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Analysis of the Effect of Chimeric Chitinase Expressed by Synthetic Promoter in T2 Generation of Transgenic Canola

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ABSTRACT

The chitinase enzymes are known to play an important role in the plant defense system against phytopathogenic fungi. The effect of chimeric chitinase, which is chitinase-42 with a chitin-binding domain (ChBD), was previously analyzed in the T0 generation of the transgenic canola. In this research, three homozygous lines (pGFC3, pGFC13, and pGFC26) containing a single copy of the transgene (chimeric chitinases) on the two homologous chromosomes were selected in the T2 generation using a kanamycin-resistant marker (*NPT*II gene). The selected homozygous plants in T2 generation were induced by chitin as an elicitor in the greenhouse. The results of the semi-quantitative RT-PCR, chitinase enzyme activity, and growth inhibition of phytopathogenic fungi demonstrated that the synthetic inducible promoter of transgenic plants was induced by chitin. The results of chitinase activity of extracted protein from all transgenic lines containing inducible promoters showed a 3.2-5.8-fold increase in chitinase activity compared to non-induced plants. The antifungal activity of the inducible expressed chitinase was examined on *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. The results showed that fungal growth inhibition increased via elicitor treatment of the inducible promoter, 82% for *S. sclerotiorum* and 62% for *R. solani*, respectively. The result of light microscopic observation demonstrated morphological changes in hyphae and that the expressed enzyme can lyse the mycelial cell walls of *R. solani*. Moreover, resistance to *S. sclerotiorum* in the intact leaves of transgenic plants (T2) was confirmed using bioassay analysis. Based on these results, it seems that the synthetic inducible promoter containing F cis-acting element driving chimeric chitinase is suitable for increasing the resistance of the canola transgenic plant when attacked by phytopathogenic fungi.

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Introduction

In the last two decades, different promising strategies have been developed to create fungus-resistant plants (Mekapogu *et al*., 2021; Khademi *et al*., 2020; Moradyar *et al*., 2016 a; Gurr and Rushton, 2005). The introduction of foreign fungal-resistant genes to important agronomic crops is one of the most popular strategies formulated in this field (Aghazadeh *et al*., 2016; Gurr and Rushton, 2005). Many attempts to genetically engineer crop plants for the overexpression of transgenes for pathogen resistance used constitutive promoters. This approach mainly produces poor-quality plants with modified development and lower growth (Hammond-Kosack and Parker, 2003). The expression patterns of the natural pathogen-inducible promoters which contain different combinations of cis-acting elements are not robust under various conditions (Gurr and Rushton, 2005; Rushton *et al*., 2002; Venter, 2007). One of the best ways to reduce this complexity is to use specific cis-acting elements in synthetic promoters to reduce the intricacy of this expression pattern (Salinas *et al*., 1992). Furthermore, synthetic inducible promoters

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provide a flexible and efficient strategy to regulate the expression of the transgene favorably for plant-pathogen interactions (Gurr and Rushton, 2005; Moradyar *et al*., 2016 b; Rushton *et al*., 2002; Venter, 2007).

The core region of the *CaM*V35S promoter has been used for producing synthetic inducible promoters (Cazzonelli and Velten, 2008; Mazarei *et al*., 2008). Among the defined pathogen-inducible cis-acting elements, only some of them are not induced by mechanical damage and wounding (Gurr and Rushton, 2005). The F element is one such example demonstrated to respond strongly to fungal attacks (Heise *et al*., 2002). Having certain characteristic features such as low background expression, rapid response, locally expressed, and non-responsiveness to mechanical damages and wounding make the F element an appropriate candidate to be used for the construction of synthetic pathogen-inducible promoters. The synthetic promoter (SP) was previously constructed by insertion of the dimerized form of the cis-acting element F (SP-FF), upstream of the core region of the *CaM*V35S promoter (Shokouhifar *et al*., 2011a). Chitinases have great potential as biocontrol agents against fungal pathogens (Chernin *et al*., 1995; Chernin *et al*., 1997; Chet and Inbar, 1994; Kobayashi *et al*., 2002; Pan *et al*., 2006). These enzymes affect hyphal tips, germ tubes (Gunaratna and Balasubramanian, 1994), and fungal growth through lysis of the cell walls (Kunz *et al*., 1992).

