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Transformation of Canola by *Chit*33 Gene towards Improving Resistance to *Sclerotinia sclerotiorum*

Tara SOLGI, Mehdi MORADYAR, Mohammad Reza ZAMANI and Mostafa *MOTALLEBI*

National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

Abstract

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An endochitinase gene (*chit*33-cDNA) from the biocontrol fungus *Trichoderma atroviride* was overexpressed under the CaMV35S constitutive promoter in canola (R line Hyola 308). Transformation of cotyledonary petioles was achieved via *Agrobacterium tumefaciens*. The insertion of the transgene was verified by PCR and Southern blotting. The transgenic over-expression approach was used in order to investigate antifungal activity of expressed Chit33 on *Sclerotinia sclerotiorum*. Antifungal activity was detected in transgenic canola using detached leaf assay. Lesion sizes of transgenic canola caused by *S. sclerotiorum* were significantly retarded when compared to non-transgenic canola plant.

Keywords: antifungal activity; *Brassica napus*; *chit*33-cDNA; fungal diseases

Fungal diseases rated as one of the most important factors contributing to the yield losses in many important crops. Contribution of the fungal diseases towards the total yield losses in some important crops is about 30% (Adams 2004). Various fungal cell wall degrading enzymes, including the chitinases and various glucanases or glucosidases are involved in the mycoparasitic process of *Trichoderma* (Chet *et al.* 1998). Among these hydrolases, chitinases are attractive molecules because they have strong antifungal activity against a wide range of fungi (Gokul *et al.* 2000; Harighi *et al.* 2006). This hydrolytic enzyme catalyses the degradation of chitin and, since this compound is abundantly present in the cell wall of many filamentous fungi (Wessels & Sietsma 1981), they are thought to be capable of inhibiting fungal growth *in planta*.

Canola (*Brassica napus* L.) is one of the most important sources of edible vegetable oil and protein-rich products in the world. Like in many other crops, the production of this crop is challenged by phytopathogenic fungi. Extensive use of chemical fungicides, which have drawbacks such as environmental pollution, producing residual poisons to the human beings and animals, and expensive cost, is the conventional method of control of fungal pathogens. Therefore, it is desirable to introduce the foreign fungal-resistant genes into the important crop plants. Theoretically, the foreign genes can be transferred into the 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, the chitinases which are potential antifungal agents through their chitin degradation activity, are excellent candidates for controlling the fungal pathogens development (Kalai *et al.* 2006; Maximova *et al.* 2006; Gentile *et al.* 2007; Jayaraj & Punja 2007; Xiao *et al.* 2007; Pasonen *et al.* 2008).

In the present study, the chitinase cDNA (*chit*33) from *Trichoderma atroviride* was introduced into canola via *Agrobacterium*-mediated transformation. The introduced gene was expressed in transgenic plants and showed to decline the *Sclerotinia sclerotiorum* growth.

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MATERIAL AND METHODS

Plant material. The rapeseed (*Brassica napus* L.) R line Hyola 308 was used as recipient, which was kindly provided by the Oilseed and Development Co., Tehran, Iran.

Microorganisms, plasmid vector, and culture conditions. *Sclerotinia sclerotiorum* was kindly provided by H. Afshari-Azad from the Iranian Research Institute of Plant Protection, Tehran, Iran. The fungus strain was grown on the potato dextrose agar (PDA) medium at 28°C. *Escherichia coli* DH5α (Cinnagen, Karaj, Iran) was used in all the molecular biological experiments and *Agrobacterium tumefaciens* LBA4404 was used for plant transformation. Bacteria were grown in the LB medium (Bertani 1951) at appropriate temperatures (37°C for *E. coli* and 28°C for *Agrobacterium tumefaciens*) with shaking (200 rpm).

General DNA procedures. Plasmid DNA preparation and electrophoresis of the DNA fragments were performed using routine procedures (Sambrook & Russell 2001). The restriction enzyme analysis of the amplified DNA was carried out as recommended by the manufacturer (Fermentas, St. Leon-Rot, Germany). The bacteria were transformed by the heat shock method (Sambrook & Russell 2001).

