein extracts were added to the wells of 0.8 % agarose plates containing 100 mM sodium acetate, pH 4.6 and 0.5 % citrus pectin. Plates were incubated for 16 h at 27 °C and the halo caused by the enzyme activity was visualized after 5 min treatment with 6 M HCl. Inhibitory activity was expressed as the ratio (in percentage) between the radius of the halo observed in the wells containing PG plus the plant protein extract and the halo observed with PG alone (Ferrari et al. 2012).

Antifungal activity on the bases of radial diffusion assay

An agar disc (5 mm diam.) containing *S. sclerotiorum,* which was derived from the fungi in an actively growing state, previously cultured on PDA, was placed

at the center of Petri dishes (100 × 15 mm) containing 25 ml PDA. After the mycelia colony had developed, 5 mm holes were made around and at a distance of 1 cm away from the rim of the mycelial colony. Equal aliquot (40–45 μg) of the crude of proteins, extracted with 50 mM Tris/HCl buffer (pH 8.8), from transgenic plants harboring Chit42 and Pgip2 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 crude of proteins from wild type plant. The inhibition rate (%) was calculated as: [(the distance between the control hyphal edges to the center—the distance between the treated hyphal edges to the center)/the distance between the control hyphal edges to the center in the control] × 100 %. The assay for each sample was performed three times.

Progeny segregation

Segregation of the transgenes in canola progeny was evaluated from the expression of the kanamycin resistance gene. 50–60 seeds from three events of T_2 transgenic lines harboring chimeric *chit42* and *pgip2* genes were cultured on MS solid medium with 30 mg kanamycin/l for 10 days. The green seedlings were considered as resistant and non-germinated/purple seedlings as sensitive. The events having only green seedling were considered as homozygous plants whereas the events having non-germinated or purple seedlings besides green seedlings were considered as hemizygous plants. Also, the events having only non-germinated or brown seedlings were considered as non-transgenic plants. The kanamycin resistant plants of each transgenic line were transferred to soil and further analyzed by semi-quantitative RT-PCR in T_2 plants.

Expression analysis

Specific mRNAs of the transgenes were checked using reverse transcriptase RT-PCR in T_2 plants. Total RNA was isolated from leaves of transgenic and control canola plants using RNX Plus kit (Cinnagen, Iran). First strand cDNA was generated using the oligo(dT) 18 primer by the "first strand cDNA synthesis kit" (Fermentas), which contained M-MuLV reverse transcriptase. PCR amplification was achieved using the first strand cDNA as template with chimeric *chit42* and *pgip2* gene specific primers (see Supplementary Table 1).

Reaction of intact plants to the pathogen

The bioassay was based on the inoculation of leaves on 30-day-old intact plants. For this purpose, a fully expanded leaf of normal color and shape was selected from each plant and marked for inoculation. The inoculation site for each selected leaf was the first one-third from the leaf tip. Leaves were inoculated with 4 mm diameter agar plugs containing hyphae of *S. sclerotiorum* which had been transferred from the margins of 3-day-old cultures. The mycelium-containing surface of the plugs was laid on the adaxial side of leaves. Plugs of ¼ PDA without the fungal mycelium were used on the control plants. All pots were immediately covered with transparent plastic bags to keep the humidity high. The experiment was established in the evening to provide dark conditions in the greenhouse. A completely randomized experimental design with three replications was used. Infection progress was measured as the diameter of necrotic areas at the inoculation points after 72 h of incubation.

Statistical analysis

The statistical differences were assessed based on the analysis of variance ANOVA using SPSS (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

The main objective of this work was to obtain transgenic canola (*Brassica napus*) plants harboring a chimeric chitinase gene along with polygalacturonase-inhibiting protein (*pgip2*) gene and to evaluate the effect of their antifungal activity.

Transformation and selection of transgenic plant

The recombinant construct, designated as pBIMZ2 (Fig. 1), was mobilized into *Agrobactrum tumefaciens* and subsequently used for *B. napus*, R line Hyola 308 transformation. The experiment was carried out with 620 explants which were co-cultivated with *A. tumefaciens* and were then transferred to selection medium containing kanamycin and cefotaxime. The number of regenerated shoots obtained at 8 mg kanamycin/l was

