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Enhancement of *Trichoderma Harzianum* Activity Against *Sclerotinia sclerotiorum* by Overexpression of *Chit42*

Mojegan Kowsari [1,2]; Mohammad Reza Zamani [1,*]; Mostafa Motallebi [1]

[1]National Institute of Genetic Engineering and Biotechnology, Tehran, I.R. IRAN
[2]Agricultural Biotechnology Research Institute of Iran, Karaj, I.R. IRAN

*Corresponding author: Mohammad Reza Zamani, National Institute of Genetic Engineering and Biotechnology, Shahrak-e Pajoohesh, Tehran-Karaj Highway, P.O. Box: 14965161, Tehran, I.R. IRAN. Tel: +98-2144580363, Fax: +98-2144580363, E-mail: zamani@nigeb.ac.ir

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Backgoround: Plant diseases, caused by a wide range of phytopathogenic fungi, could be managed using of *Trichoderma* sp, as a biocontrol agent. Cell wall degrading enzymes like chitinase from *T. harzianum* are important means for fungal pathogen inhibition. Overexpression of these chitinase enzymes can improve the antagonistic potential of *Trichoderma* sp. strains.

Objectives: This study aimed to produce a new enhanced biocontrol system of *Trichoderma harzianum*, overexpressing *chit42* gene. The improved *T. harzianum* could be an appropriate biocontrol agent for controlling the stem rot disease of canola caused by *Sclerotinia sclerotiorum*.

Materials and Methods: *T. harzianum* protoplast cotransformation was carried out by pLMRS3-*Chit42* and p3SR2 plasmids. The transformants were selected based on their growth on colloidal chitin containing medium. The improvement of transformants was investigated by quantification of mRNA using real-time quantitative polymerase chain reaction (RT-PCR) and measurement of chitinase activity in the medium containing colloidal chitin as the carbon source. Furthermore, the antagonistic activity of transformants against *S. sclerotiorum* was assessed by dual culture experiments.

Results: The overexpressing transformants of Chit42 displayed higher levels of chitinase activity to inhibit *S. sclerotiorum* growth compared with the wild type. The results indicated that the value of the chitinase activity ($126.42 + 0.07$ U.ml^{-1}) of Chit42-9 increased 64.17 fold. Transcriptomic analysis demonstrated that Chit42-9 transformant expressed 5.2 fold of *chit42* transcript as compared with the parent strain. Biocontrol inhibition of this transformant was 4.98-fold more compared with the non-transformant type.

Conclusions: The improved Chit42-9 transformant can be used for biocontrolling *S. sclerotiorum*, cause of stem rot disease in canola.

Keywords: Chitinase; Real-Time Polymerase Chain Reaction; *Sclerotinia sclerotiorum*; *Trichoderma*

1. Background

The genus *Trichoderma*, a soil borne group of fungi, acts as important biocontrol agents against phytopathogenic fungi. More than 50 bioproducts of *Trichoderma* have been registered and available on the market (1). *Trichoderma* strains are ubiquitous biocontrol agents because of their high reproductive capacity, strong aggressiveness against pathogens, ability to survive under difficult conditions, efficiency in promoting plant growth and defense mechanisms (2).

Parasitism, competition and antibiosis are the main mechanisms of biocontrol in *Trichoderma* sp (3). Parasitism is a common cause for pathogen death (4). Hydrolytic enzymes are important factors during parasitism for cell wall degradation of pathogens. *Trichoderma* sp. are efficient sources for the production of destructive enzymes like chitinases (5).

These enzymes, including chitinases are capable of reducing or stopping spore germination and hyphal growth of fungal pathogens (6). Among chitinases, chitinase 42 plays a major role in the inhibition of pathogen growth (7). The expression of *chit42* gene is strongly induced during fungus-fungus interaction and when the colloidal chitin is the sole carbon source in growth media (8, 9).

The biocontrol activities of *Trichoderma* sp. can be enhanced through overexpressing the chitinolytic enzymes by genetic engineering (10-12). Overexpression of single chitinase can improve the mycoparasitic potential of the biocontrol agent (13-15). The improved *Trichoderma* sp could reduce the development of *S. sclerotiorum*, the causal agent of stem rot disease in canola plants.

