Transformation of potato (*Solanum tuberosum* cv. Savalan) by chitinase and β-1,3-glucanase genes of mycoparasitic fungi towards improving resistance to *Rhizoctonia solani* AG-3

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Abstract
Potato (*Solanum tuberosum* L.) an agro-economically important food crop in the world, is sensitive to many fungal pathogens including *Rhizoctonia solani* (AG-3), the causal agent of stem and root rot diseases. Chitinase and glucanase are cell wall degrading enzymes which have been shown to have high antifungal activity against a wide range of phytopathogenic fungi. In the present study, plasmid pBIKE3 harboring a double-gene cassette containing the chitinase (*chit*42) and β-1,3-glucanase (*bgn*13.1) genes was constructed. In this construct, the *chit*42 gene is located between the *CaMV* 35S promoter and *nos* terminator derived from pBI121, while the *bgn*13.1 gene is downstream of a modified *CaMV* 35S promoter, followed by the *nos* terminator both of which were derived from the pRTL plasmid. Micro-tubers of potato plants (the Savalan cultivar) were transformed with the pBIKE3 construct via the *Agrobacterium* delivery system. Integration of these two genes into the potato genome and their expression at the transcriptional level was confirmed by polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR). The radial diffusion assay showed that the heterologous expressed chitinase and glucanase enzymes demonstrated antifungal activity on *R. solani* (AG-3).
Keywords: Chitinase; β-1,3-glucanase; Potato; *Rhizoctonia solani*; *Trichoderma*; Fungal disease

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the major crops of many countries like Iran with an annual global production of approximately 300 million tones (Banerjee *et al*., 2006). Because of its high nutritional quality and ease of production, the potato is a suitable candidate crop for genetic engineering-related improvement projects. Like many other crops, the production of this crop is challenged by fungal pathogens.

Fungal diseases are rated as one of the most important factors contributing to yield losses in many economical crops including the potato. The mycoparasitic process in *Trichoderma* involves various fungal cell wall degrading enzymes, including hydrolytic enzymes (Chet *et al*., 1998). Among these enzymes, chitinases and β-1,3-glucanases are attractive molecules because they have activity against a wide range of fungi (Walsh *et al*., 2000; Lorito, 1998; Stone and Clarke, 1993). Chitinases and β-1,3-glucanases from *Trichoderma* sp. have been shown to have strong antifungal activities when used individually and highly synergistic in combination (Lorito *et al*., 1998). Chitinases also confer broad resistance to other biotic and abiotic stresses, such as bacterial pathogens, salinity and heavy metals (Dana *et al*., 2006).

In this work the chitinase gene (*chit*42) from *Trichoderma atroviride* and the β-1,3-glucanase gene (*bgn*13.1) from *Trichoderma virens* are used to prepare a double gene construct containing these two genes. Potato plants are transformed by this construct via *Agrobacterium*-mediated transformation method. The putative transgenic plants are analyzed by PCR and RT-PCR, and tested for their antifungal activity on *Rhizoctonia solani*, the causal agent of potato root and stem rot.

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MATERIALS AND METHODS

Enzymes and chemicals: All chemicals, culture media, plant growth regulators and antibiotics were purchased from Merck (USA) and Sigma (USA) at the highest purity available, unless stated otherwise. Restriction enzymes and other DNA-modifying enzymes were obtained from Roche (Switzerland) and Fermentas (CA).

Plant material: An economically important potato (*S. tuberosum*) of the Savalan cultivar, which was released by the Seed and Plant Improvement Institute, Karaj, Iran, was used as the receptor.

Microorganisms and growth conditions: *R. solani*, (AG-3), the causal agent of potato root and stem rot was propagated on potato dextrose agar (PDA) and subcultured as needed. *Escherichia coli* DH5α (Cinnagen, Iran) was used in all molecular experiments and *Agrobacterium tumefaciens* LBA4404 was used for the plant transformation procedure. Bacteria were grown in Luria-Bertani (LB) medium at appropriate temperatures (37ºC for *E. coli* and 28ºC for *A. tumefaciens*) with shaking (150 rpm).

