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ORIGINAL ARTICLE

Potential of endochitinase gene to control Fusarium wilt and early blight disease in transgenic potato lines

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Abstract

Potato (*Solanum tuberosum* L.), an important food crop in the world, is susceptible to many fungal pathogens including *Alternaria solani* and *Fusarium oxysporum* causing Fusarium wilt and early blight diseases. Mycoparasitic fungi like *Trichoderma* encode chitinases, cell wall degrading enzymes, with high antifungal activity against a wide range of phytopathogenic fungi. In this study, a binary vector harboring endochitinase gene of ~1,000 bp was constructed and used to transform potato nodes through *Agrobacterium*-mediated transformation. Out of several primary transformants, two transgenic potato lines were verified for transgene insertion and integration by Southern blot. In a pot experiment for *Fusarium* resistance, the transgenic potato lines didn't show any symptoms of disease, instead they remained healthy post infection. The transgenic potato lines exhibited 1.5 fold higher mRNA expression of endochitinase at 7 days as compared to 0 day post fungus inoculation. It was evident that the mRNA expression decreased over days of inoculation but was still higher than at 0 day and remained stable upto 30 days post inoculation. Similarly, for *A. solani* infection assay, the mRNA expression of the endochitinase gene was 3 fold higher 7 days post inoculation compared to expression at 0 day. Although the expression decreased by1.2 fold during subsequent days post infection, it remained stable for 30 days, suggesting that protection in transgenic potato plants against fungal pathogens was achieved through an increase in endochitinase transcript.

Keywords: early blight disease, *Fusarium*, potato, *Trichoderma*

Introduction

Agricultural crops are constantly at the risk of different plant pathogens that influence the development of the individual plant and eventually the whole crop, resulting in the reduction of yield as well as affecting the quality of the crop (Christou and Twyman 2004). The loss in the total production of a certain major crop due to plant pathogen attack turns out to be very destructive for the economy since the pathogens compromise food security (Kumar and Rawat 2011).

Potatoes are affected by many pathogens (Oerke and Dehne 2004; Toufiq *et al.* 2017), of which fungal pathogens are considered to be the most damaging because they cause 40% loss in global potato yield (Jeger *et al*. 1996). The use of chemical fungicides to get rid offungal pathogens is an effective solution but their after effects are devastating for both the environment and the crop they are being sprayed on. These chemical fungicides pollute the environment due to the components they are composed of. Furthermore, some fungicides have been shown to have a negative impact on the fertility of the plants (Pavlik and Jandurova 2000). Their excessive use is also giving rise to different resistant fungal biotypes. To avoid all such concerns associated with the use of fungicides or any other control measures a biotechnological approach (Hermosa *et al.* 2000) can be the

best solution i.e. by introducing a transgene that possesses, in the case of fungi, a fungicidal effect, into plant DNA. This could be a safer way to treat disease. *Alternaria,* a genus of ascomycete, belong to class Dothideomycetes while Fusarium belong to class Sordariomycetes.

Chitinases are proteins which degrade chitin which is the second most abundant polymer and is an integral component of fungal cell walls (Perrakis *et al.* 1994). Chitinase is believed to be present in all organisms that include plants, viruses, bacteria, insects, mammals and fungi.

Endochitinases (endo-β-N-acetylglucosaminidase; EC 3.2.1.14) cleave the chitin molecules into to N-acetyl glucosamine by either endo or exo cleavages of the 1–3 and 1–4 bond (Van Aalten et al. 2000) randomly or a single residue from the non-reducing end of the chitin molecule (Suzuki et al. 1999). Chitinases have a very important role since they attack directly the fungal structural component.

Chitinases from different origins are found to bring resistance against fungal pathogens in a number of plants. Jabeen *et al.* (2015) reported generation of transgenic tomato plants harboring chitinase RCG3 gene to bring resistance against *Fusarium oxysporium* and *Alternaria solani.* In a similar study, endochitinase genes CHIT33 along with CHIT42 originating from *Trichoderma harzianum* were overexpressed in tobacco plants and showed enhanced resistance against fungal attack (de las Mercedes Dana *et al.* 2006). Chitinase producing transgene is a potential candidate to create resistance against fungal pathogens in transgenic plants.