Trichoderma atroviride endo-chitinase (Chit42) plays an essential role in the antagonistic effect against fungal pathogens (Harighi *et al*., 2006; Limón *et al*., 2004), but lacks the chitin-binding domain (ChBD), which is important in increasing chitinase activity. Accordingly, in a previous study the authors produced a chimeric chitinase with stronger chitin-binding ability by fusing *Serratia marcescens* chitinase B chitin-binding domain (ChBD) to Chit42. The results revealed that in comparison to chitinase42 (without ChBD), chimeric chitinase showed higher antifungal activity toward fungal pathogens (Matroodi *et al*., 2013).

The present study deals with the production of the homozygous transgenic lines harboring the synthetic inducible promoter SP-FF driving a chimeric chitinase containing ChBD and evaluating the genetic stability of the trait in the T2 generation. The research described here demonstrates stable inheritance of the transgene in the T2 generation of transgenic canola as a commercially important crop.

Materials and Methods

Organisms, plasmids, and media

Seed of canola (*Brassica napus* L.) R line Hyola 308, was generously prepared by Oilseed and Development Co. Tehran, Iran. The fungal strains, *S. sclerotiorum* and *R. solani* were also kindly provided by Dr. H. Afshari-Azad (AREE Organization, Tehran, Iran). *Escherichia coli* DH5α and *Agrobacterium tumefaciens* LBA4404 were used in the cloning procedure and plant transformation, respectively. Moreover, plasmid pJET1.2 (Roche) was used as a cloning vector. Plasmids pGFF (Shokouhifar *et al*., 2011 b), pGMPC, pGFC (Moradyar *et al*., 2016 b), and pBISM2 (Matroodi *et al*., 2013) were also utilized as binary plant expression vectors (Fig. 1). Finally, LB broth and PDA (potato dextrose agar) media were used for bacterial and fungal strains growth, respectively.

Identification of homozygous transgenic plants

T0 transgenic plants harboring the single copy of the transgene were self-pollinated and then, T1 seeds were gathered. The T1 seeds were germinated and grown to maturity and were allowed to self-pollinate and produce a subsequent generation of progeny (T2 seeds). T2 seeds (30 seeds from each T1 plant) were germinated on MS agar medium containing 35 mg/l Kanamycin and Mendelian segregation analysis was used to identify homozygous T1 parent lines. Seedlings from homozygous T1 plants were completely resistant to Kanamycin, while T2 progeny of hemizygous T1 plants segregated 3:1 kanamycin resistance to sensitivity.

Expression assay

Total RNA was isolated from leaves of transgenic and control plants, before and 24 h after *S. sclerotiorum* treatment (as an elicitor) (RNX-Plus™ kit, Cinnagen, Tehran, Iran). First-strand cDNA was synthesized using the oligo

(dT) 18 primer and M-MuLV reverse transcriptase by the BFirst-Strand cDNA Synthesis Kit (MBI Fermentas, Flamborough, ON, Canada). Semi-quantitative RT-PCR was used to detect the mRNA expression, using gene-specific (*Chit42*F3/*Chit42*R3) and tubulin primers as controls (Table 1).

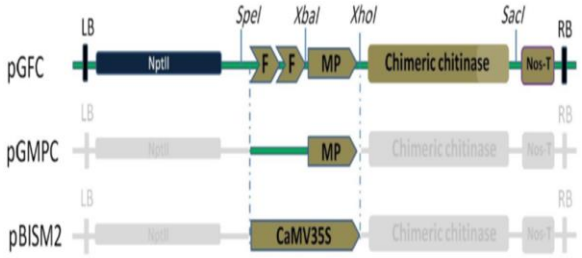

Fig. 1. T-DNA region of constructed synthetic promoters. pGFC contains the SP-FF synthetic promoter (carrying FF elements upstream of the *CaM*V35S minimal promoter) (Shokouhifar et al., 2011 a), pGMPC contains only the *CaM*V35S minimal promoter (as a negative control), and pBISM2 contains the constitutive *CaM*V35S promoter (as a positive control). LB - left border, *Nos*-T - nopaline synthase terminator, *NptII* - neomycin phosphotransferase II, *Nos*-P - nopaline synthase promoter, MP - sequence of -46 to +8 from the *CaM*V35S promoter as a minimal promoter, chimeric chitinase (*chit42* gene + ChBD (chitin-binding domain)) RB - right border. Some parts of pGMPC and pBISM2 constructs, which are the same as pGFC, were lightly painted to bold the different segments of all three constructs (in normal color) which are important in the experiment.

PCR was carried out as explained: initial denaturation at 94 °C for 10 min followed by 35 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.

Table 1. Oligonucleotides (primers) used in this study.