Construction of the binary vector. The *A. tumefaciens* strain LBA4404 harbouring the recombinant binary vector pBITS1 with the *chit*33 gene was used in the experiments. The *chit*33-cDNA from *T. atroviride* was transferred from pUCSM2 (Matroudi *et al.* 2008) to pBI121GUS$^-$ (Esfahani *et al.* 2012) under the control of the *Cauliflower mosaic virus* 35S promoter and the nopaline synthase terminator. The obtained construct pBITS1 was transferred into *E. coli* DH5α using $CaCl_2$ method (Sambrook & Russell 2001). Enzyme digestion of pBITS1 was done according to manufacturer's recommendations (Fermentas). PCR analysis was carried out to confirm the new construct using a combination of MFCH33, MRCH33, 35SF, and NOSR primers (Table 1).

Preparation of the explants and bacterial strain for transformation. Seeds were surface sterilised in 70% ethanol for 5 min and then in 0.1% $HgCl_2$ for 8 minutes. Then they were rinsed several times with the sterilised water and plated on the ½ MS medium (Murashige & Skoog 1962) in the incubator for 5 days. After germination, the cotyledonary petioles were cut and placed on the MS solid medium with 3.5 mg/l benzylaminopurine (BAP) for pre-culture. After 2 days, the explants were used for transformation.

Single colonies of the *A. tumefaciens* strain harbouring pBITS1 containing the *chit*33 gene were grown in the 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 into a fresh medium with the amount of 1% and cultivated till OD_{600} = 0.4 with liquid medium. The bacterial cells were collected by centrifugation and re-suspended in ½ MS medium for use.

Transformation and selection procedure. The explants were immersed in the bacterial suspension for 1.5 min, and then placed onto the sterile filter paper to remove the excessive moisture, and placed on the MS solid medium containing 3.5 mg/l BAP in the Petri dishes for co-cultivation at 25°C for 2 days.

After co-cultivation, the explants were washed with the sterile water containing 200 mg/l cefotaxime (Jaber Ebne Hayyan, Tehran, Iran) to inhibit the growth of *A. tumefaciens* attached to the explants and then transferred to the MS solid medium with 3.5 mg/l BAP, 15 mg/l kanamycin, and 200 mg/l cefotaxime. After shoot initiation, the explants were transferred to MS solid medium with 25 mg/l kanamycin and 200 mg/l cefotaxime. The regenerating shoots (about 3 cm in length) were excised from the explants

Table 1. Names and sequences of primers used in the experiments

Primer name	Sequence
MFCH33	5' GCTCTAGAATGCCTTCATTGACTGCTCTTGCG 3'
MRCH33	5' CGTCTAGATTACCTCAAAGCATTGACAACC 3'
MSR33	5' GTGATTCCGTTTAAAGCAATGTC 3'
MF2CH33	5' CTAACGGCAAGACCATCC 3'
35SF	5' GGCGAACAGTTCATACAGAGTCT 3'
NOSR	5' CGCGATAATTTATCCTAGTTTGC 3'
virGf	5' ATGATTGTACATCCTTCACG 3'
virGr	5' TGCTGTTTTTATCAGTTGAG 3'

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and transferred to the MS solid medium containing 100 ml/l of coconut milk, 25 mg/l kanamycin, and 200 mg/l cefotaxime for rooting and recovering of the complete plants. All the above mentioned media contained 3% (w/v) sucrose with pH 5.8 and all the explants were cultured under 23–25°C and 16 h photoperiod with light provided by high pressure metal halide lamps (60 $\mu m^2/s$).

Molecular analysis of the transgenic canola. The leaf materials from the transgenic (T0) and non-transgenic canola plants grown *in vitro* were harvested, lyophilised, and grinded into the fine powder for extraction of genomic DNA by the CTAB method (Doyle 1990). PCR amplification was used for initial evidence of the presence of the transgene in the putative transgenic plants. The DNA fragment containing the *chit*33 gene was amplified by PCR and nested PCR using the transgenic canola genomic DNA and a combination of 35SF/MSR33 and MFCH33/MSR33 primers, respectively (Table 1).

Dot and Southern blot analysis. As a probe in hybridisation experiments, a fragment (655 bp in size) was obtained from PCR amplification of the *chit*33 gene, using the MF2CH33/MRCH33 primers and plasmid pBITS1 containing the *chit*33 gene as a template and subjected to the DIG DNA labelling (Roche Applied Science, Basel, Switzerland).