General procedures: Plasmid DNA preparation and electrophoresis of DNA fragments were performed by routine procedures (Sambrook and Russell, 2001). Enzymatic treatments of DNA molecules were carried out as recommended by the manufacturer (Fermentas). *E. coli* DH5α was transformed using the $CaCl_2$ method (Sambrook and Russell, 2001).

Bacterial strain for transformation: Single colonies of the *A. tumefaciens* LBA4404, harboring pBIKE3 containing the *chit*42 and *bgn*13.1 genes, were grown in LB medium supplemented with 50 mg/l of kanamycin and allowed to grow overnight at 28ºC with constant shaking (200 rpm) to mid-log phase. The bacterial cells were collected by centrifugation and resuspended in infection medium (Murashige and Skoog (MS) medium, 50 mg/l of sucrose, pH 5.5) and were then shaken for 2 h at 28ºC. This culture was subsequently used for co-cultivation.

Plant transformation and selection procedure: Micro-tuber slices (approximately 6 months old), containing at least one bud, were infected with *A. tumefaciens* LBA 4404. Infected explants were co-cultivated for 3 days on MLS medium (Linsmaier and Skoog, 1965) containing MS macro salts, LS micro salts, 100 mg/l of inositol, 8 mg/l of thiamine-HCl, 8 mg/l of adenine sulfate, 2 mg/l of 6-benzylaminopurine (BAP), 1 mg/l of zeatin, 20 g/l of sucrose and 7.5 g/l of agar (Sigma), without any antibiotics in dim light (white fluorescent light; 15 mE/m/s) at 22ºC (± 2ºC). The MLS medium was adjusted to pH 5.7 with 1 N HCl or NaOH before adding agar. The growth regulator BAP was added to the medium before autoclaving, but zeatin was filter sterilized and then added to the autoclaved medium.

After co-cultivation, infected slices were rinsed by MS medium and distilled water, which had an appropriate concentration of cefotaxime (500 mg/l) for *Agrobacterium* removal. They were then transferred to MLS selection medium containing kanamycin (100 mg/l) and cefotaxime (500 mg/l). Shoots were regenerated during the week after transformation. Most of the early shoots were not transgenic and unable to grow on selective media. Subsequent emerging shoots on antibiotic containing media (kanamycin 100 mg/l) were transferred to MS medium containing kanamycin (100 mg/l) and cefotaxime (500 mg/l). After 15 days, the rooted plantlets were transferred to soil.

Molecular analysis of transgenic potato: Leaf material from transgenic and non-transgenic potatoes was harvested, lyophilized and grinded into fine powder for extraction of genomic DNA (Doyle and Doyle, 1987). PCR amplification was used for initial molecular confirmation of the presence of transgenes in kanamycin-resistant putative transgenic plants. DNA fragment containing the *chit*42 and *bgn*13.1 genes was amplified by PCR using specific primers (Table 1). PCR was carried out as follows: an initial denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 60°C for 1 min, extention at 72ºC for 2 min and a final extension at 72ºC for 10 min. The resulting PCR products were separated by electrophoresis on 1% (w/v) agarose gel.

Expression analysis: Specific mRNAs of the transgene were checked using reverse transcriptase RT-PCR. Total RNA was isolated from leaves of transgenic and control potato 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 chitinase and glucanase gene specific primers (F3/R3 and F/R357).

Table 1. The sequence of primers used in PCR and RT-PCR analysis.