Name	Oligonucleotides sequence (5'→3')
*Chit*42F3	GCCTACGCCGATTATCAGAAGC
*Chit*42R3	CGCCTCCGTTGATATAAGCC
*βtubu*F	TTCTTGCATTGGTACACTAGCG
*βtubu*R	ATCGTTCATGTTGGACTCAGCC

Elicitor treatment of leaf discs

Synthetic promoters were induced by chitin, as a potential defense elicitor in plants (Nürnberger and Brunner, 2002). The Leaf discs (leaf segments 3cm in diameter) were placed inside 600 µg cm-3 of colloidal chitin or ddwater (as a negative control) and vacuum-infiltration (10 kPa) for 20 min (Qing *et al*., 2000) and negative pressure was applied suddenly.
Leaf discs were then incubated on the sterilized filter paper moistened with MS medium at 22 °C for 24 h. Colorimetric assay was then used for analyzing the discs.

Enzyme activity assay

Colorimetric assay was done according to the protocol described by Moradyar *et al*. (2016 a).

Antifungal activity of the expressed enzyme

Radial diffusion assay

Pathogenic fungi, *R. solani* and *S. sclerotiorum*, were employed for the assay of antifungal activity. Fungal strains were cultured on potato dextrose agar (PDA) and then a 5-mm-diameter of PDA plug was cut from actively growing margins of fungal colonies, placed upside down in the mid-point of petri plates (100 × 15 mm) containing PDA, and allowed to grow. After 48 h, wells (0.5 cm in diameter) were created at a distance of 1 cm away from the rim of the mycelial colony.
Consequently, crude protein from the transgenic and control plants (containing 50 µg of total protein) was added to the holes. The plates were then incubated at 28 °C for 48 h and fungal growth inhibition was monitored daily after the treatment.

Light microscopy

Mycelial discs (3 mm3) of *R. solani* were cut from actively growing margins of fungal colonies, placed on clean glass microscope slides, and maintained overnight in a moist chamber. By 24-48 h after inoculation, hyphae were treated with a 100 µl sample of crude extract from transgenic and control plants and maintained for one hour. Slides were examined and photographed at 30-min intervals, using a Nikon light microscope at 100x magnification.

Microspectrophotometric assay

Crude protein extracts from transgenic and non-transgenic plants treated with elicitor and water were tested for fungal growth inhibition in 96-well microtiter plates at 595 nm according to a microspectrophotometric method adapted from Broekaert *et al*. (2006). Approximately 2000 spores or mycelial fragments and crude extracts (40 µg) were added to the wells containing potato dextrose broth (16 mg/ml), in a total volume of 200 µl. Growth was recorded at 30 min and 24 h of incubation at 27 °C. The absorbance at 595 nm served as a measure of fungal growth. The equation [(ΔC – ΔT)/ΔC] 100 x was used to calculate the fungal growth inhibition, where ΔC = (OD595 at 48 h - OD 595 at 30 min) was treated with water and ΔT = (OD 595 at 48 h - OD 595 at 30 min) with chitin.

Intact plant bioassay

The reaction of transgenic lines and control genotypes to fungal pathogen infection was evaluated using a greenhouse assay. For this experiment, young, fully expanded leaves of 30-day-old WT and T2 generation transgenic lines were used for inoculation. Mycelial plugs (5 mm diameter) were taken from the edges of actively growing colonies (5-day-old) on PDA plates and placed upside down on the center of the adaxial leaf surface. All pots were immediately covered with transparent plastic bags, incubated for 5 days at 25 ± 2 °C to favor high relative humidity, and kept in greenhouse conditions. Infection progress was measured as the diameter of necrotic areas at the inoculation points after 36 h and 72 h of the incubation.

Statistical analysis

The experiments were conducted in a completely randomized design with three replicates. The data obtained were subjected to analysis of variance test by SPSS software. Duncan's test was also applied to determine the differences between the groups.

Results

Production of T2 generation and selection of homozygous plants

In this study, transgenic canola plants containing the chimeric chitinase gene under the control of a synthetic pathogen-inducible promoter with two copies of the F cis-acting regulatory elements (SP-FF) (Moradyar *et al*., 2016 b) was used for the generation of homozygous line and further analysis.