For dot blot analysis, the extracted genomic DNA (15 µg) from the fresh leaves of the putative transgenic plants (T0) and untransformed control ones grown *in vitro* was denatured for 10 min in boiling water and chilled on ice. The denatured genomic DNAs were spotted onto a nylon membrane (Hybond N^+; Amersham, Buckinghamshire, UK), and hybridised to Dig-dUTP labelled *chit*33 probe. For Southern blot analysis, 15 µg of genomic DNA was digested with *Eco*RI. The digested genomic DNAs were fractioned on 0.7% (w/v) agarose gel, transferred onto a nylon membrane (Hybond N^+), and hybridised to Dig-dUTP labelled *chit*33 probe.

Bioassay of the transgenic canola plants. Susceptibility of the transgenic and non-transgenic canola to *S. sclerotiorum* was evaluated using a detached leaf assay described by Carstens *et al*. (2003). The leaves of the 5- to 6-week-old T0 greenhouse plants were detached and placed in the Petri dishes, positioned over the wet filter papers. The upper surface of the leaves was inoculated with an agar disc (5 mm in diameter) of the actively growing *S. sclerotiorum*. Petri dishes were incubated at 28°C and the lesion growth was measured 48 h after inoculation and then photographed.

RESULTS

In order to transform *Brassica napus*, R line Hyola 308, a chitinase gene (*chit*33-cDNA) originated from *T. atroviride*, was used. The DNA fragment containing the *chit*33 cDNA was isolated from pUCSM2 (Matroudi *et al.* 2008) and subcloned into plant binary expression vector pBI121. Due to the presence of a *Sac*I site in *chit*33 cDNA, the fragment containing the *chit*33 open reading frame (ORF) was subcloned into *Xba*I site of pBI121GUS$^-$ (originated from pBI121 vector which has lost its *gus* gene and *Sac*I site) (Esfahani *et al.* 2012).

Cloning of the fragment into this expression vector was confirmed by digestion with appropriate enzyme and amplified patterns using combination of the gene and vector specific primers (Figure 1).

The open reading frame of *chit*33 gene in pBIGUS (pBITS1 construct), which is between the CaMV 35S promoter and nopaline synthase terminator, was confirmed by DNA sequencing (data not shown).

The pBITS1 was delivered into *Agrobacterium tumefaciens* (LBA4404) and subsequently used for transformation of 5-day-old cotyledonary petioles of *B. napus*, R line Hyola 308. The shoots were regenerated from

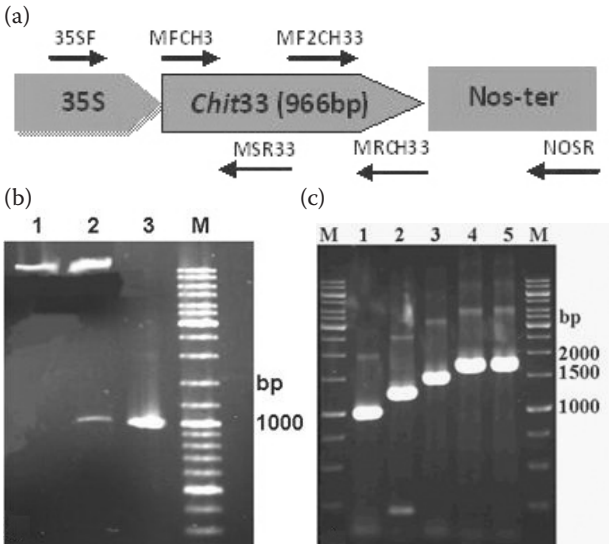

Figure 1. Confirmation of pBITS1 containing *chit*33 cDNA: (a) schematic representation of various primers used for PCR analyses, (b) digestion pattern (1 – digested pBI121GUS$^-$ using *Xba*I; 2 – digested pBITS1 using *Xba*I; 3 – PCR product with gene specific primers (MFCH33/MRCH33); M – ladder mix), and (c) PCR analysis confirming pBITS1 as template (1 – PCR product using the MFCH33/MRCH33 as primers; 2 – MFCH33/NOSR as primers; 3 – 35SF/MRCH33 as primers; 4 and 5 – 35SF/NOSR as primer; M – 1 kb ladder)