Primer name	Sequence
35S	5' –GGCGAACAGTTCATACAGAGTCT- 3'
FE35S	5' -CGGAATTCGCATGCCTGCAGGTCCCCAG- 3'
NOS-TER	5' -GTGAAGCTTCCCGATCTAGTAACAT- 3'
RENOS	5' -CCAGTGAATTCCCGATCTAGTAAC- 3'
F	5' -GCTCTAGAATGTTGAAGCTCACGGCGC- 3'
R	5' -GCTCTAGATTAAGTAGTATAACGGGCAACG- 3'
F2	5' -AGAGCTTTCTTTCGCCGTAGCTATTAAG- 3'
R2	5' -CTGAAGTTCTCAGCGATGTGATCCG- 3'
Pf1	5' -GCTCTAGAATGTTGGGTTTCCTCGGAAAG- 3'
Prx	5' -GCTCTAGACTAGTTGAGACCGCTTCGGAT- 3'
F3	5' -GCCTACGCCGATTATCAGAAGC- 3'
F4	5' -CGTTCCCGCAAGCAAGATCG- 3'
R3	5' -CGCCTCCGTTGATATAAGCC- 3'
18SF	5' -CTTCGGGATCGGAGTAATGATTAA- 3'
18SR	5' -GCCCAGAACATCTAAGGGCATCACAGA- 3'
RGLU327	5' -GGTATCAGTGTTGAATCTCAG- 3'

Bioassay of the transgenic plants: The antifungal activity of crude extracts from the transgenic plants was tested using a modification of the radial diffusion method (Broglie *et al*., 1991). The leaf material (3 g) was grounded to a fine powder in liquid nitrogen using a mortar and pestle. Two volumes of 1 M NaCl in 20 mM NaOAc (pH 4.7) were added to the leaf material. The extracts were then shaken for 1 h at 4ºC and subsequently centrifuged at 13000 g for 20 min at 4ºC. Protein content of extracts was determined using Bradford assay (1976). An agar disc (5 mm in diameter) containing *R. solani* (AG-3), which was derived from the fungus in an actively growing state, previously cultured on PDA, was placed at the center of a Petri dish containing PDA. The plates were then incubated at 28ºC for 2 days. Wells were subsequently punched into the agar at a distance of 25 mm from the center of the plates. The samples (containing proteins) to be tested were mixed with 10 µl of 15 mM acetate sodium buffer, pH 4 and placed in the wells.

RESULTS

In this study we used the *Agrobacterium*-mediated transformation method to transform potato (*S. tuberosum*) c.v. Savalan with chitainase (*chit*42) and β-1,3-glucanase (*bgn*13.1) genes.

Construction of plant expression cassettes: To produce the double gene construct pBIKE3 containing the *chit*42 and *bgn*13.1 genes, the *Hind*III fragment (3.3 kb) containing the modified *CaMV* 35S promoter/*bgn*13.1/*nos*- terminator was isolated from

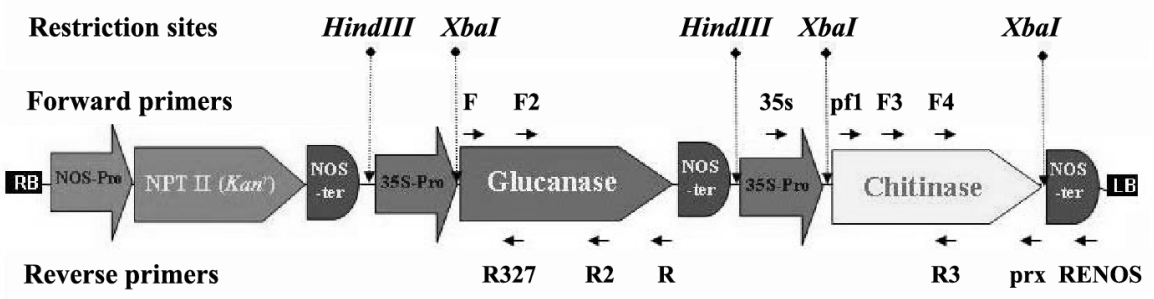

Figure 1. Schematic representation of T-DNA region of pBIKE3 containing *chit*42 and *bgn*13.1 genes with expected restriction patterns and positions of different primers used for PCR confirmation. Name of forward and reverse primers are shown with the arrows.