The steps of selecting these plants were carried out by considering that obtaining homozygous plants containing a single copy of the transgene on the two homologous chromosomes is important in the next experiments. Since segregation is observed in obtaining T1 generation, it is expected that some plants in this generation contain the transgene in both homologous chromosomes (homozygous), some contain a copy of the transgene in one of the homologous chromosomes (hemizygote), and some of them have no copy of the transgene. The progeny test was used to identify and isolate homozygous plants. The T0 plants containing a single copy, which were previously identified by the Southern blot test, were self-fertilized and the resulting seeds (T1) were collected. From each plant, 10 seeds (T1) were cultivated. To obtain the seeds, these plants were grown in greenhouse conditions and T2 seeds from these plants were collected separately. To obtain homozygous lines, from each T1 generation plant, 60 seeds (T2) were cultivated in the selective culture medium (MS medium containing 30 mg/liter kanamycin). The difference in resistance to kanamycin was used as an indicator of parental genotype. If all the seeds showed sensitivity to kanamycin (whitening or purple leaf color and abnormal growth), it indicates the absence of the resistance gene in their parent plant (Fig. 2). In a number of plants, resistant and sensitive plants were observed with a ratio of nearly 3:1, demonstrating that their parents were hemizygous (Fig. 2). However, resistance to kanamycin was detected in all of the T2 progeny from the homozygous parents (Fig 2). These plants were used to evaluate the resistance to the phytopathogenic fungi.

Expression analysis of the transgenes in the T2 generation

The expression level of the transgene in T2 generation plants was evaluated in response to fungal elicitors using semi-quantitative RT-PCR.

Leaf discs of T2 generation plants were treated with *S. sclerotiorum* elicitors. Lines pGFC3, pGFC13, and pGFC26 contain inducible promoter (which are independent events from transformation), line pMPC11 harbor minimal promoter without cis-acting element and wild-type canola plant (as negative controls), and pBISM2 contain *CaM*V35S constitutive (as a non-inducible) promoter used for evaluation of inducibility of the synthetic promoter.

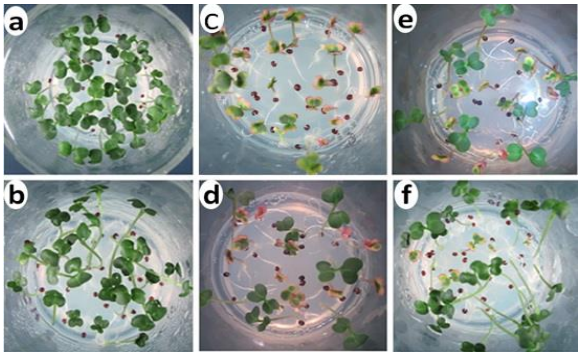

Fig. 2. Examination of T2 generation plants on the selected medium (MS medium containing 30 mg/L kanamycin) to distinguish homozygous plants from hemizygous ones. a) Non-transgenic plants (wild-type) on MS medium without kanamycin as a control b) homozygous pGFC3 plants (all plants are green and resistant) c) pGFC3 plants lacking the transgene (all sensitive to kanamycin) d, e, and f) pGFC3 hemizygous plants (both resistant and susceptible plants are observed).

After 24 hours, the results indicated that in the induction condition, a significant response of the SP-FF promoter to the elicitors was observed (Fig. 3). Furthermore, the expression levels of both inducible (in induction condition) and constitutive promoters (pBISM2) were approximately the same (Fig. 3), and no difference was observed in the expression level of pGMPC11 and wild-type lines before and after induction.

Enzyme assay

The effect of the chitin elicitor on the expression of the chimeric chitinase gene was examined in the T2 generation of transgenic canola, using the chitinase enzyme activity assay. The results indicated that chitinase activity in transgenic plants carrying SP-FF promoter was significantly higher when compared to the plant harboring minimal promoter (pGMPC11) or those of the untreated and wild-type plants (WT) (Table 2).

The activity of the chimeric chitinase was measured using the total crude protein from the transgenic and wild-type plant leaves treated with chitin or water.

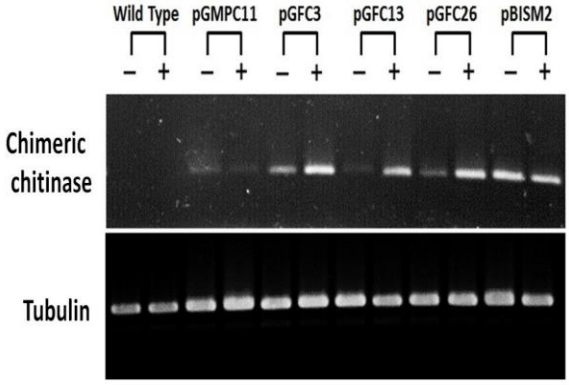

Fig. 3. Analysis of chimeric chitinase gene expression by semi-quantitative RT-PCR. Total RNA was isolated from leaves of transgenic and control plants, both before (-) and 24 h after (+) *S. sclerotiorum* elicitors treatment and analyzed for mRNA expression by semi-quantitative RT-PCR, using Gene-specific primers (*chit*42F3/*chit*42R3) or tubulin primers as controls. Construct pGMPC11 contains only a minimal promoter without a cis-acting element, while pGFC3, pGFC13, and pGFC26 are three independent transgenic lines containing cis-acting element F upstream of a minimal promoter. The pBISM2 is a transgenic line with *CaM*V35S (constitutive) promoter.