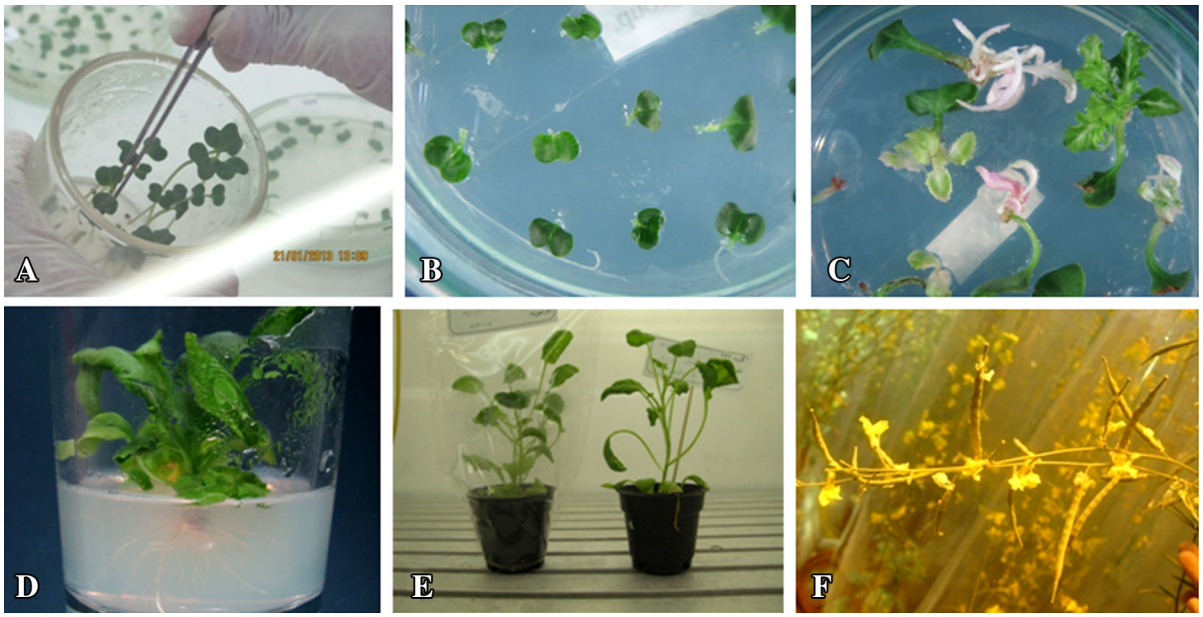

Fig. 2 Transformation and regeneration of transgenic canola plants: **a** Germinated seeds**, b** Cotyledons cultivated on co-culture medium with *A. tumefaciens*, **c** Regenerated shoots in growth and selective medium, **d** Regenerated plantlets with well-developed roots and leaves, **e** Covered regenerated plantlets in pots, **f** Pod development of transgenic plants in the greenhouse

248 for this cultivar. Shoots were transferred to a medium containing 15 mg kamamycin/l and only 135 green shoots were obtained. Then only 60-rooted plantlets were transferred to pots (Fig. 2). The putative transgenic lines were phenotypically analyzed and compared with the untransformed controls and they did not show any abnormalities with regard to the growth, size or reproduction.

Molecular analysis of regenerated plants

The rooted plantlets were subjected to PCR analysis for confirmation of the integration of chimeric chitinase and *pgip2* transgenes. All clones showed amplified fragments of 1484 bp for chimeric *chit42* and 855 bp for *pgip2* genes using 35SF/C42PR and 35SF/17DR2 as specific primers, respectively (Supplementary Table 1) in multiplex PCR (Fig. 3). This analysis confirmed the presence of these genes in 26 kanamycin-resistant putative transgenic plants with 4.1 % transformation efficiency. The *pgip2* and chimeric *chit42* specific primers did not amplify the corresponding fragments in the untransformed plants.

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

PGIP assay

The crude protein was extracted from leaves of all transgenic plants harboring *pgip2* and was then characterized by agarose diffusion assay against polygalactronase from *Sclerotinia sclerotiorum*. Inhibitory activity of the *pgip2* gene against PG, varied in all different line of the PCR positive transgenic plants (3.5–75 %) (Fig. 4a). Seven out of 26 transgenic plants (T26, T16, T9, T8, T4, T3 and T1) showed high level of PGIP expression ($\geq$60 % inhibition) (Fig. 4b).The wild type canola extracts displayed negligible inhibitory activity against *S. sclerotiorum* PG (Fig. 4a, b).