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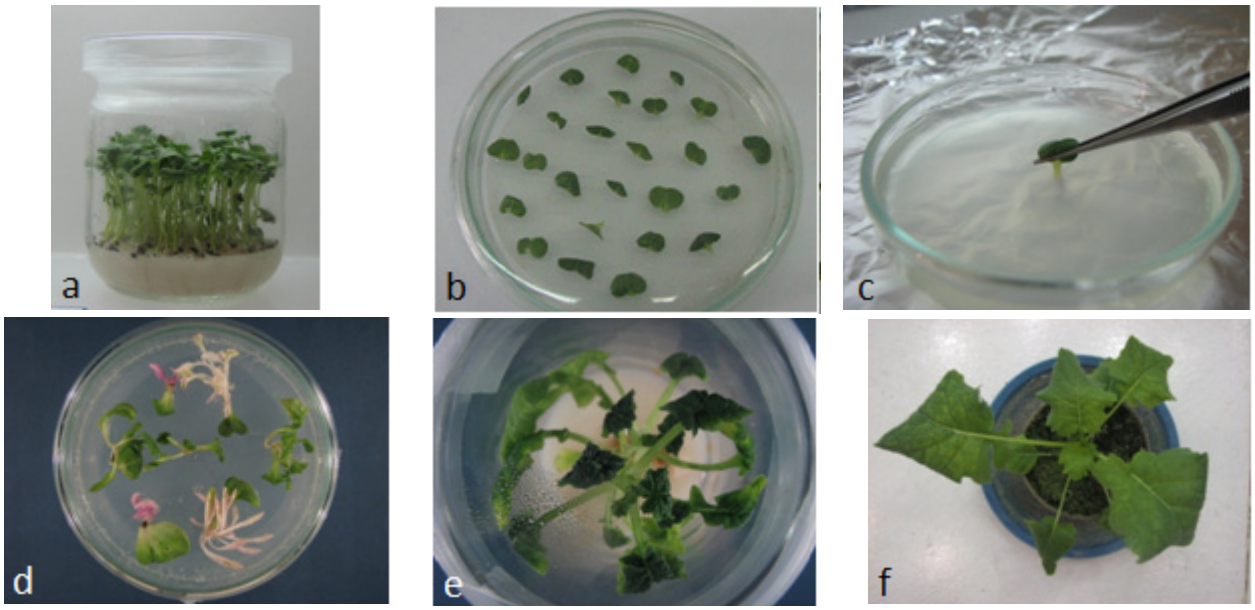

Figure 2. Transformation and regeneration of transgenic canola plants: (a) germination of the R line Hyola 308 seeds, (b) cotyledon explants on preculture medium, (c) transformation of canola explant with *Agrobacterium tumefaciens* LBA4404, (d) regenerated transgenic (green) and non-transgenic (pink or white) shoots on selective medium, (e) shoot and root of established plant, and (f) regeneration plantlet in the pot and acclimated to the non-aseptic environment

the cotyledonary petioles 1–2 weeks after planting on kanamycin selective medium. Independent transgenic canola lines were successfully rooted on the MS medium containing coconut milk and then transferred to the greenhouse (Figure 2). The putative transgenic plants showed to contain the *chit33* transgene using PCR pattern (Figure 3). PCR results were confirmed by nested PCR via gene specific primers (MFCH33 and MSR33). The presence of the expected 200 bp fragment confirmed the transformation of PCR positive plants by *chit*33 cDNA. The *chit33* specific PCR primers did not amplify the corresponding *chit*33 fragment in the untransformed sample.

A set of *vir*G primers (virGf and virGr) was used for detection of any *Agrobacterium* contamination that might have escaped the selection (Table 1). The PCR detection under various conditions showed no detectable band using the transgenic plants DNA as template. Using virG primers an expected 738 bp band was detected when the *Agrobacterium* DNA was used as control (data not shown).