Twenty-four hours after the induction of leaves, the transgenic lines showed a 3.2 – 5.8-fold increase in chitinase activity, when compared to that of leaves induced with water (Table 2). The average fold change in chitinase activity was up to 4.1-fold higher than that of the negative control, which was statistically significant ($p < 0.01$). Among three transgenic lines, pGFC13 showed the lowest and pGFC3 exhibited the highest induction levels.

Moreover, chitinase activity in response to the chitin elicitor in transgenic lines carrying an inducible promoter was approximately the same as a positive control pBISM2 harboring constitutive *CaM*V35S promoter (Table 2). A comparison of the enzyme activity of transgenic plants harboring SP-FF with those containing SP-MP (Synthetic Promoter containing Minimal Promoter) or non-transgenic plants demonstrated

that they have a higher enzyme activity. Additionally, the results indicated that the amount of enzyme expressed from the inducible promoter shows no significant differences with a minimal promoter from the wild-type plants.

Table 2. Characterization of transgenic and non-transgenic canola.

	Enzyme Activity (µmol/h)*			Fungal Inhibition (%)**	
	Water	**Chitin**	**Fold change**	***R. solani***	***S. sclerotiorum***
pGFFC3	0.64 ± 0.04	3.73 ± 0.08 a	5.82	62.82 ± 4.31 a	82.61 ± 8.30 a
pGFFC13	0.63 ± 0.08	2.04 ± 0.15 b	3.23	48.70 ± 1.78 b	38.32 ± 2.50 c
pGFFC26	0.76 ± 0.16	2.51 ± 0.21 b	3.30	61.51 ± 4.30 a	66.54 ± 1.70 b
pBISM2(+)	2.67 ± 0.19	3.22 ± 0.24 a	1.20	54.77 ± 3.18 ab	78.34 ± 3.47 a
pGMPC (-)	0.74 ± 0.20	1.16 ± 0.19 c	1.56	30.89 ± 2.40 c	28.25 ± 3.20 d
WT	0.59 ± 0.03	1.28 ± 0.14 c	2.16	23.55 ± 0.91 c	25.82 ± 1.60 d

***** Analysis of chitinase activity of the synthetic promoters in canola plants exposed to chitin. The leaf discs were exposed to 600 µg/ml of colloidal chitin and incubated at 22 °C for 24 h. Leaf discs treated with water were used as the negative control. Production of 1 µmole of colorful product per hour was considered as one unit of activity (U). $P < 0.01$; ** Growth inhibition of *S. sclerotiorum* and *R. solani* by crude extracts of stable transgenic and non-transgenic plants following chitin elicitor treatment. Each value represents the mean of three independent experiments; ±, standard deviation

Antifungal activity of the expressed enzyme

The antifungal activity of total proteins was assessed on *S. sclerotiorum* and *R. solani* using the microspectrophotometric assay. The effects of total protein extracted from the leaf discs of the transgenic and wild-type plants on the growth inhibition of these fungi were also compared. The results showed that fungal growth inhibition can be substantially increased via elicitor treatment of the SP-FF inducible promoter in both *S. sclerotiorum* and *R. solani* (Table 2). Among all transgenic plants, leaf extracts from the pGFC3 line showing the highest enzyme activity had the strongest inhibitory effects on the growth of *S. sclerotiorum* and *R. solani*, resulting in more than 82% and 62% reduction in fungal mycelial growth, respectively.