pRTLKE2 and cloned into the *Hind*III site of pBIKE1 (Fig. 1). For construction of pRTLKE2, the *bgn*13.1 coding region from pUCSO2 (Raoufzadeh, 2007) was subcloned into the pRTL vector. To construct pBIKE1, a fragment containing the *chit*42 gene from pMJH1 (Rezanezhad *et al*., 2009) was cloned between the *CaMV* 35S promoter and *nos* terminator in the T-DNA region of pBI121. The pBIKE3 construct was confirmed by PCR patterns using different combinations of gene specific and vector specific primers (Fig. 2). The T-DNA region of this plasmid harbors both the chitinase and β-1,3-glucanase genes. This allows them to be integrated together when transferred into plant cells.

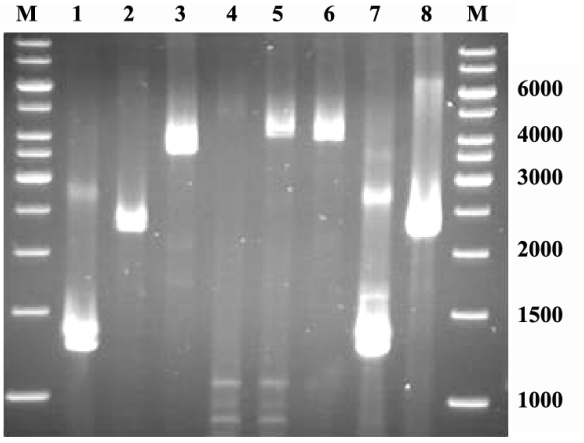

Figure 2. PCR analysis confirming the accurate pBIKE3 binary vector construction: 1) Pf1/Prx primers (1300 bp); 2) F/R primers (2300 bp); 3) F2/R3 primers (3700 bp); 4) F/Prx primers (4150 bp); 5) F/R3 (4200 bp); 6) F2/Prx primers (4100 bp); 7) Pf1/Prx primers (1300 bp); 8) F/R primers (2300 bp); M: 1Kb DNA ladder (Fermentas, CA). The position of primers are shown in Figure 1.

Transformation and selection of transgenic plants: The pBIKE3 introduced into the *A. tumefaciens* strain LBA4404 was subsequently used for potato micro-tuber transformation. These explants were co-cultivated and after 3 days, the slices were rinsed and transferred to selection medium containing kanamycin and cefotaxime. Most early shoots not growing on selective media were shown to be non-transgenic. Nineteen well established shoots (4-5 cm long) were transferred to new MLS medium containing appropriate antibiotics. Finally eight independent transgenic lines were successfully rooted on kanamycin-containing selection media and then transferred to soil after 20 days (Fig. 3). Only one well-grown shoot from each infected micro-tuber was transferred to rooting medium. All rooted plants exhibited a normal phenotype capable of tuber formation.