Sclerotinia stem rot is a destructive disease which in serious epidemic years may reduce the canola yield by 20-25% (16).

Implication for health policy/practice/research/medical education:
Production of a new enhanced biocontrol system of *Trichoderma harzianum*, by overexpressing *chit42* gene could benefit crop yield production of canola.

2. Objectives

The objectives of this study was to improve the antagonistic effect of the biocontrol agent *T. harzianum* by overexpressing the *chit42* gene, derived from *T. atroviride* under the control of a constitutive promoter. The improved strain may be useful to control the stem rot disease of canola.

3. Materials and Methods

All chemicals were purchased from Merck (Germany), unless otherwise stated.

3.1. Microorganisms and Plasmids

Trichoderma harzianum and *Sclerotinia sclerotiorum* were provided by the Agricultural Biotechnology Research Institute of Iran, type collection culture. Plasmid p3SR2, which carries the *amdS* gene (coding for acetamidase as a selectable marker) from *Aspergillus nidulans* was used. The pLMRS3 plasmid, which carried the constitutive promoter *pki1* from *T. reesei* and the *cbh2* terminator from *T. reesei*, cellobiohydrolaseII was used as the expression vector.

3.2. Growth Media and Culture Condition

T. harzianum and *S. sclerotiorum* were maintained on PDA (potato dextrose agar) slants. The colloidal chitin agar (CCA) selective medium was composed of following compounds: colloidal chitin 5.0 g.l $^{-1}$; sucrose 1.0 g.l $^{-1}$; $NaNO_3$ 2.0 g.l $^{-1}$; K2HPO_4 1.0 g.l $^{-1}$; KCl 0.5 g.l $^{-1}$; $MgSO_4$ 0.5 g.l $^{-1}$; $FeSO_4$ 0.01 g.l $^{-1}$; agar 15 g.l $^{-1}$; and distilled water 1000 ml at pH 6.5. *Trichoderma* minimal media (MM) contained the following chemicals (in mg/mL): (NH_4) 2SO4, 5; KH_2PO_4, 15; $MgSO_4$, 0.6; $CaCl_2$, 0.6; $CoCl_2$, 0.002; $FeSO_4.7H_2O$, 0.005; $MnSO_4$. H_2O, 0.0016; and $ZnSO_4$.$7H_2O$, 0.0014.

The selective medium for *amdS* expression was MM containing 10 mM acetamide as the sole nitrogen source and 12.5 mM CsCl (MMA). The *E. coli* DH5α was grown in a Luria-Bertani (LB) medium at 37°C; the media were supplemented with ampicillin (SIGMA, 100 g.ml $^{-1}$). DNA modifying enzymes were obtained from Fermentase and Roche Biochemical.

3.3. Construction of Expression Cassette

The RNA from powdered mycelia was isolated using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations. Molecular biology procedures were performed following the standard protocols (17, 18). The 1278 bp *chit42* cDNA from *T. atroviride* (DQ022674) was amplified using primers Pf1 (5'GCTCTAGAATGTTGGGCTTCCTCGGAAAG3') and Prx (5'GCTCTAGACTAGTTGAGACCGCTTCGGAT3'), which were designed along with the XbaI recognition sites, by Pfu DNA polymerase. The PCR mixture contained the standard concentrations of DNA, dNTPs, primers, and DNA polymerases. The PCR program consisted of 35 repetitive cycles at 94°C for one minute, 62°C for one minute and 72°C for one minute and a separate step for 10 minutes at 72°C. Amplified fragment was purified using a PCR product purification kit (Roche). The blunt-ended fragment was ligated to vector pJET (pJEchit42) and sequenced. The XbaI digested fragment was subcloned into the XbaI site of the pLMRS3 expression vector to create pLMRS3-*chit42*, which was used for protoplast transformation.

3.4. Cotransformation

Protoplast preparation and transformation were carried out according to the method of Penttila et al. (19). *T. harzianum* wild type was cotransformed with chitinase-containing plasmid pLMRS3-chit42 and p3SR2 plasmid. Plasmid p3SR2 carries the *amdS* gene from *Aspergillus nidulans*, which codes for acetamidase as the selectable marker.