Dot blot analysis on the PCR positive transgenic plants was performed with a 655 bp probe representing an internal fragment of *chit*33 coding region and the results verified the integration of the exogenous gene into the host genome (data not shown). No hybridisation signal occurred in the non-transgenic control plants.

In order to test the antifungal activity of the expressed Chit33 from transgenic canola lines against the phytopathogenic fungus *S. sclerotiorum,* the experiment was performed using the greenhouse acclimated transgenic lines in a detached leaf assay. The lesion expansion occurred on leaves and proceeded

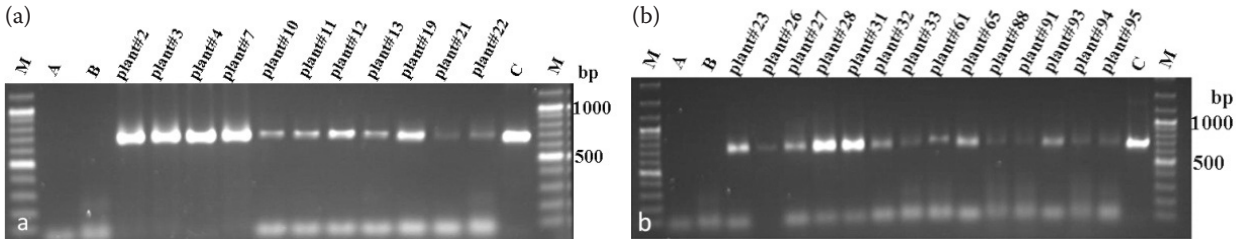

Figure 3. PCR analysis of 25 putative transgenic canola plants; (a) 655bp band was amplified using the specific primers (35SF/MSR33) and (b) DNA of different putative transgenic plants as template: A – negative control (master PCR); B – wild type plant; C – pBITS1 construct as a positive control; M – ladder mix

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Figure 4. *Sclerotinia sclerotiorum* lesion development on detached leaves of selected transgenic (plant Nos 12, 28, 65, and 88) and non-transgenic (Wt) canola plants. Photographs were taken 48 h after inoculation showing confined lesion growth in the transgenic plants as compared to the wild type ones

aggressively in untransformed control (Figure 4). Lesion sizes were significantly retarded in some of the transgenic canola lines (4 out of 25 lines). The transgenic line 28 exerted a stronger inhibition against lesion expansion with inhibition rate of 62.5%, while transgenic lines 88, 65, and 12 exhibited lower inhibition with inhibition rate of 55, 32.5, and 30%, respectively (Figure 4). However, inhibition from other transgenic lines was negligible. The transgenic lines were phenotypically analysed and compared to the untransformed controls and did not show any abnormalities with regards to the growth, size or reproduction.

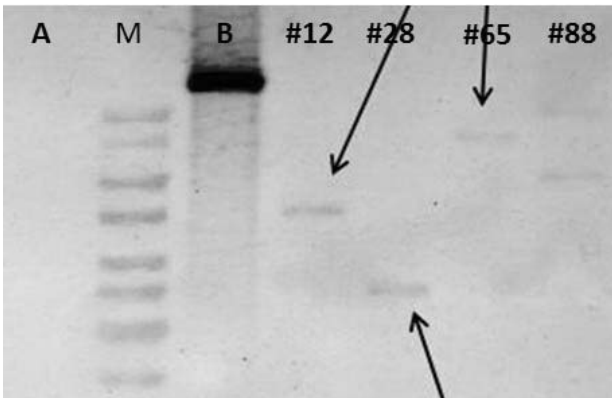

Figure 5. Southern blot analysis of the transgenic canola plants transformed with the *chit33* encoding gene. Genomic DNA from the canola plant was digested with EcoRI and hybridised with the digoxigenin-labelled 655 bp partial internal fragment of the *chit33* gene as a probe, to show integration of the DNA into the plant genome and the number of integrations (arrows). The numbers identify each independent transgenic plant tested. Untransformed canola genomic DNA digested with EcoRI is shown in lane A; B – pBITS1 DNA as positive control; M – molecular marker

Southern blot hybridisation of the transgenic lines 28, 88, 65, and 12 was conducted with the same probe as dot blot. Plant genomic DNA was digested with *Eco*RI enzyme. By the presence of only one *Eco*RI restriction site between right and left borders, fragments longer than 3 kb would be detected by Southern blot. The *chit*33 transgene copy number was estimated as one copy in lines 12, 28, and 65 and two copies in line 88 (Figure 5).