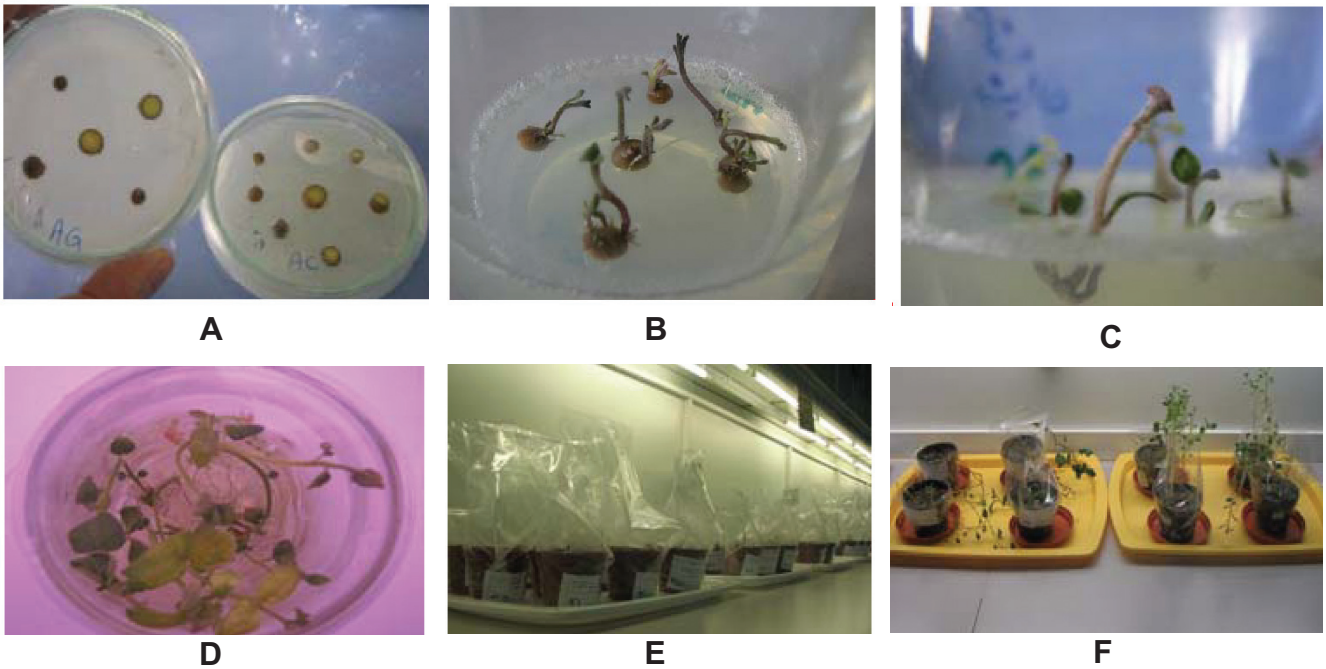

Figure 3. Transformation and regeneration of transgenic potato plants: A) Infected micro-tuber slices transformed with *Agrobacterium*; B) Regenerated shoots in growth medium; C) Regenerated shoots transferred to new selective medium; D) Regenerated plantlets with well developed roots and leaves; E) Covered regenerated plantlets in pots; F) Transformed potato plants in pots acclimated to non-aseptic environment.