Cotransformation was conducted with a 1:10 (p3SR2/pLMRS3-chit42 plasmid ratio, and 200-1000 μL aliquots of the transformed protoplasts were plated in 0.75% (w/v) selective top agar containing one M sorbitol as the osmotic stabilizer.

The selective medium for *amdS* expression was MM glucose containing 10 mM acetamide as the sole nitrogen source instead of $(NH_4)_2SO_4$ and 12.5 mM CsCl. Individual colonies were randomly chosen for *amdS* in the selective medium and incubated at 28°C for 5 days. Protoplasts were placed on a 2% (w/v) CCA selective medium. The regeneration of protoplast and the colony developments were observed on the plates that were incubated at room temperature.

Regenerated transformants were selected based on their growth rate on the selective medium. One mycelial disk (5 mM) of each transformant was inoculated on 0.5% (w/v) CCA and PDA media and incubated at 28°C for four days.

3.5. Transcriptomic Analysis by Quantitative Real-Time RT-PCR

Expression levels of *chit42* were quantified by real-time RT-PCR assay. Total RNA was isolated from 100 mg of freeze-dried mycelia powder of single spore of selected transformants and wild type using the RNeasy Plant Mini Kit (Qiagen). The cDNA were synthesized from 1 μg of total RNA using a cDNA synthesis kit with an oligo (dT) primer. One microgram of the cDNA was used in the PCR reaction (with chiF/chiR and βtubuF/βtubuR as specific primers).

Real-time PCR was performed using an ABI system with a SYBR green master mix. All PCRs were performed in triplicate, and in a total volume of 10 μl for 40 cycles under the following conditions: denaturation, 95°C, 45 seconds; annealing, 60°C, one minute; and extension, 72°C, one minute.

The number of cDNA transcripts was normalized

against the expression of the housekeeping β-tubulin gene (20). The expression level was calculated by the formula $2^{-\Delta\Delta CT}$ (21).

3.6. Chitinase Activity

Chitinase activity was assayed according to the Boller and Mauch method (22). *N*-Acetyl-D-glucosamine (NAG) standard curve was prepared according to the procedures set out by Reissig and associates (23). Colloidal chitin was prepared from crab shell chitin (24). Mycelium was prepared by inoculating the spores into PDB (potato dextrose broth) medium and incubated on a rotary shaker at 28°C for three days under aerobic conditions.

The mycelium (1.0 g) was transferred into 100 ml fermentation medium ($MgSO_4$. $7H_2O$, 0.6 g.l $^{-1}$; $FeSO_4$. $7H_2O$, 0.1 g.l $^{-1}$; NH_4NO_3, 3.0 g.l $^{-1}$; KH_2PO_4, 2.0 g.l $^{-1}$; and colloidal chitin 20% w/v) using 10 g.l $^{-1}$ colloidal chitin as the sole carbon source.

The supernatant was obtained by removing the mycelium from filtrate after 48 hours. Each experiment was performed in triplicates, and the mean value was taken. Standard deviation was calculated using functions of MS Excel application (Microsoft Corporation). The released *N*-acetylglucosamine (GlcNAc) was measured according to the procedures set out by Reissig et al. (23). A unit was defined as the amount of the enzyme that releases 1 μmol GlcNAc per 60 minutes.

3.7. Test for Antagonism

Antagonistic activity of *Trichoderma* wild-type and its transformants against *S. sclerotiorum* were assessed using the dual culture technique (10). One mycelial disk (5 mm) of transformants and one disk (5 mm) of test pathogen were simultaneously placed on opposite sides of a PDA Petri dish and incubated at 26°C. Three plates (replications) were used for each transformant and test pathogen based on a completely randomized design. The plates that only received the mycelial disk of pathogens are served as control ones.

The colony interaction was assayed as the percentage of the inhibition on the PDA plate after five days incubation based on the formula suggested by Sundar et al. (25). Inhibition of growth is calculated by the formula;

$(\%) = (X–Y/X) \times 100$,

where X = mycelial growth of pathogen in the absence of *Trichoderma* (control), and Y = mycelial growth of pathogen in the presence of transformants.