Antifungal activities of extracted protein from three transgenic lines varied, yet they were high enough to be significantly different ($P < 0.01$) from the negative controls. Different levels of inhibition were observed against *S. sclerotiorum*, 38 - 82 %, and *R. Solani*, 48- 62 %, (Table 2). In order to confirm that the observed growth inhibition was due to the biological activity of the chimeric chitinase gene in the transgenic lines and not the natural defensive compounds produced by the plants, the antifungal activities of extracts from plants harboring the pGMPC construct and non-transgenic plants were assessed. The results indicated that the difference between the growth rate inhibition of the SP-FF promoter containing transgenic lines and the negative control plants was significant. Moreover, the total protein extracts from leaves of transgenic and non-transgenic plants were tested using the radial diffusion assay to examine the antifungal activity of the crude protein from transgenic plants on the actively growing culture of *R. solani* and *S. sclerotiorum*. Antifungal activity was tested using three lines, pGFC3, pBISM2, and pGMPC11, harboring a single copy of the transgene (Fig. 4). The inhibition of fungal growth was observed on agar plates by using an aliquot (50 µg) of the crude protein extract. Leaf extract from the transgenic line (pGFC3) was treated with chitin as elicitor and pBISM2 (as positive control), which inhibited fungal growth in all plates. However, there was no observable difference between negative control samples (pGFC3) induced with water, pGMPC11, and non-transgenic canola (Fig. 4).

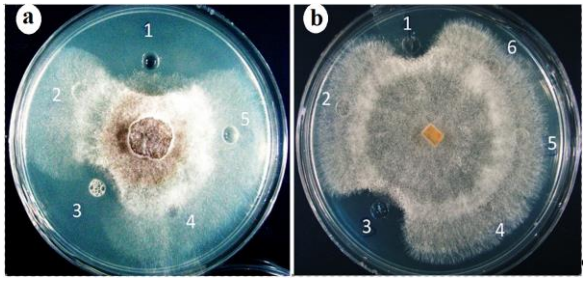

Fig. 4. Radial diffusion assay of crude protein extracts of transgenic canola induced by chitin, against a) *S. sclerotiorum* and b) *R. solani*.1) pBISM2 crude protein extract as a positive control, 2) pGFC3 induced by water, 3) pGFC3 induced by elicitor, 4) pGMPC11, 5) non-transgenic canola, 6) protein extraction buffer. (2,4,5 and 6 as negative control).

Morphological changes in hyphal pathogenic fungi using light microscopy were discernible within 60 min after exposure of hyphal samples to the crude extract of the leaf discs from transgenic plants (pGMPC11 and pGFC3) induced with chitin as an elicitor. After 2, 4, and 6 hours of incubation at 25ºC, the effect of crude extract was assessed. The results showed that the crude extract from the pGFC3 leaf treated with the elicitor could lyse the *R. solani* mycelial cell walls (Fig. 5).

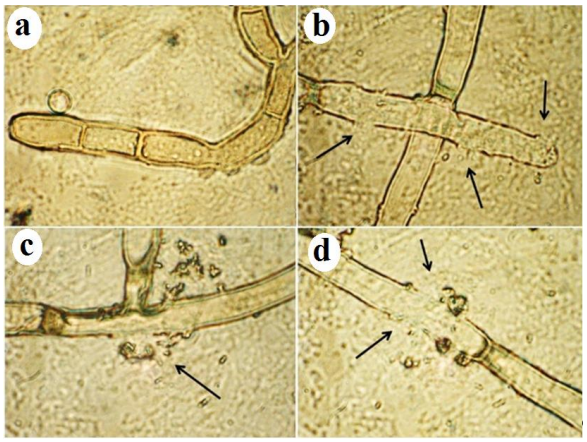

Fig. 5. Morphological changes induced in R. solani hyphae after exposure to crude protein extract. (a) Control hyphae exposed to induced pGMPC11 crude extract showing a regularly septate mycelium. (b-d) Mycelium of *R. solani*, 2h (b), 4h (c), and 6h (d) after the addition of the induced pGFC3 crude extract. Cell wall lysis was observed (as indicated by arrows).

By contrast, hyphae grown under the control conditions (exposure to the crude extract from pGMPC11, containing minimal promoter) did not show any sign of destruction.

Greenhouse assay of homozygous T2 transgenic lines

The inoculation of intact leaves on transgenic plants was used for bioassay. Three homozygous T2 plants pGFC (lines 3, 13, and 26) with pBISM2 plant containing *CaM*V35S constitutive promoter and pGMPC11 as positive and negative controls, respectively, and non-transgenic plants (wild-type) were used in bioassay. Mycelial agar plugs of *S. sclerotiorum* excised from the margin area of 2-day-old PDA cultures (28 °C) were laid on the adaxial side of leaves. Leaves of wild-type plants and pGMPC11 line inoculated with *S. sclerotiorum* exhibited visible signs within the first 36 hours post-inoculation (hpi), which increased in size in the next 72 hours (Fig. 6 and Table 3).