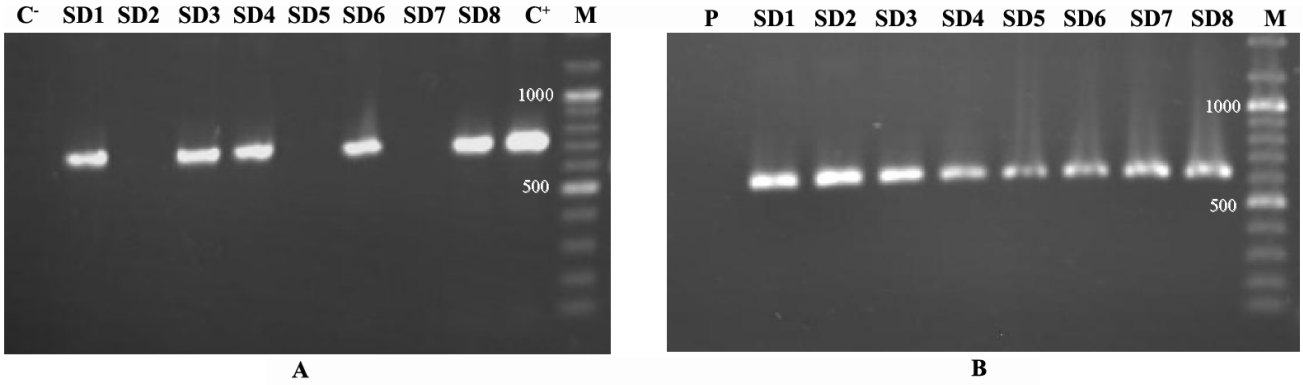

Figure 4. PCR analysis of different putative transgenic lines: A) Expected 700 bp fragment amplified by PCR from the DNA isolated from putative transgenic plants using F4/RENOS primers. C^+, pBIKE3 plasmid DNA template as positive control; Five lines out of eight plants showed to contain the 700 bp fragment. B) 650 bp fragment amplified using 18SF/R primers and putative transgenic plant's DNA was used as internal control. P, pBIKE3 plasmid DNA template as negative control; M, DNA ladder Mix (Fermentas, CA).

Molecular analysis of regenerated plants: Five lines out of eight putative transgenic plants showed to contain the end part of the *chit*42 transgene and *nos* terminator. The corresponding fragment, 700 bp of the *chit*42 gene, was amplified using specific primers (F4/RENOS) (Fig. 4). For further confirmation, a 589 bp fragment within the *chit*42 gene was detected using F3/R3 specific primers (Fig. 5). These putative transgenic lines were also assayed for the presence of the *bgn*13.1 gene by PCR using F/R357 as specific primers. A fragment with expected size (357 bp) was amplified from the transgenic plants (Fig. 5). The results indicated that the transgenic plants were transformed by these two genes. The *chit*42 and *bgn*13.1 specific primers did not amplify the corresponding fragments in the untransformed samples.

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).

Expression of both chitinase and glucanase genes at the transcriptional level in the transformed potato lines were proven by means of RT-PCR. RNA was isolated from leaf tissues for generation of cDNA. The expected size of the amplified cDNA fragments were detected in the transformed lines, SD1 and SD3 (Fig. 6) confirming the stable integration and expression of T-DNA in the genome of kanamycin-screened plants. Non-transformed plants were used as negative controls and no PCR products were detected.

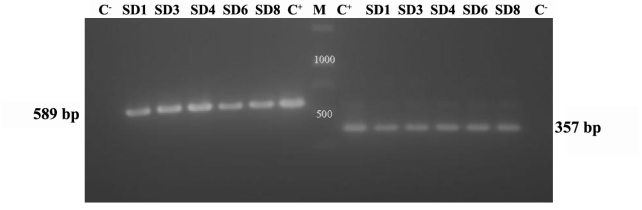

Figure 5. PCR analysis of transgenic lines (SD1, SD3, SD4, SD6 and SD8) with gene specific primers. 589 bp fragment within the *chit*42 gene using F3/R3 primers and 357 bp fragment within the *bgn*13.1 gene using F/R357 primers were amplified by PCR using putative transgenic plant's DNA. C^+, pBIKE3 plasmid DNA template used as a positive control; C^-, non-transformed plant DNA as negative control; M, 1Kb DNA ladder (Fermentas, CA).

Evaluation of antifungal activity: In order to examine the antifungal activity of expressed *Chit*42 and *Bgn*13.1 from transgenic plants on the actively growing phytopathogenic fungus *R. solani,* total protein extracts from leaves of transgenic and non-transgenic plants were tested using the radial diffusion assay. Antifungal activity was detected in extracts from two RT-PCR positive lines, SD1 and SD3. Antifungal activity was not observed in other potato leaf extracts (Fig. 7).