4. Results

Since chit42 is an essential component of chitinolytic system in *T. harzianum* and plays an important role in terms of mycoparasitism, we transformed *T. harzianum* with *chit42* gene in order to reinforce the expression of this enzyme.

4.1. Transformation of T. Harzianum by Chitinase Gene

T. harzianum was cotransformed with the plasmid p3SR2 (containing acetamidase gene) and pLMRS3-chit42. For construction of pLMRS3-chit42, the *chit42* cDNA fragment was placed between the *pki1* promoter and *cbh2* terminator, which is confirmed by PCR and digestion pattern (Figure 1) and DNA sequencing (Data not shown).

Stable transformants were initially selected using a selective medium containing acetamide. Among 350 transformants, 80 were selected based on their ability to grow on the selective medium containing 2% colloidal chitin (2% CCA). The selected stable *amdS* transformants were

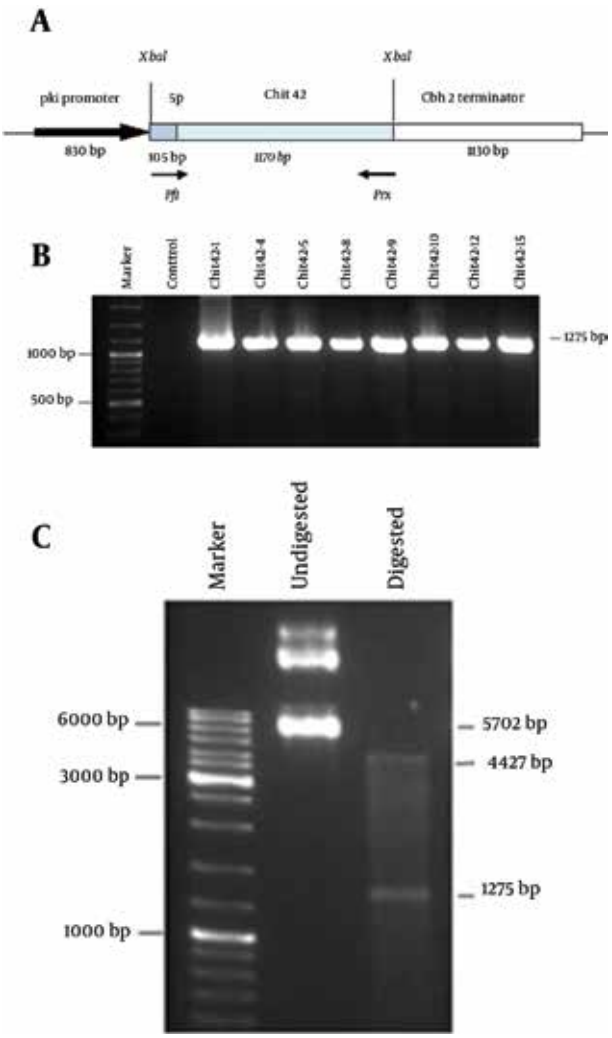

Figure 1. A) Shematic Representation of the Over Expression Cassette for Chit42 *pki* Pyruvate Kinase Promoter, *cbh2 CellobiohydrolaseII* Terminator. B) PCR amplification of the *Chit42* gene using Pfi/prx specific primers (lane 3-10,eigth transformants, approximately 1275 bp), Lane 1, DNA ladder., lane 2, negative control. C) Confirmation of pLMRS3 containing *chit42* gene by digestion pattern using *XbaI* enzyme, Lane 2,Undigested pLMRS3-Chit42 (5702bp), Lane 3, Digested pLMRS3-Chit42 using *XbaI* (4427 bp & 1275 bp fragments)

found to have chitinase activity. Among these transformants, 16 fast growing colonies, designated *Chit42*-1 to *Chit42*-16, were selected for further study. The growth rate of the selected colonies was examined on a 0.5% CCA medium for 48 hours. Based on the mycelial growth, eight fast growing transformants (*Chit42*-1, -4, -5, -8, -9, -10, -12, and -15) were selected for subsequent study (Table 1).