However, disease severity was significantly lower in transgenic plants carrying inducible promoters (pGFC3, pGFC13, pGFC26) and pBISM2 as a positive control. Additionally, the results showed that the level of resistance at 72 hpi was approximately the same in pBISM2, pGFC3, pGFC13, and pGFC26 lines (Table 3). No significant differences in disease severity were observed between the non-transgenic controls (wild-type) and line pGMPC11 containing minimal promoter.

Table 3. Reaction of wild-type and homozygous T2 transgenic lines to *S. sclerotiorum* after 36 and 72 hours.

Transgenic and non-transgenic lines	Lesion diameter (mm)	
	36 hours*	72 hours*
pGFC3	8.7 ± 0.5 b	19.5 ± 1.9 ab
pGFC13	10 ± 1.2 b	22.1 ± 1.3 b
pGFC26	7.8 ± 0.7 ab	17 ± 1 ab
pBISM2 (positive control)	5.7 ± 1.1 a	14.8 ± 2.4 a
pGMPC11 (negative control)	17.1 ± 0.7 c	31.3 ± 2.7 c
WT (negative control)	15.5 ± 0.9 c	29.7 ± 3.1 c

Means within columns followed by different letters are significantly different ($P < 0.05$). * Incubation time; ±, standard deviation.

Discussion

Expression of resistant genes derived by an inducible promoter activated in response to the pathogen has well-defined advantages for crop improvement.

The W-box motifs are an essential part of pathogen-inducible plant promoters. The F element has three copies of a core sequence (GTCA), which are separated by a 19-nucleotide spacer (Heise *et al*., 2002). This core sequence (GTCA) which belongs to the W-box cis-acting element family is a target site for WRKY

transcription factors and is required for promoter activation in response to elicitor treatment or pathogen infection (Gurr and Rushton, 2005; Heise *et al*., 2002).

Previously, it was observed that the SP-FF synthetic promoter (containing F elements) was induced in response to fungal infection and certain elicitors, such as chitin in the transient expression of the *GUS* gene (Shokouhifar *et al*., 2011 a). In this research, the SP-FF pathogen-inducible promoter was used to drive the expression of a novel chimeric chitinase gene of fungal origin, with stronger chitin-binding capacity in the stable transformation of canola, to examine the potential of the synthetic promoter to inhibit pathogen growth.

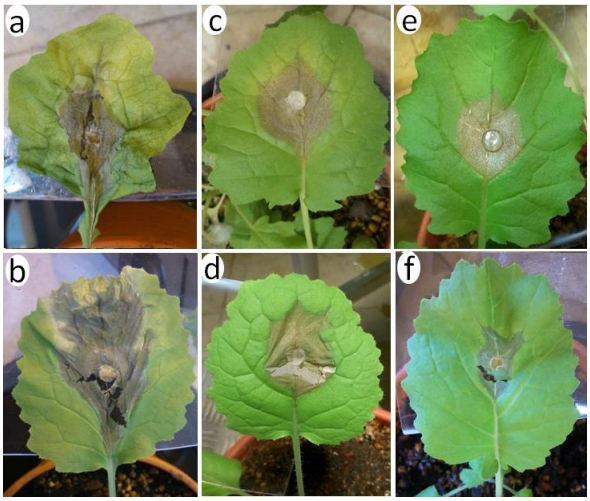

Fig. 6. Greenhouse assay of homozygous T2 transgenic lines with S. sclerotiorum to evaluate the level of resistance. Controls and T2 transgenic plants were inoculated with S. sclerotiorum and pictures were taken a 72-hour post-inoculation (hpi). (a) pGMPC11 transgenic line carrying minimal promoter as negative control; (b) non-transgenic (wild-type) plant as negative control; (c) pGFC3 transgenic line; (d) pGFC13 transgenic line; (e) pGFC26 transgenic line; (f) pBISM2 transgenic line carrying constitutive promoter as positive control.

Transgene integration and inheritance have been investigated in T2 plants, using PCR (data not shown). Additionally, semi-quantitative RT-PCR was used to evaluate fungal elicitor-mediated responses in T2 generation plants. The results indicated that induced chitinase expression levels directed by the inducible promoter varied in independent transgenic lines. This is known to come from the position effect and copy number of the transgene (Tiwari *et al*., 2011), which were the same as the *CaM*V35S constitutive promoter. In a similar study by Zou *et al*. (2014), it was confirmed that transgenic plants harboring pathogen inducible promoters (ppp1, hsr203J, and gst1 promoters) were activated by inducer and the reporter gene (GUS) was expressed.