Chitinase activity assay

Chitinase activity from leaf tissues of the PCR positive transgenic plants, harboring chimeric chitinase and *pgip2*, and wild type plant was assayed in the presence

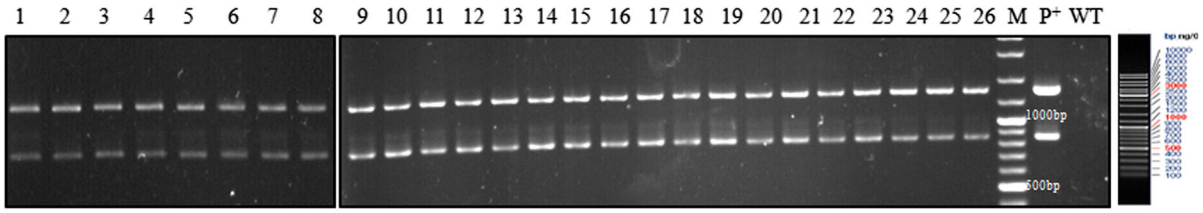

Fig. 3 Multiplex PCR analysis of different putative transgenic lines: Expected 1484 bp fragment amplified by PCR from DNA isolated of putative transgenic plants for chimeric *chit42* using 35SF/C42PR primers and expected 855 bp fragment amplified for *pgip2* using 35SF/17DR2 primers. P^+ pBIMZ2 plasmid template as positive control, *WT* untransformed sample, *M* DNA ladder Mix (Fermentas, CA)

of colloidal chitin. The specific enzyme activity of different transgenic plants varied from 0.52 ± 0.14 to 5.69 ± 0.27 U/μg (Fig. 4c). There was a significant difference between enzyme activities in transgenic lines with untransformed plant. Among all transgenic lines, T26, T16, T9, T8, T4 and T3 showed the highest specific enzyme activity (3.46 ± 0.32 to 5.69 ± 0.27 U/μg) (Fig. 4c).

Southern blot analysis

Southern blot analysis was performed in six PCR positive plants (T26, T16, T9, T8, T4 and T3) demonstrating higher chitinase activity and PGIP inhibition and the results showed that the transgenes were integrated into the transformed plants (Fig. 5). Amplified CaMV35S promoter was labeled as probe. Genomic DNA from transformed lines containing chimeric chitinase and *pgip2* genes were digested with *Sal*I (Fig. 1). The integrated genes were cut ones by *Sal*I and the other *Sal*I site was localized in the plant genome. The transgenes copy number was estimated as one in T16, T3 and T8 lines; two bands in T26 and T9, also three bands were indicated in T4 Line. The size of hybridization bands from all tested transgenic plants were more than 4.7 kb. The *Sal*I digested pBIMZ2 plasmid was used as positive control. No transgene insertion was detected in untransformed plant.

Antifungal activity on the basis of radial diffusion assay

In order to study antifungal activity of transgenes, the crude proteins from single copy transformant leaves having high chitinase and PGIP activity (T16, T8 and T3) were used in radial diffusion assay. The inhibitory effect was evaluated against *S. sclerotiorum* (Fig. 6). According to this result, the restriction of hyphal growth in T16, T8 and T8 with inhibition rate was of 44, 40 and 36 %, respectively. Protein extraction buffer and crude protein from untransformed plant, as negative control, showed no or negligible inhibition against the fungal pathogens tested (Fig. 6).

Greenhouse assay for resistance to *S. sclerotiorum*

Transgenic lines of T16, T3 and T8 were permitted for self-pollination to produce T_1 and T_2 generations. T_2 seeds were then cultured on medium containing 30 mg kanamycin/l to select homozygous plants. The result indicated inheritance in Mendelian fashion (3:1) and the events having only green seedlings were considered as homozygous plants. PCR and RT-PCR analyses showed stable integration and mRNA expression of the chimeric chitinase and *pgip2* genes in each of three transgenic lines (Fig. 7). These lines were transferred to the soil for evaluating the reaction of intact plants to *S. sclerotiorum.* After 72 h inoculation, all leaves showed symptoms of necrosis and gray-colored lesions. The lesions had a mostly necrotic soft and ovoid-shaped expansion (Fig. 8). Results revealed that there were significant difference between the lesion diameter of fungus on T16, T3 and T8 transgenic lines (11.01 ± 0.48, 11.35 ± 0.5 and 11.41 ± 0.49 mm, respectively) with untransformed plant (27.35 ± 0.25 mm).