4.2. Expression Analysis by Quantitative Real-Time RT-PCR

To quantify the expression of *chit42* in the selected transformants, quantitative RT-PCR was performed using Ct values in the real-time PCR. The cDNA was prepared from the RNA of transformants and non-transformant (as negative control) grown in inducing liquid culture medium containing colloidal chitin (2% w/v) as the sole carbon source. Adjustment of *chit42* expression levels was achieved by using β-tubulin as an internal control. Based on calculations using the $2^{-\Delta\Delta CT}$ method differential expression folds of *chit42* (ranging from 3.3 to 5.2) were detected in transformants, with the highest level of expression for *Chit42*-9 transformant (Table 2).

Expression analysis was performed by real-time quantitative RT-PCR using chiF/chiR primers on cDNA from *T. harzianum* transformants that have been grown on medium containing colloidal chitin. Values ($2^{-\Delta\Delta CT}$) correspond to comparative measurements against the *chit42* transcript in the wild-type *T. harzianum* ($2^{-\Delta\Delta CT} = 1$). *T. harzianum* β-tubulin was used as an internal reference gene.

4.3. Chitinase Activity

The overexpression of *chit42* was investigated with colloidal chitin as the carbon source. While the enzyme activity in the non-transformant was 1.97 U.ml $^{-1}$, the *Chit42* transformants showed improved chitinase activity from 48.72 to 126.42 U.ml $^{-1}$ (Table 1). These results indicated that the highest chitinase activity of 126.42 + 0.07 U/mL in the case of *Chit42*-9 showed 64.17-fold increase as compared to the parent strain.

4.4. Antifungal Activity

To determine whether an increase in chitinase activity correlates with the antifungal activity of different transformants, dual culture tests were performed using *T. harzianum* and *S. sclerotiorum* (Figure 2). When phytopathogenic fungus and wild type or transformants of *T. harzianum* were grown in the same plates, a zone of lysis was produced in the pathogenic fungal mycelia.

Different transformants showed varied reductions with the minimum of 29% and the maximum of 89.7%, in the growth rate of this pathogen (Figure 3). Among these transformants, *Chit42*-9 (showing the highest enzyme activity) was the best at inhibiting the growth of the pathogen tested (Figure 3). This transformant showed 4.98 fold more inhibitions when compared with the non-transformant. No growth was detected when pieces of the overgrown area of lysed and killed *S. sclerotiorum* mycelia were transferred to fresh medium (data not shown).

Table 1. Growth Rate and Chitinase Activity of the *Chit42* Transformants + SE Standard Error of Three Replicates

Isolates	Diameter, mm/48h	Chitinase Activity, U.ml $^{-1}$
Control (nontransformed)	17.2 ± 0.3	1.97 ± 0.02
***Chit42*-1**	29.5 ± 1.0	97.02 ± 0.56
***Chit42*-4**	30.0 ± 0.5	105.00 ± 1.01
***Chit42*-5**	25.0 ± 0.3	49.06 ± 0.91
***Chti42*-8**	26.5 ± 0.4	67.62 ± 1.12
***Chit42*-9**	30.5 ± 0.9	126.42 ± 2.5
***Chit42*-10**	25.4 ± 0.2	49.14 ± 0.82
***Chit42*-12**	28.5 ± 0.7	90.72 ± 2.35
***Chit42*-15**	25.5 ± 0.3	48.72 ± 1.6

Table 2. Analysis of *Chit42* Expression of Different *T. harzianum* Transformants

Row	Isolates	$2^{-\Delta\Delta CT}$
1	Control	1.00
2	*Chit42-1*	4.1
3	*Chit42-4*	4.6
4	*Chit42-5*	3.8
5	*Chti42-8*	4.0
6	*Chit42-9*	5.2
7	*Chit42-10*	3.6
8	*Chit42-12*	4.4
9	*Chit42-15*	3.3

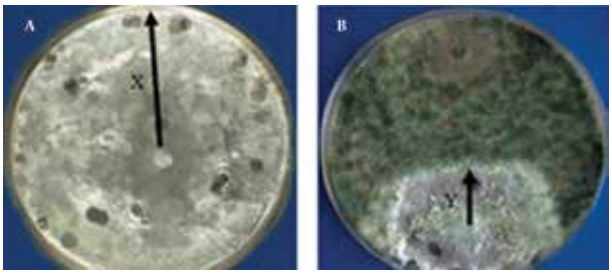

Figure 2. Dual Culture Test Using *T. harzianum* and *S. sclerotiorum*. Inhibition of growth (%) = (X-Y/X) × 100 where, X = mycelial growth of *S. sclerotiorum* in the absence of *Trichoderma* (A), and Y = mycelial growth of pathogen in the presence of *T. harzianum*

