



Agrobacterium-Mediated Transformation of Potato (*Solanum tuberosum* L. cv. Desiree) with *Trichoderma harzianum* Chitinase Gene for Enhanced Fungal Resistance

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Abstract

Solanum tuberosum L. (Potato) is a food crop of global significance, as it is ranked fourth after maize, rice, and wheat. It is, however, highly susceptible to fungal infections, namely late blight (*Phytophthora infestans*) and early blight (*Alternaria solani*), with a combined effect of the two causing tremendous losses to world food production. The use of conventional fungicides is being limited by pathogen resistance, environmental toxicity, and cost, necessitating the development of viable genetic resistance measures. Chitinases, an enzyme that breaks down chitin polymers in fungal cell walls, are a promising antifungal enzyme that can be used in transgenic crops to enhance crop resistance. In the current research, 1000 bp of the chitinase gene of *Trichoderma harzianum* was cloned and inserted into the binary vector pCambia1301 under the constitutive CaMV 35S promoter and inserted at the NcoI and BglII cut-off sites. Recombinant construct pCAM-trid was transferred into *Agrobacterium tumefaciens* by the freeze-thaw transformation process and then used to transform the potato cultivar Desiree, which is a highly regenerable cultivar. Among 37 explants, 26 regenerated, and molecular confirmation in the form of PCR and dot blot hybridisation revealed two transgenic lines with the insert of the *T. harzianum* chitinase gene, leading to a transformation efficiency of 7.69%. The study provides an underpinning for creating genotypes of *Solanum tuberosum* with superior resistance to fungal pathogens with the help of the heterologous expression of microbial chitinase genes, which offers a foundation for sustainable and environmentally friendly disease control in potato production.

Keywords

Solanum tuberosum, *Trichoderma harzianum*, chitinase, *Agrobacterium tumefaciens*, transgenic plants, fungal resistance

1. Introduction

Potato (*Solanum tuberosum* L.) is among four vital food harvests globally, after maize, wheat, and rice, that offer a significant supply of carbohydrates, vitamins, and dietary fibre to the world population [1]. Although the potato is economically and nutritionally valuable, it is the productivity of the crop that is frequently limited by the presence of fungal diseases, which greatly influence the level and quality of tubers [2]. The most destructive ones among them are late blight inflicted by *Phytophthora infestans*, Fusarium wilt, and early blight inflicted by *Alternaria solani*, which result in significant economic losses each year [1]. The traditional methods of control are associated with the use of fungicides, but the described processes are quite expensive, unsustainable, and can even give rise to developed resistant strains of pathogens [3].

Genetic engineering provides a high and sustainable approach to increasing resistance to fungal pathogens in potato. *Agrobacterium tumefaciens*-mediated transformation is still amongst surest and suitable approaches of transferring foreign genes to dicotyledonous plants, potato being one of them [4]. Through this method, genes encoding defence-related proteins like chitinases, glucanases, and pathogenesis-related (PR) proteins, which boost plant immunity, can be stabilised and expressed [5].

Chitinases (EC 3.2.1.14) which are able to break down chitin, which is one of the most important structural constituents of fungal cell walls. Such enzymes are essential in the defense of plants, limiting the invasion of fungi and their colonization [6]. Of microbial sources, *Trichoderma harzianum* has received a significant level of attention, owing to its good efficiency in chitinases and other mycolytic enzyme production, which provide the source with a broad-spectrum antifungal activity (Cloning and characterisation of a chitinase (chit42) cDNA from *Trichoderma harzianum* [7]. It has been reported that the expression of *T. harzianum* chitinase genes in transgenic plants has enhanced resistance to a variety of fungal pathogens by direct action in degrading fungal hyphae and directly through the host defence pathways [8].

Earlier research showed that expression of chitinases in transgenic plants like tobacco, rice and canola improves resistance of plants against fungal infections [9] [10]. Nonetheless, the use of the strategy on potatoes is relatively low. The Desiree cultivar has been a popular plant species in transformation research due to its regeneration ability and sensitivity to *Agrobacterium* infection [11]. Thus, in the given study, the aim was to clone a 1000 bp *T. harzianum* chitinase gene with the CaMV 35S promoter, insert it into the binary plasmid pCambia1301 and then transform it into the potato cultivar Desiree and verify the stability of transgenic integration using molecular and hybridisation techniques. The study aims to provide a basis to design fungal-resistant potato hybrids using molecular genetic techniques to enhance the management of the disease and crop yields sustainably.

2. Materials and Methods

2.1. Plant Material



In vitro *S. tuberosum* cv. Desiree plantlets were grown in Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962) with 3% sucrose and agar solidification. A 16 h photoperiod was used to maintain the cultures at 25 ± 2 °C

2.2. Source of Gene and Vector

The TA clone that carries 1000 bp chitinase gene (GenBank: KY290959) of *Trichoderma harzianum* was acquired at Seed Biotech Lab, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore. Plant transformation was done with binary vector pCambia1301 and propagated in *Escherichia coli* Top10. This expression plasmid has CaMV 35S promoter, hygromycin phosphotransferase gene to be selected and β -glucuronidase (GUS) reporter gene.