Discussion

Canola (*Brassica napus*) is a major oilseed crop and the production of this plant is challenged by different fungal diseases. Sclerotinia stem rot (SSR), which is caused by *Sclerotinia sclerotiorum,* is one of the most important fungal diseases of canola which leads to serious crop losses in Iran (Aghajani

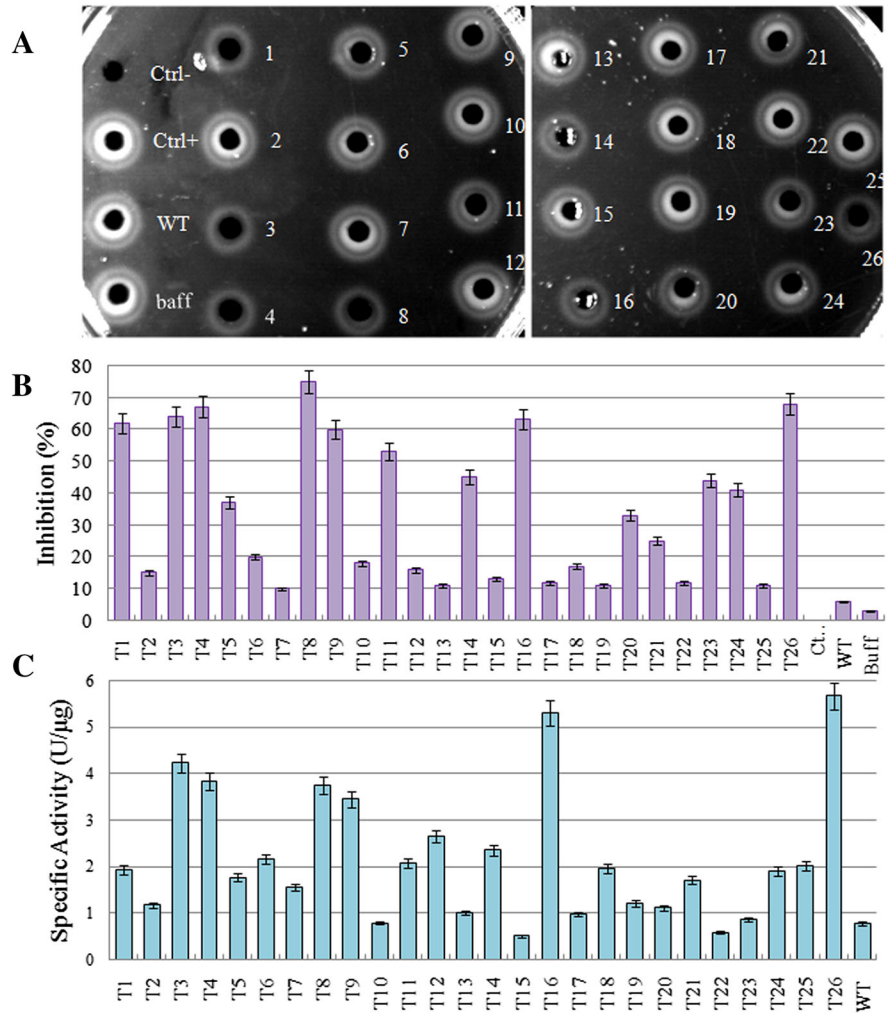

Fig. 4 Comparison of PGIP inhibition and chitinase specific activity in all transformants harboring chimeric *chit42* and *pgip2*. **a** PGIP inhibition assay against polygalacturonases from *S. sclerotiorum* using semi-quantitative agarose diffusion assay. $Ctrl^{+}$; PG enzyme alone as positive control, $Ctrl^{-}$; boiled PG enzyme alone as negative control, Baff; Extraction buffer of protein plus PG enzyme as negative control, WT; PG enzyme plus 35 μg of protein extract from untransformed canola that do not display any inhibitory activity against PGs. 1–26 (T1–T26); PG plus 35 μg of protein extracts from different transgenic canola plants. The samples T26, T16, T9, T8, T4, T3 and T1 exhibit high inhibitory activity (>60 %). Bar indicates 10 mm. **b** The PGIP inhibition (%) diagram. **c** Chitinase specific activity in leaf tissues of transgenic canola lines (T_0) and untransformed control plant (WT). One unit of activity is defined as the enzyme activity catalyzing the formation of one μmol of *N*-acetyl-glucosamine h^{-1} (μg protein)$^{-1}$. Difference was significance at $P < 0.05$ using least significant difference test (LSD) and Duncans multiple range tests. Results represent the average and standard deviation of three biological replicates

et al. 2013). Some investigations have been conducted to develop transgenic canola with enhanced SSR-resistant varieties (Liu et al. 2011; Solgi et al. 2015). Cell wall-degrading enzymes, such as chitinase (*chit42*) from *Trichoderma atroviride* and polygalacturonase inhibiting protein (*pgip2*) from *Phaseolus vulgaris,* have demonstrated high antifungal activity against a wide range of phytopathogenic fungi specially *S. sclerotiorum* (Harighi et al. 2006; Hegedus et al. 2008). The *chit42* lack a chitin-binding domain (ChBD). This domain permits tight interaction with the polymeric substrate and facilitates chitinase binding, thus allowing efficient degradation of chitin (Kojima et al. 2005).