Materials and Methods

Gene source and amplification

Endochitinase gene of ~1,000 bp (GenBank accession # KY290959) derived from *T. harzianum* was kindly obtained from the Seed Biotechnology Lab, CEMB Punjab University Lahore-Pakistan. The gene was amplified with modified forward primer 5′-CGC AGATCTCTCGACGCCAGCTTTCTG-3′ and reverse primer 5′-CGCAGATCTCTCGACGCCAGCTTT CTG-3′ to add *NcoI* and *BglII* restriction sites for directional cloning in plant binary vector, pCAMBIA1301. The PCR mix was comprised of 10 X PCR Buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs 1 pmoles each of forward and reverse primer, 50 ng plasmid DNA and 3U of *Taq*DNA Polymerase (Thermo scientific). The amplification was performed in aVeriti cycler (ABI) under the following conditions; 95°C as initial denaturation for 4 min followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 30 s. The final extension was at 72°C for 7 min.

Construct details

Amplified endochitinase gene of ~1000 bp was directionally cloned at *NcoI* and *BglII* sites in pCAMBIA1301 vector under the control of CaMV35S promoter (Fig. 1). The gene was fused with Gus exon. Successful cloning resulted in pCAM-trid construct that was used to transform potato.

Plant transformation

The binary construct was moved into *Agrobacterium tumefaciens* strain LB4404 by electroporation. The transformation method was adopted from Tabassum *et al.* (2016). Nodes from potato variety Desiree were used as explant and co-cultivated with Agrobacterium harboring pCAM-trid construct at 25°C for 45 min in a gyratory shaker at a speed of 80 rpm. After co-cultivation, the explants were blot dried and shifted onto MS media (Murashige and Skoog 1962) supplemented with 3% sucrose and solidified with 2.5 $g \cdot l^{-1}$ phytageland placed at 25 ± 2°C for regeneration. The regenerated potato plantlets were shifted in test tubes onto

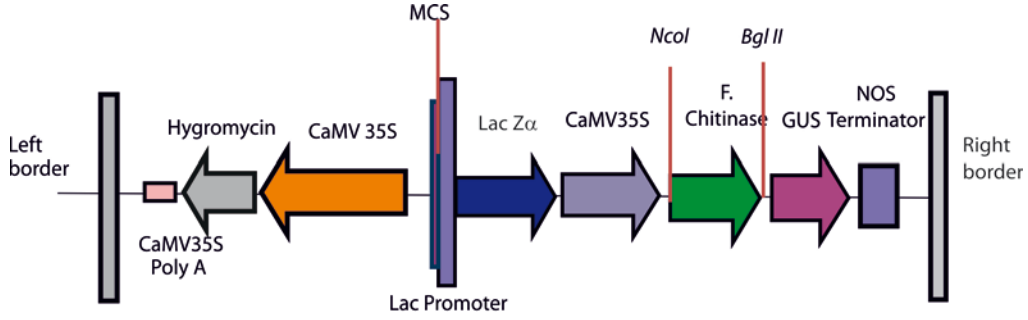


Fig. 1. Schematic representation of T-DNA region of pCAMBIA1301 containing endochitinase gene. The gene was cloned directionally immediately after CaMV35S promoter at *NcoI* and *BglII* restriction sites and was in fusion with Gus exon

MS media. The transformation was performed without selection for an antibiotic R gene on the construct.

Confirmation studies to reveal transgene insertion in regenerated potato plants

Regenerated transformed potato plantlets were analyzed for the presence of endochitinase transgene. Genomic DNA was isolated by using Genomic DNA purification kit (thermo scientific) as per instructions. Specific forward and reverse primers were used to amplify the fragment. The amplified fragments were resolved onto 0.8% agarose gel and visualized after staining with ethidium bromide.

Southern blot

To screen transformed potato plants for transgene integration, Southern blot was performed. The endochitinase gene fragment was labelled with digoxigenin (DIG) using a DIG DNA Labelling and Detection Kit (Roche) as per manual. Twenty µg of genomic DNA for each tested potato plant was digested with *NcoI* and *BglII* restriction enzymes. The construct pCAM-tridwas used as positive control that was digested with similar enzymes and proceeded with test samples. The denatured fragments were blotted onto Hybond N membrane (Amersham, USA) and probed with DIG-labelled probe of fungal chitinase gene. Further, hybridization was carried out at 42°C for 16 h and detection was done through an enzymatic reaction where BCIP/NBT tablets were used as a substrate against the alkaline phosphatase enzyme.

Pot experiment for stress assays on transgenic potato plants

The transgenic potato plants were analyzed for biotic stress tolerance. Two fungal pathogens of potato: *F. oxysporum* and *A. solani* were used in these assays. These fungi were kindly obtained from the Institute of Agricultural Sciences (IAGS), Punjab University-Lahore. Cultures were maintained on potato dextrose agar (PDA) plates. Twenty-one day old transgenic and non-transgenic potato plants grown in soil pots were used in these assays. For each treatment, three biological replicates were used. The fungal spore-suspension consisting of 10^4 spores/1 ml were prepared in potato dextrose broth media for each *F. oxysporum* and *A. solani* separately. The plants were sprayed with the prepared spore suspension of the pathogen and this practice was repeated twice at 3 day intervals. The inoculated potato plants were placed at 25 ± 2°C with 16/8 h photoperiod.