2.3. Chitinase gene amplification and Cloning

PCR-amplification of the chitinase gene involved the use of primers that have been created with the restriction sites of *Nco*I and *Bgl*II (Forward: 5'-CGCAGATCTCTCGACGCCAGCTTTCTG-3', Reverse: 5'-CGCCC ATGGTGTGCGCAAGTGTCCAGTTA-3'). PCR was carried out with the following profile: denaturation at 95 °C (4 min), 35 cycles of denaturation at 95 °C (30 s), 61 °C (30 s) and 72 °C (30 s) and 72 °C final extension (7 min). This amplified 1 kb product was gel-purified and digested with *Nco*I and *Bgl*II followed by the ligation with pCambia1301 downstream of the CaMV 35S promoter to produce pCAM-trid.

2.4. Transfection of *E. coli* and Screening of Recombinants

The ligation mix was transferred into the chemically capable *E. coli* Top10 cells by the method of calcium chloride. A selection of transformants was done on LB agar, with kanamycin (50 μ g mL⁻¹) and tetracycline (25 μ g mL⁻¹). The recombinant plasmids were isolated on the GeneJet plasmid miniprep kit (Thermo Scientific) and verified by restriction digestion and PCR.

Agrobacterium tumefaciens is transformed to allow the introduction of plasmids into the target organisms. The transformation of *Agrobacterium tumefaciens* is required to introduce plasmids into the target organism.

The verified construct pCAM-trid was transferred into a *A. tumefaciens* strain LBA4404 through the freeze-thaw practice (Hofgen & Willmitzer, 1988). Colonies were picked on YEP agar that had kanamycin (50 μ g mL⁻¹) and rifampicin (25 μ g mL⁻¹). Presence and integrity of the plasmids were verified via PCR with the aid of chitinase-specific primers and hygromycin resistance gene amplification as a control.

2.5. Transformation of Potato by *Agrobacterium*

Healthy in vitro-grown *S. tuberosum* cv. Desiree node was submerged in the suspension of *Agrobacterium* (OD₆₀₀ $\approx$ 0.6) containing pCAM-trid and shaken gently after 10 min. The explants were dried out on sterile filter paper and co-cultured on MS media with 100 μ M acetosyringone in the dark and left to dry 48 h. Thereafter, the explants were inoculated into regeneration medium (MS + 1 mg L⁻¹ BAP + 250 mg L⁻¹ cefotaxime + 50 mg L⁻¹ hygromycin) and sub-cultured every two weeks until the formation of shoots and roots.

2.6. Genomic DNA Isolation and Molecular Confirmation

The CTAB method was used in extracting genomic DNA of putative transgenic and control plants (Doyle and Doyle, 1990). Detection of transgene integration was done by PCR screening using primers specific to chitinase. PCR products were separated on agarose 1% gels. To further confirm, dot blot hybridization was carried out with DIG labeled chitinase gene probes (Roche Diagnostics, Germany). The presence of hybridization was identified by the application of alkaline phosphatase-conjugated anti-DIG antibodies and NBT/BCIP substrate.

2.7. Transformation Efficiency

Transformation efficiency was calculated as:

Transformation Efficiency (%) = Number of transgene-positive plants / Total regenerated plants × 100

3. Results

Plasmid DNA of pCAMBIA1301 was the small plasmid that was interested in this study and was successfully isolated and seen as a supercoiled band on agarose 1% gel, which indicated purity and was suitable to use in the downstream applications (Figure 4.1). Before gene insertion, restriction digestion and gel electrophoresis identified the desired 13 kb band of the vector, which showed the integrity of the constructs.

The *Trichoderma harzianum* gene chitinase (~1000 bp) was amplified with gene-specific primers that had constructed NcoI and BglII restriction sites. Only one, sharp amplicon with the desired size was seen on agarose gel (Figure 4.2), which is indicative of specific amplification with no non-specific byproducts. The purified product of the PCR, along with pCAMBIA1301, were digested and further ligated to create the recombinant construct, named pCAM-trid. Digestion of the ligated plasmid with restriction showed that the genome had been successfully inserted into the plasmid by yielding two separate fragments of 13 kb (vector) and 1 kb (insert) (Figure 4.3).

After the recombinant construct was transformed into *E. coli* Top10, several colonies resistant to kanamycin and tetracycline were obtained. Isolated plasmid DNA samples of randomly picked colonies had distinct supercoiled agarose gel bands (Figure 4.4). It was found that the presence of recombinant clones was confirmed by a diagnostic digestion with both NcoI and BglII showing the presence of both the 13 kb pCAMBIA1301 backbone and the 1 kb chitinase insert (Figure 4.5).