The present study describes the transformation of *pgip2* and chimeric *chit42* genes under CaMV35S

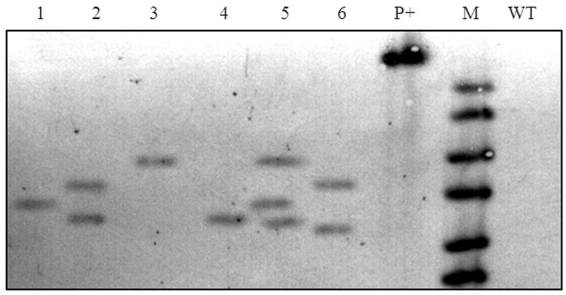

Fig. 5 Southern blot analysis of the transgenic canola plants transformed with the chimeric chitinase and *pgip2* encoding genes. Genomic DNA from the canola plant was digested with *Sal*I (Fig. 1) and hybridized with the digoxigenin-labelled 631 bp partial internal fragment of the CaMV35S as a probe, to show integration of the DNA into the plant genome and the number of integrations. The numbers identify each independent transgenic plant tested. Lane 1, *Sal*I digested DNA from transgenic plant containing these genes (T3); lane 2 (T26); lane 3 (T16); lane 4 (T8); lane 5 (T4); lane 6 (T9); Untransformed canola genomic DNA digested with *Sal*I is shown in lane WT. P^+; pBIMZ2 plasmid as positive control. *M* molecular marker

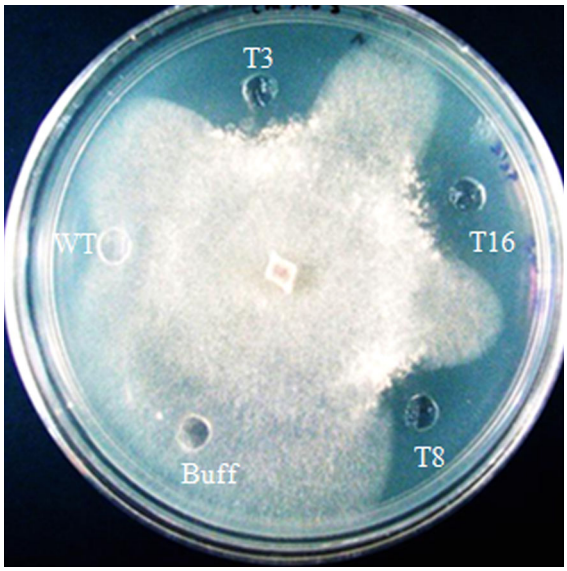

Fig. 6 Radial diffusion assay of crude protein extracts of transgenic canola against *S. sclerotiorum*. T16, T3 and T8 indicate the crude of proteins from transgenic lines with high chitinase and Pgip2 expression and have only one copy of the genes. Extraction buffer of protein as negative control (Buff); the crude of proteins from wild type or untransformed canola plant as negative control (WT)

promoter into canola plants using *Agrobacterium* as a vector. *Agrobacterium*-mediated transformation and use of CaMV35S promoter have been reported by several research groups as effective parameters. These studies indicate that the promoter were used to ensure high level of gene expression in all tissues and also *Agrobacterium* mediated method were used for increasing the rate of transformation in canola plants (Kahrizi et al. 2007; Liu et al. 2010). This novel gene combination could be complementary to broaden the antifungal genes spectrum for developing resistance against *S. sclerotiorum* infestation in transgenic plants.

Regeneration and transformation system are two important keys in success of transgenetics in canola plants. There are a number of factors such as hormone combination, co-culture time, antibiotic concentration and type of plant species which affect this intricate process for a specific cultivar (Cogbill et al. 2010). In this experiment, 8 and 15 mg kanamycin/l were used as suitable concentrations for 3 weeks and the time of co-culture was 72 h. All the lines that demonstrate positive results in the PCR analyses were further confirmed by the use of *vir*G primers. This indicates that the PCR product results were obtained from stable T-DNA integration into the canola genome and not from *Agrobacterium* contamination. The transformation efficiency of the transgenic lines achieved as 4.10 % which was similar to those reported in *B. napus* by Liu et al. (2011) and other studied *Brassica* species, *B. rapa* and *B. juncea* (Cho et al. 2001; Das et al. 2006).