DISCUSSION

Cultivated plants often suffer from various diseases caused by microbes including phytopathogenic fungi, resulting in reduced yield and quality of crop products. Potato (*S. tuberosum*), one of the most important food crops in the world, is susceptible to several fungal, bacterial and other pathogens, leading to considerable

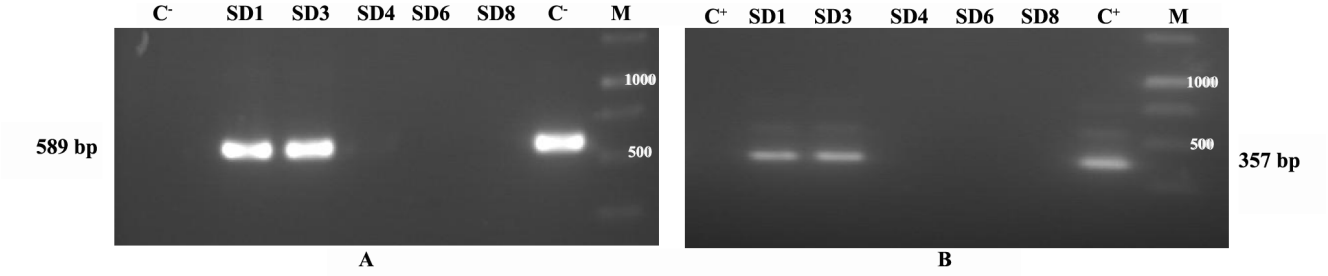

Figure 6. RT-PCR analysis of transgenic potato lines:A) Expected 589 bp DNA fragment was amplified from SD1 and SD3 potato lines using F3/R3 primers. C^+, pBIKE3 plasmid DNA template used as a positive control; C^-, non-transformed plant DNA used as a negative control; M, 1Kb DNA ladder. B) Expected 357 bp DNA fragment was amplified from SD1 and SD3 potato lines using F/R357 primers. C^+, pBIKE3 plasmid DNA template used as positive control; C^-, non-transformed plant used as a negative control; M, 1Kb DNA ladder (Fermentas, CA).

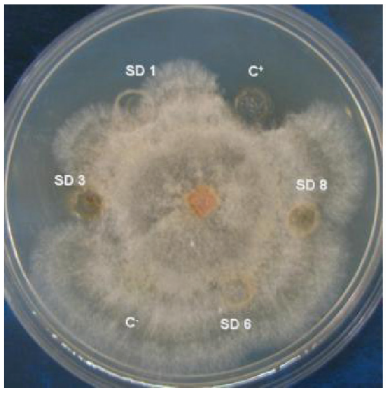

Figure 7. Radial diffusion assay of crude protein extracts of transgenic potato against *Rhizoctonia solani.* SD1, SD3, SD6 and SD8 indicate crude protein extracts from transgenic potatos. C+, heterologous expressed *Chit*42 in *E. coli* used as a positive control; C^-, protein extract from non-transgenic potato used as a negative control.

losses in yield and quality of products (Khan *et al*., 2008; Walter *et al*., 2001). Root and stem rot are one of the most important fungal diseases in potato, both of which are caused by *R. solani* (AG-3). This fungus causes serious losses in this crop, especially in the potato fields of Iran. Cell wall degrading enzymes such as chitinases and glucanases from mycoparasitic fungi including *Trichoderma* sp. have demonstrated high antifungal activity against a wide range of economically important phytopathogenic fungi (Harighi *et al*., 2006; Gokul *et al*., 2000; Carsolio *et al*., 1999; Leubner-Metzger and Meins, 1999; Zhu *et al*., 1994). Some researches have been conducted to develop transgenic crop plants that have elevated expression levels of the cell wall degrading enzymes in hopes of producing fungal disease-resistant varieties (Datta *et al*., 2001; Chai, 1999; Nishizawa *et al*., 1999; Asao *et al*., 1997; Tabei *et al*., 1997; Stuiver *et al*., 1996; Broglie *et al.,* 1991).