Koch's postulate

For the confirmation of a disease pathogen in an experiment, Koch`s postulate needs to be fulfilled. After inoculation of *A. solani* and *F. oxysporum* to transgenic potato plants, a leaf from each inoculated plant was separated and placed on a PDA plate to confirm the pathogen and the onset of infection.

mRNA expression of fungal chitinase gene in transgenic plants during biotic stress

To reveal the mRNA expression of endochitinase gene in transgenic potato lines during biotic stress, semi-quantitative RT-qPCR assays were performed. Gene specific forward 5′-AGCCCAGGGTA ACAAACCAT-3′ and reverse primer 5′-CCAAGCAT ACCGCAATACCT-3′ were used to amplify 210 bp fragment of chitinase gene. Beta actin was used for normalization (adopted from Khan *et al.* 2017) and as reference in the RT-qPCR. The reaction was performed on a thermal cycler with Piko real 3.1 software (Thermo scientific) by using Syber Green qPCR Master Mix 2X (Thermo scientific). The reaction was performed by the followingprofile with initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation for 30 sec at 94°C, annealing at 61°C for 30 s and extension at 72°C for 30 s. The relative gene expression was calculated by using Cq values obtained in different samples. Each assay had three biological replicates.

Results

Generation of transgenic potato plants

The aim of this study was to evaluate the potential of a fungus derived chitinase gene (endochitinase) in biotic stress assays. For this, we successfully generated transgenic potato lines having chitinase gene integrated in their genome.

Potato variety 'Desiree' was transformed with recombinant binary construct, pCAm-trid. The endochitinase gene was fused with GUS exon and was under the control of CaMV 35S promoter for constitutive expression. The transformation was facilitated by *Agrobacterium*. A total of 37 nodal explants were transformed, of which 26 regenerated and converted into small plantlets. Initial screening of regenerated potato plants was performed with histochemical GUS staining. It was found that three potato lines namely T1ps2, T3ps3 and T6ps9 exhibited a bluish green color specific for GUS exon. The color was clearly visible in subjected potato plants, both in leaf and stem portions (Fig. 2C–E).

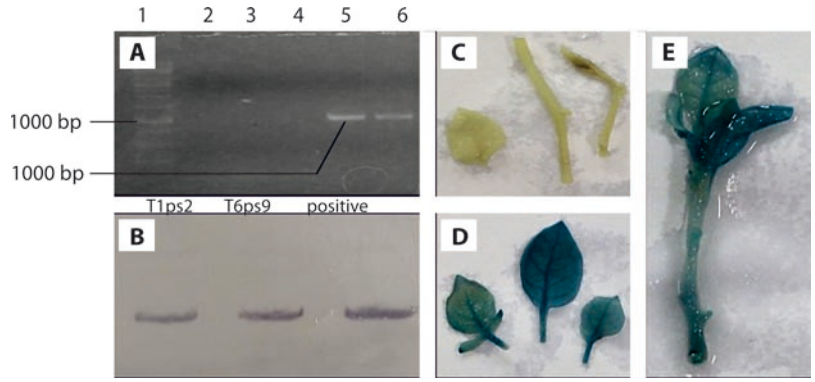
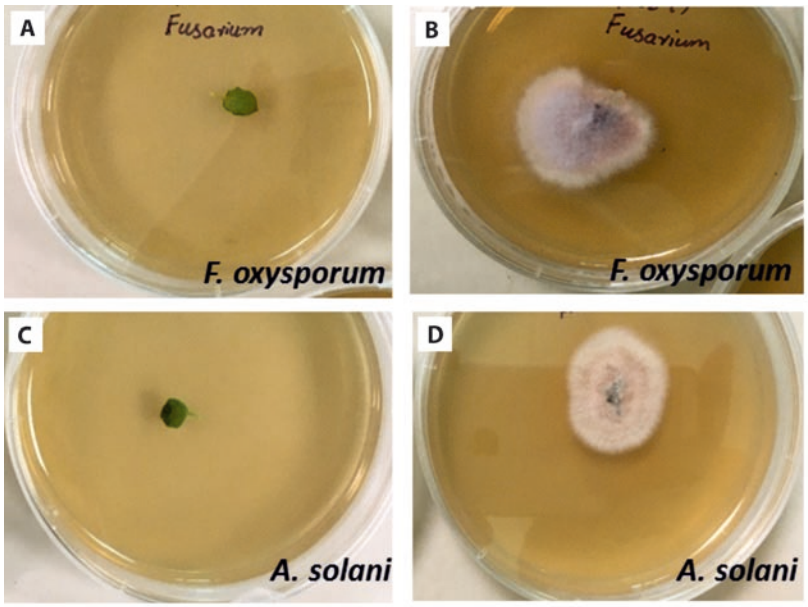