This remarkable evidence shows that pathogen infections can result in the elevation of defense signal levels. In this study, to investigate the inducibility of transgenes in stably transformed canola, a chitin elicitor as a major component of fungal cell walls and a general plant defense elicitor were used to treat leaf discs from transgenic and wild-type plants. The amounts of chitinase activity in transgenic plant with an inducible promoter (SP-FF) were measured quantitatively in response to the elicitor, using an enzyme activity assay. The transgenic lines containing the inducible promoter (SP-FF) responded strongly to chitin, confirming the findings of the previous study of this research team, in which GUS expression was studied (Shokouhifar *et al*., 2011 a).

The results of chitinase activity in this study indicated that there are significant differences between plants harboring the inducible and minimal promoter. The induction of plants harboring minimal promoter (pGMPC) with chitin, increased chitinase activity (from 0.74 to 1.16 units). This background expression may come from the basal-induced expression of plant endogenous chitinase genes in response to the elicitor.

A comparison of enzyme activity between transgenic lines harboring the inducible promoter (SP-FF), plants containing the minimal promoter (pGMPC), and wild-type plants (non-transgenic), all treated with water, revealed that there were no statistically significant differences between their expressions. This means that the inducible promoter has no detectable background expression, and thus, it is suitable for using the expression of different fungal-resistant genes in transgenic plants.

The fact that elicitors can induce tightly controlled pathogen-inducible promoters is a highly desirable and advantageous characteristic. The results of this study show that not only synthetic promoter containing SP-FF is tightly

controlled and highly responsive to the chitin elicitor, but when the transgene under the control of the synthetic promoter is induced, it can inhibit germination and growth of different phytopathogens. All transgenic lines demonstrated normal growth and morphology, even in the lines showing the highest chimeric chitinase expression (pGFC3). Thus, the antifungal activity of the crude extract in transgenic plants, even in pGFC13 which showed the lowest chimeric chitinase activity, was sufficient for the fungal growth inhibition, without causing any phenotype abnormality to the host.

Strong growth inhibition of fungal pathogens by leaf extracts from elicitor-treated transgenic lines, when compared to the elicitor-treated controls, confirmed that the chimeric chitinase gene itself rather than other compounds was responsible for antifungal activity. On the other hand, plant chitinases usually lack the ability to efficiently degrade spores or hard chitin structures and affect only the hyphal tip (Joosten *et al*., 1995; Neuhaus *et al*., 1991). However, fungal chitinases can lyse not only the hyphal tip but also the hard chitin of the cell wall of the mature hyphae and other preserving and protective structures in fungi (Lorito *et al*., 1996, Lorito *et al*., 1998). In this research, the chitinase gene with fungal origin was used for antifungal activity. Microscopic observation in this study demonstrated the ability of leaf extracts from transgenic lines to degrade the cell walls of *R. solani*, not only in the hyphal tip but also all over the mycelium. Examination of fungal cells exposed to the crude protein for 2-6 hours revealed that most hyphae were disrupted and depleted of their protoplasm. By contrast, hyphae grown under controlled conditions did not show any sign of destruction, as compared by the normal shape.

Radial diffusion assay results indicated that leaf extracts from transgenic plants containing the *CaM*V35S constitutive and inducible promoters treated with an elicitor strongly inhibited the growth of fungal pathogens, especially that of *S. sclerotiorum*. The results show that the inhibition rate in transgenic plants containing the SP-FF promoter was as good as those harboring the *CaM*V35S constitutive promoter, in response to chitin.

Finally, to evaluate the resistance of transgenic lines to *S. sclerotiorum*, the causal agent of white mold disease, the greenhouse assay was used. The results demonstrated that all transgenic plants carrying inducible and constitutive promoters, showed essentially increased levels of disease tolerance when compared to control plants. The observation of SP-FF promoter responding to *S. sclerotiorum* confirms our previous findings (Shokouhifar *et al*., 2011a). The same finding was also reported by this research group using DDEE cis-acting elements (SP-DDEE) in chitinase activity and fungal growth inhibition when induced with elicitors and fungal induction (Moradyar *et al*., 2016 a).

In conclusion, our study confirms that synthetic promoters could be used as a robust tool for the regulated expression of heterologous genes. Moreover, the use of SP-FF synthetic pathogen-inducible promoter for the expression of the fungal resistant gene can be an effective approach for achieving successful engineering of plant tolerance against fungal diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

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