There are many reports about the advantages of using chitinases for plant protection (Jayaraj and Punja, 2007; Xiao *et al*., 2007; Dana *et al*., 2006; Liu *et al*., 2004). However, there are almost no reports demonstrating that chitinase genes originating from plants or bacteria confer resistance to several fungi in transgenic plants, indicating that these enzymes have a narrow spectrum of antifungal activity (Dana *et al*., 2006; Lorito *et al*., 1998; Joosten *et al*., 1995; Neuhaus *et al*., 1991). The unsuccessful use of plant chitinases for plant protection probably comes from the fact that the gene codes for enzymes that usually affect only the hyphal tip and lack the ability to efficiently degrade spores or hard chitin structures (Joosten *et al*., 1995; Neuhaus *et al*., 1991; Mauch *et al*., 1988). Whereas fungal chitinases are able to lyse not only the hyphal tip, but also the hard chitin wall of the mature hyphae and other fungal preservation structures (Lorito *et al*., 1998; Lorito *et al*., 1996a; Lorito *et al*., 1994).

Also, there are some reports on the development of transgenic crop plants with glucanase genes in hopes of producing fungal disease resistant varieties (Datta *et al.,* 2001; Chai, 1999; Nishizawa *et al*., 1999; Asao *et al*., 1997; Tabei *et al*., 1997; Stuiver *et al*., 1996; Broglie *et al*., 1991).

Several reports describe the synergic interaction effects of chitinase and β-1,3-glucanase in antifungal defense *in vitro* (Moravcikova *et al*., 2007; Mauch *et al*., 1988; Leah *et al*., 1991) and *in vivo* (Anand *et al*., 2003; Melchers and Stuiver, 2000; Jach *et al*., 1995; Jongedijk *et al*., 1995). These enzymes are non-toxic to plants animals and higher vertebrates (Dana *et al*., 2006; Lorito *et al*., 1998; Lorito *et al*., 1996a; Lorito *et al*., 1996b). The previous study has shown that heterologous expressed *chit*42 in the prokaryotic system has antifungal activity against *R. solani* (Harighi *et al*., 2006). In this study, under experimental conditions, the potato cultivar Savalan (economically important cultivar, which is suitable for crisp production in Iran)

was transformed with a combination of chitinase (*chit*42) and glucanase (*bgn*13.1) genes isolated from different *Trichoderma* species. Application of multiple resistant transgenes with different mechanisms to plant genetic manipulations could widen the spectrum and increase the level of resistance in transgenic crops (Zhu *et al*., 2007; McDowell and Woffenden, 2003; Punja, 2001).

The potato micro-tuber slice, as an explant for inoculation was found to be effective for transformation, while internodal transformation showed not to be very efficient (data not shown). The leaf disc system, which is routinely used, demonstrates the known problem of somaclonal variations (Beaujean *et al*., 1998) and thus was not used in this research. Slice transformation was carried out in this study via the *A*. *tumefaciens* strain LAB4404, which plays a significant role in determining the efficiency of infection (Banerjee *et al*., 2006) and has been commonly used for transformation of potato (Banerjee *et al*., 2006; Ducreux *et al*., 2005; Trujillo *et al*., 2001; Wenzler *et al*., 1989; Sheerman *et al*., 1988; Stiekema *et al*., 1988).

All the lines that gave 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 potato genome and not from *Agrobacterium* contamination. Some PCR-positive chitinase and glucanase-containing lines failed to express the corresponding mRNA when further analyzed by the RT-PCR method, thus indicating the silencing of these genes. Silencing of transgenes, have also been reported in some other transgenic lines (Khan *et al*., 2008; Chang *et al*., 2002; De Buck *et al*., 1998; Jorgensen, 1995).

The ability of the introduced chitinase and β-1,3-glucanase genes to enhance the antifungal potential of transgenic potato plants was studied by the fungal bioassays. Crude protein extracts from the transgenic lines were able to delay the hyphal growth of the fungal pathogen *R. solani* in the *in vitro* assay.

The research presented here demonstrates a successful transformation of potato plants with T-DNA containing the double hydrolytic genes from mycoparasitic fungi and exhibit antifungal activity against *R. solani* AG-3. Meanwhile, the expression of these genes had no deleterious phenotypic effects on the transgenic plants.

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