Chitinase and PGIP activity of chimeric *chit42* and *pgip2* were measured in all PCR positive transgenic plants and six transgenic lines (T3, T4, T8, T9, T16 and T26) showing the highest chitinase and PGIP activity were selected for checking the copy number of transgenes through Southern blot hybridization. The genes were integrated into all seletced PCR positive plants. The transgenic lines carried one to three copies of these genes. This finding is in agreement with the results of Moloney et al. (1989), who reported single or multiple copy insertion into the canola genome. The lines (T3, T8 and T16) have only one copy of the transgene which were selected for further studies.

The ability of the introduced transgenes to enhance the antifungal potential of transgenic canola plants was studied by antifungal bioassays. Various in vitro inhibition assays reveal that different chitinases and PGIPs show inhibitory activity against a broad range of fungal pathogens. The inhibitory activity of PGIP2 from *P. vulgaris* against *Rhizoctonia solani* (Akhgari et al. 2012), AtPGIP1 and AtPGIP2 from *Arabidopsis thaliana* against a flower pathogen *Fusarium graminearum* (Ferrari et al. 2012), a novel chitinase gene

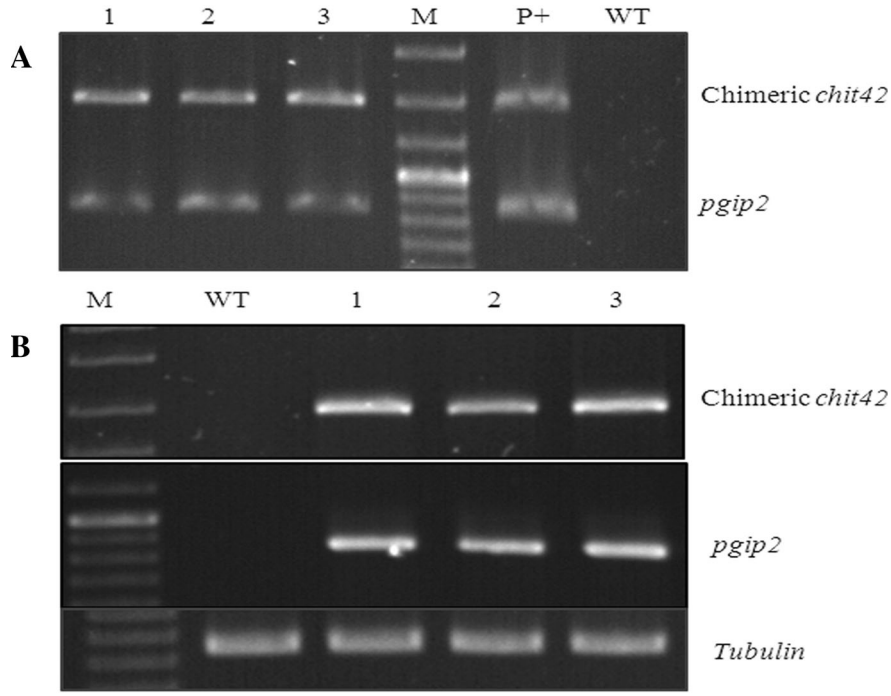

Fig. 7 PCR and RT-PCR analyses of T_2 transformed and untransformed canola. **a** Multiplex PCR was performed to confirm stable integration of transgenes using specific primers (35SF/C42PR for chimeric *chit42* and 35SF/17DR2 for *pgip2* genes) in T_2 transformants. **b** RT-PCR analysis was carried out to confirm expression of the transgenes at the mRNA level using gene specific primers (F3/R3 for chimeric *chit42* and 35SF/17DR2 for *pgip2*, respectively) in T_2 transgenic plants. The canola Tubulin gene was amplified in RT-PCR as a loading control. *M* DNA ladder Mix; *WT* Untransformed plant DNA; Lane 1 (T16); Lane 2 (T8) and Lane 3 (T3)