Fig. 2. Molecular analysis of potato plants transformed with pCAM-trid construct containing endochitinase gene, A – PCR analysis of different putative transgenic potato lines: expected 1000 bp fragment amplified by PCR from the DNA isolated from putative transgenic plants specific primers. Lane 1: DNA marker, Lane 2–6: test samples from transformed potato plants; B – southern blot assay of transgenic potato plants confirming integration of endochitinase in the genome of subject plant. The digested fragments were hybridized with DIG-labelled chitinase probe; C–E – histological GUS staining of leaf and stem regions of transgenic potato plants in comparison with non-transgenic potato plant. Non-transgenic potato plant (C) didn't exhibit any colour as compared to transgenic lines where blue colour particular of Gus was clearly visible

The transformed, regenerated potato plants were analyzed for transgene insertion by PCR. It was found that three potato plants namely; T1ps2, T3ps3 and T6ps9 were positive for transgene amplification (Fig. 2A). Further, in Southern blot assay, two potato plants T1ps2 and T6ps9 were detected chromogenically on membranes when hybridized with agene specific probe (Fig. 2B). The transgenic potato lines, T1ps2 and T6ps9 were subjected to biotic stress assays. The overall transformation efficiency was 7.69% on the basis of Southern blot.

Alternaria and *Fusarium* resistance assay

In the resistance assay, three plants with three biological replicates for each of the two transgenic potato lines were inoculated with fungi while three control potato plants were also infected. mRNA expression of the chitinase gene in transgenic potato plants was analyzed post inoculation with *F. oxysporum*. The infection was confirmed by applying Koch's postulate (supplementary Fig. 1). It was revealed that at 7 days post *F. oxysporum* inoculation, the mRNA expression was 1.5 fold higher than the mRNA expression at 0 day. Further, at 15 and



Supplementary Fig. 1. Koch's postulate to verify the pathogen. The small leaf portion was taken from pathogen infected plant (either *Fusarium* or *Alternaria*) was taken and cultured on PDA plate

30 days post inoculation, the mRNA expression of the transgene was approximately 0.9 and 1 fold higher and remained stable (Fig. 3). This indicates that the fungus derived chitinase responded to biotic stress positively and helped the plants to overcome this stress by increasing the transcript level. Also, the expression of the transcript remained comparatively high and stable for up to 30 days post inoculation with *F. oxysporum*. It was also found that no visible infection symptoms particular to *Fusarium* appeared on transgenic potato lines. Similar findings were revealed in transgenic potato plants against inoculation of *A. solani*, where mRNA expression of the chitinase gene was enhanced 3.2 fold at 7 days post inoculation as compared to 0 day of inoculation (Fig. 4). Although at 15 days post inoculation of *A. solani*, the mRNA expression of the transgene was reduced by approximately 1.2 fold it was still higher than at 0 day and remained stable for up to 30 days of infection (Fig. 4).The results were significant at $p < 0.05$ (n = 3) when one-way ANOVA was applied in combination with Bonferroni's multiple comparison test.

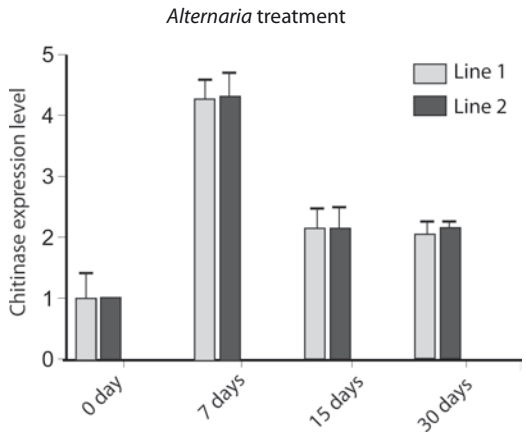


Fig. 3. Relative quantification of endochitinase gene at various time intervals to detect mRNA expression in transgenic potato plants, T1ps2 (line 1) and T6ps9 (line 2), inoculated with *Alternaria solani*. Beta actin was used as interval control for normalization