DISCUSSION

Canola is a major oilseed crop and *S. sclerotiorum* is associated with the stem rot disease of this economically important crop. Stem rot is one of the most important fungal disease in canola which can cause serious yield losses, especially in warm and humid areas which often occur on canola fields in Iran (Afshari-Azad 2001). Cell wall degrading enzymes including chitinases from biocontrol fungi belonging to the genus *Trichoderma* have been demonstrated to have high antifungal activity against a wide range of economically important aerial and soil-borne plant pathogenic fungi (Carsolio *et al.* 1999; Gokul *et al.* 2000; Adams 2004; Harighi *et al.* 2006). The chitinase enzymes are fungicidal (Jayaraj & Punja 2007; Xiao *et al.* 2007) and non-toxic to plants, animals, and higher vertebrates (Lorito *et al.* 1996). Some research has been conducted to develop transgenic crop plants that have increased expression levels of chitinase genes in hopes of producing fungal disease resistant varieties (Liu *et al.* 2004; Deng *et al.* 2007; Gentile *et al.* 2007; Jayaraj & Punja 2007; Xiao *et al.* 2007). It has been shown that chitinase enhances fungal disease resistance in different species

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including carrot against foliar pathogens (Jayaraj & Punja 2007), lemon against *Phoma tracheiphila* and *Botrytis cinerea* (Gentile *et al*. 2007), and rice against *Rhizoctonia solani* and *Magnaporthe grisea* (Liu *et al*. 2004). Also tobacco transformants overexpressing *chit33* showed a noticeable tolerance to biotic stress like fungal and bacterial pathogens as well as abiotic stress such as high salinity and heavy metal contamination (Dana *et al*. 2006).

Therefore in this study, canola R line Hyola 308 was stably transformed by the *chit*33 cDNA to evaluate the role of the chitinase originated from *T. atroviride* in resistance against phytopathogenic fungus *S. sclerotiorum*. The transgenic lines were able to delay formation of lesions caused by fungal pathogen *S. sclerotiorum* in an *in vitro* assay. The ability of the introduced *chit*33 gene to enhance the antifungal potential of transgenic canola plants was evaluated by detached leaf assay (Carstens *et al.* 2003).

Transformation of canola was mediated by *Agrobacterium* and the cut surfaces of cotyledonary petioles containing the target cells. Results showed that this target is a vigorous source of new shoot material leading to very rapid shoot development. The origin of these shoots has been shown by Sharma (1987) to be cells located around the cut end of the petioles.

All the lines which showed positive results in the PCR analyses were further confirmed by the use of *vir*G primers, which showed that the PCR product resulted from stable T-DNA integration into the canola genome and not from *Agrobacterium* contamination.

Beside PCR analysis, Southern blot analysis of four transgenic lines provided additional evidence for the T-DNA integration. The transgenic lines showed to carry one or two copies of the *chi*33 gene. The same findings have been reported for transformation of *B. napus* by Kahrizi *et al*. (2007). Many factors such as the transgene localisation and the copy number (Finnegan & McElroy 1994; Iyer *et al.* 2000; Matzke *et al.* 2000) can contribute to the variation in the transgene expression.

Some PCR-positive *chit*33 containing lines failed to show the antifungal activity when further analysed by detached leaf assay, thus indicating the silencing of this gene. Silencing of transgenes, which can occur at the transcriptional level, may result from the block of transcription. This type of silencing is mediated by surrounding heterochromatin, endogenous repetitive sequences, transgene repeats, and aberrant promoter transcripts (Fagard & Vaucheret 2000). Transgene silencing has also been reported in some other transgenic plants (De Buck *et al.* 1998; Chang *et al.* 2002; Khan *et al.* 2008).

In conclusion, the heterologous expressed Chit33 in canola showed to be able to delay the lesion formation by *S. sclerotiorum,* which is an important first step in disease control strategies.

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Corresponding author:

Prof Mohammad Reza Zamani, National Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14965/161, Tehran, Iran, E-mail: zamani@nigeb.ac.ir