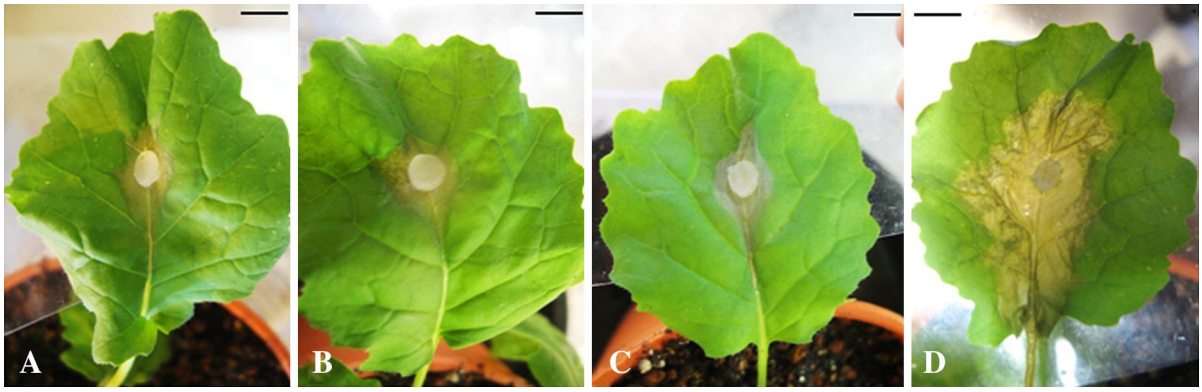

Fig. 8 Green house assay for resistance to *S. sclerotiorum* in T_2 generation. **a** T16, **b** T3 and **c** T8 transgenic lines; **d** untransformed plant. *Scale bar* 1 cm

Trchi1 from *Trichothecium roseum* against *Alternaria alternata* and *Colletotrichum nicotianae* (Xian et al. 2012) and rice chitinase gene against *Cercospora arachidicola* (Iqbal et al. 2012) are some of the examples in this issue. In the present study, crude protein extracts from T_0 transgenic lines (T3, T8 and T16) restricted *S. sclerotiorum* hyphal growth ranging from 36 to 44 % over the wild type plants in radial diffusion assay. The inhibition of hyphal growth of *S. sclerotiorum* may be due to degradation of chitin, a

major structural polysaccharide of growing hyphae, by chitinase and inactivation of fungal PGs by PGIP2 (Hegedus et al. 2008; Das and Rahman 2012).

Greenhouse assay of homozygous T_2 intact plants demonstrated that the produced lesions by *S. sclerotiorum* in transgenic plants were restricted to about 2.5-fold in diameter as compared to non-transgenic ones. Also the transgenic plants showed a significant delay in disease development. Similar results have been documented by several reports on different host-pathogen systems which have been shown resistance in the form of reduced lesion size due to restricted growth of fungal pathogens. For instance, co-expression of sporamin and chitinase *PjChi-1* genes into *B. napus* indicated high levels of resistance to *S. sclerotiorum* and reducing the size of leaf spot in transformants when compared to untransformed wild-type plants (Liu et al. 2011). In the other study, the transgenic *Brassica juncea* carrying barley class II chitinase and type I ribosome inactivating protein was sprayed with *Alternaria brassicae* spores in green house screening. The results showed significant resistance through delayed onset of the disease and restricted number, size, and expansion of lesions as compared to wild type plants (Chhikara et al. 2012).

Until now, there have been some evidence that the simultaneous deployment of multiple fungal resistant genes has a greater antifungal activity against a range of different fungi compared to a single gene (Liu et al. 2011; Chhikara et al. 2012). The novelty of this study is introducing different fungal pathogen-resistant genes, chimeric *chit42* and *pgip2,* into *B. napus*. The expression of these genes had no deleterious phenotypic effects on the transgenic plants.

In conclusion this study is a good precedent for introducing the simultaneous use of the several genes with the enhanced resistant effects on phytopathogenic fungi. These transgenic lines demonstrate a promising potential for variety development of canola lines with enhanced resistance against *S. sclerotiorum*.

Acknowledgments We would like to acknowledge National Institute of Genetic Engineering and Biotechnology (Project No. 407-M) for providing financial for this work.

Supporting Information Supplementary Table 1—The sequence of primers used in PCR and Southern blot analysis.

References

Aghajani MA, Safaie N, Alizadeh A (2013) Yield loss assessment of sclerotinia stem rot of canola in Iran. J Crop Prot 2:229–240

Akhgari AB, Motallebi M, Zamani MR (2012) Bean polygalacturonase-inhibiting protein expressed in transgenic *Brassica napus* inhibits polygalacturonase from its fungal pathogen *Rhizoctonia solani*. Plant Protect Sci 1:1–9

Benedetti M, Leggio C, Federici L, De Lorenzo G, Pavel NV, Cervone F (2011) Structural resolution of the complex between a fungal polygalacturonase and a plant polygalacturonase-inhibiting protein by small-angle X-ray scattering. Plant Physiol 157:599–607