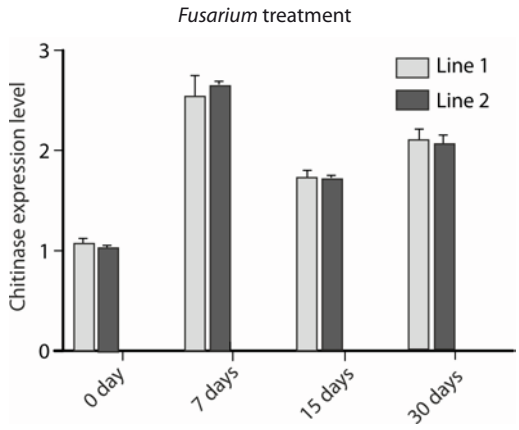


Fig. 4. Relative quantification of endochitinase gene at various time intervals to detect mRNA expression in transgenic potato plants, T1ps2 (line 1) and T6ps9 (line 2), inoculated with *Fusarium oxysporum*. Beta actin was used as interval control for normalization

Discussion

Plant diseases caused by microbes, including phytopathogenic fungi, are the major cause of yield reduction in cultivated plants (Savary *et al.* 2012; Khan *et al.* 2017). Potato, one of the most important food crops in the world, is susceptible to a number of fungal, bacterial and viral pathogens (Keen 2000). Fusarium wilt and early blight of potato are the most important fungal diseases in potato, which are caused by *F. oxysporum* and *A. solani,* respectively. In modern agriculture, crop protection from multiple fungal pathogens relies on chemical fungicides who seexcessive use is no longer a sustainable approach. The sustainable method currently available includes genetic engineering of plants to make them resistant towards a particular pathogen. Microorganisms exhibiting inhibitory activity against pathogens can be a potential source of disease resistant genes (Pinnamaneni *et al.* 2010). Chitinases are cell wall degrading enzymes, translated from chitinase genes and induced by the presence of chitin (Gentile *et al.* 2007) from mycoparasitic fungi, including *Trichoderma* sp., which have demonstrated high antifungal activity against a wide range of economically important phytopathogenic fungi (Harighi *et al.* 2006; Emani *et al.* 2003; Gokul *et al.* 2000). Fungal chitinases are reported to have the ability to lyse the hyphae tip and the hard chitin wall of the mature hyphae (Lorito *et al.* 1998). There are many reports about the successful use of chitinases for plant protection including utilization of barley derived plant chitinase in generation of transgenic potato plants for effective resistance against soil fungi *A. solani* (Khan *et al.* 2017). In another report Tariq *et al.* (2018) generated transgenic sugarcane plants over expressing plant chitinase gene for protection against Sugarcane Mosaic Virus.

In the current study, we generated endochitinase expressing transgenic potato lines to provide protection against Fusarium wilt and early blight. Full length chitinase gene of 1000 bp was integrated in the genome of potato variety Desiree and two transgenic lines were obtained. Several reports have demonstrated the use of pathogenesis related proteins to overcome pathogenic fungi in crop plants. Esfahani *et al.* (2010) transformed potato (*S. tuberosum* cv. Savalan) with chitinase and β-1,3-glucanase genes of mycoparasitic fungi origin to create resistance against *Rhizoctonia solani* AG-3.

Similarly, the transformation of canola by an endochitinase gene, chit33 from *T. atroviride*, increased resistance towards *Sclerotinia sclerotiorum* (Solgi *et al*. 2015). It was found that both transgenic plants expressing endochitinase showed no morphological abnormalities. This result was consistent with previous studies on broccoli (Mora and Earle 2001) where endochitinase expression did not adversely affect the plants. The study correlate with Khan *et al*. (2017) where barley derived chitinase II gene was over expressed in transgenic potato lines and up to 7 fold higher mRNA expression of transgene was achieved that was sufficient to create resistance against *A. solani*.

The two endochitinase expressing lines were subjected to *F. oxysporum* and *A. solani* infection in independent pot experiments. The results demonstrated that transgenic plants exhibited significant protection against the inoculated fungi even when the infection inoculum was sprayed for 30 days at 3 day intervals. It was revealed that mRNA expression of the transgene was significantly high post pathogen inoculation and remained stable for up to 30 days. The study suggested that resistance of transgenic plants against pathogen was because of high expression of endochitinase. Apart from the role of chitinase in chitinolytic activity, it has an indirect mechanism of protection in plants. Upon degradation of a fungal pathogen's cell wall, glycosidic components are released which elicit the induction of plant defense mechanisms (Shibuya and Minami 2001).

The study suggest that endochitinases in combination with recombinant technology can be a promising tool for improving plant resistance to fungal diseases.

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