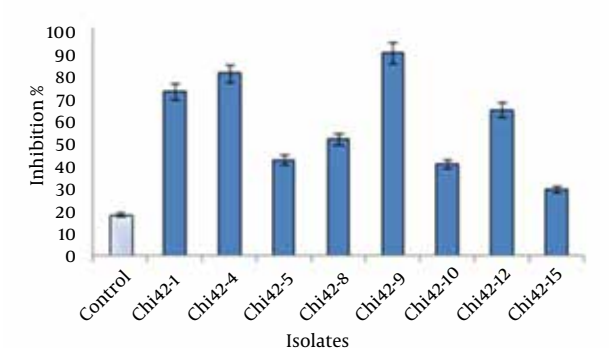

Figure 3. Analysis of the Antifungal Activity. inhibition (%) of selected *Chit42* transformants against *S. sclerotiorum*. Each value represents the mean (± standard error) of three independent experiments. Columns are significantly different at $p < 0.05$.

5. Discussion

Canola stem rot caused by *S. sclerotiorum* is an important and destructive disease (26, 27). The disease causes high yield losses of canola in Iran (28). Due to the limitation of canola germplasm resistant to *S. sclerotiorum*, canola cultivars with high level of resistance are not easy to obtain by classical methods. Although application of fungicides is necessary for agricultural productivity, they have undesirable impacts on health and environment (29).

Biological control is an alternative method for managing fungal diseases (30). Biological control agents have specific advantages over synthetic fungicide, including, fewer non-target, and environmental effects, as well as lower probability of resistance development (31). *Trichoderma* sp. is a suitable biocontrol candidate for protection of crop yields against fungal pathogens.

Cell wall degrading enzymes such as chitinases, have been reported to have an essential role in the ability of *Trichoderma* to control phytopathogenic fungi (32). Chitinases produced by *T. harzianum* play an important role in the antagonistic effect of this fungus (13). Improvement of fungal strains by overexpression of chitinase gene in *Trichoderma* has contributed to intensive increased antifungal activity against fungal pathogens (13, 33). In this research *chit42* gene from *T. atroviride*, previously reported as a strain showing high chitinase activity (34), was overexpressed in *T. harzianum* under the control of a constitutive pki promoter.

Use of this promoter is an advantage for overexpression of a transgene without applying specific inducer if the transformants are to be released to control a plant disease. Overexpression of *chit42* in Chit42-9 transformant (as the highest enzyme producer) increased the enzyme activity by 64 fold. A similar finding has also been reported by Limon et al. (13) using the same promoter. Carsolio et al. (9) reported a 42 fold overexpression of a chitinase gene under the control of trpC promoter from *Aspergilus nidulans*.

Dual culture experiment showed that the efficiency of biocontrol of transformants is higher than the wild type. This finding demonstrates the involvement of the overexpression of *chit42* in biocontrol activity of *T. harzianum* against the *S. sclerotiorum*. A correlation between *Trichoderma* sp. biocontrol activity and cell wall degrading enzyme production has been reported in antagonistic studies (35, 36).

In this study, different transformants exhibited various levels of mRNA expression, chitinase activity and antagonistic effects. This fact may be due to the effect of copy numbers of the transgene, and the reflect of the transgene location on the chromosome as they have been described for other genes (37).

Our results offered positive evidence that a transgenic approach to engineer *Trichoderma* strains transformed with *chit42* can offer much more antagonistic effect on *S. sclerotiorum* than the wild type, indicating that the overexpression of chitinase is necessary for strain improvement. Among all the transformants, *Chit42-9* can be used for field assessment of antagonistic activity against Sclerotinia disease in canola.

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Authors' Contribution

Mohammad Reza Zamani and the authors conducted the study; Mostafa Motallebi: Data analysis and interpretation; and Mojegan Kowsari: contributed in all aspects of the work.

Financial Disclosure

None reported.

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