Casasoli M, Federici L, Spinelli F, Di Matteo A, Vella N, Scaloni F, Fernandez-Recio J, Cervone F, De Lorenzo G (2009) Integration of evolutionary and desolvation energy analysis identifies functional sites in a plant immunity protein. Proc Natl Acad Sci USA 106:7666–7671

Ceasar SA, Ignacimuthu S (2012) Genetic engineering of crop plants for fungal resistance: role of antifungal genes. Biotechnol Lett 34:995–1002

Chhikara S, Dhankher OP, Chaudhury D, Jaiwal PK (2012) Combined expression of a barley class II chitinase and type I ribosome inactivating protein in transgenic *Brassica juncea* provides protection against *Alternaria brassicae*. Plant Cell Tissue Organ Cult 108:83–89

Cho HS, Cao J, Ren JP, Earle ED (2001) Control of lepidopteron insect pests in transgenic Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) transformed with a synthetic *Bacillus thuringiensis cry1C* gene. Plant Cell Rep 20:1–7

Cletus J, Vashisht D, Balasubramanian V, Sakthivel N (2013) Transgenic expression of plant chitinases to enhance disease resistance. Biotechnol Lett 35:1719–1732

Cogbill S, Faulcon T, Jones G, McDaniel M, Harmon G, Blackmon R, Young M (2010) Adventitious shoots regeneration from cotyledonary explants of rapid-cycling fast plants of *Brasscia rapa* L. Plant Cell Tissue Organ Cult 101:127–133

Das DK, Rahman A (2012) Expression of a rice chitinase gene enhances antifungal response in transgenic litchi (cv. *Bedana*). Plant Cell Tissue Organ Cult 109:315–325

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

Dong X, Ji R, Guo X, Foster SJ, Chen H, Dong C, Liu Y, Hu Q, Liu S (2008) Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape. Planta 228:331–340

Doyle JJ, Doyle JL (1991) Isolation of plant DNA from fresh tissue plant. Plant Mol Biol 9:340–342

Ferrari S, Sella L, Janni M, De Lorenzo G, Favaron F, D'Ovidio R (2012) Transgenic expression of polygalacturonase-inhibiting proteins in *Arabidopsis* and wheat increases resistance to the flower pathogen *Fusarium graminearum*. Plant Biol 14:31–38

Harighi MJ, Motallebi M, Zamani MR (2006) Antifungal activity of heterologous expressed chitinase 42 (*Chit*42) from *Trichoderma atroviride* PTCC5220. Iran J Biotech 4:95–103

Hegedus DD, Li R, Buchwaldt L, Parkin I, Whitwill S, Coutu C, Bekkaoui D, Rimmer SR (2008) *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment. Planta 228:241–253

Iqbal MM, Ali S, Iqbal J, Asif MA, Nazir F, Zafar Y, Ali GM (2012) Over expression of rice chitinase gene in transgenic peanut (*Arachis hypogaea* L.) improves resistance against leaf spot. Mol Biotechnol 50:129–136

Janni M, Sella L, Favaron F, Blechl AE, De Lorenzo G, D'Ovidio R (2008) The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. Mol Plant Microb 21: 171–177

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

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 Tiss Organ Cult 106:143–151

Matroodi S, Motallebi M, Zamani MR, Moradyar M (2013) Designing a new chitinase with more chitin binding and antifungal activity. World J Microbiol Biotechnol 29:1517–1523

Mohammadzadeh R, Zamani MR, Motallebi M, Norouzi P, Jourabchi E, Benedetti M, Lorenzo GD (2012) *Agrobacterium tumefaciens*-mediated introduction of polygalacturonase inhibiting protein 2 gene (PvPGIP2) from *Phaseolus vulgaris* into sugar beet (*Beta vulgaris* L.). Aust J Crop Sci 6:1290–1297

Moloney MM, Walker JM, Sharma KK (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep 8:238–242

Solgi T, Moradyar M, Zamani MR, Motallebi M (2015) Transformation of canola by *Chit33* gene towards Improving resistance to *Sclerotinia sclerotiorum*. Plant Protect Sci 51:6–12

Sweetingham MW, Cruickshank RH, Wong DH (1986) Pectic zymograms and taxonomy and pathogenicity of the Ceratobasidiaceae. Trans Brit Mycol Soc 86:305–311

Taylor RJ, Secor GA (1988) An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopathology 78:1101–1103

Xian H, Li D, Li J, Zhang L (2012) Cloning and functional analysis of a novel chitinase gene *Trchi1* from *Trichothecium roseum*. Biotechnol Lett 34:1921–1928

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