The validated pCAM-trid construct was moved into *A. tumefaciens* strain LBA4404 through the freeze-thaw method. On antibiotic selection (rifampicin and kanamycin), there was a differentiation of cream-colored colonies typical of *Agrobacterium* (Figure 4.6). The construct was maintained by producing a clear 13 kb band when plasmid DNA was isolated on agarose gel in the colonies that were transformed (Figure 4.7a). Successful transformation was verified by PCR amplification with chitinase-specific primers resulting in the appearance of a single PCR amplification product of about 1000 bp (Figure 4.7b).

PCR analysis on hygromycin-specific primers was done to verify the change in the control strain with the empty vector (Figure 4.8). The outcomes of all these were the confirmation of the presence of both recombinant and control constructs and their stability in *Agrobacterium*.

Co-culture with recombinant *Agrobacterium* was done on 37 nodal explants of *S. tuberosum* cv. Desiree. Twenty-six regenerated successfully on MS medium with the addition of cefotaxime and hygromycin, which implies the successful bacterial suppression and selection (Figure 4.9a-c). The morphology of



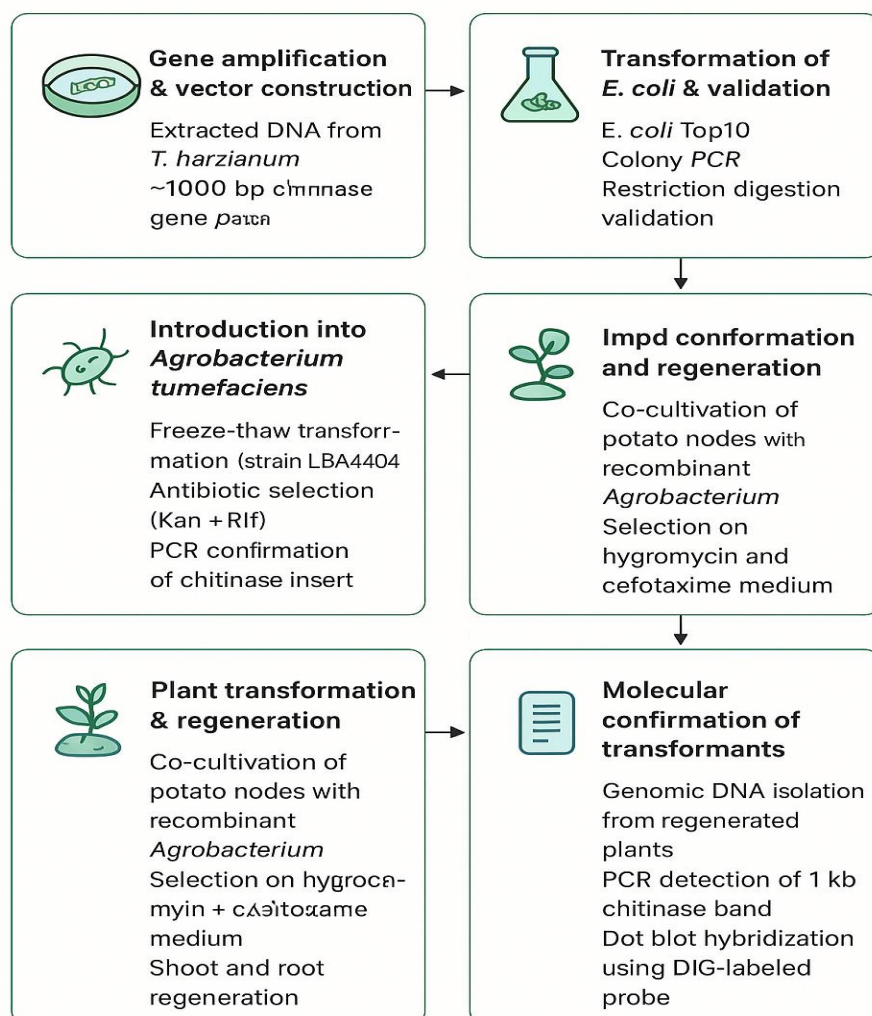
regenerated plantlets was normal and tissue necrosis was not evident, which proved that transformation and selection was not toxic to potato tissues.

The agarose gel with genomic DNA of regenerated plants gave clear and intact bands, which confirmed quality DNA to be used in the molecular analysis (Figure 4.10). Figure 4.11a-b: PCR amplification with the specific chitinase primers found three transformants (T1ps2, T3ps3, and T6ps9) that had a clear band size of about 1000 bp that corresponded to the inserted chitinase gene.

The presence of the transgene in two of the PCR-positive lines (T1ps2 and T6ps9) was further validated by dot blot hybridization with DIG-labeled chitinase probe. The non-transformed controls did not show any indication of hybridization, which confirms that the assays are specific (Figure 4.12).

Of 37 explants, 26 regenerated successfully and produced plantlets, and two were identified as true transgenics through the dot blot hybridization, which had a transformation efficiency of 7.69% (Table 4.1). Such a high efficiency is comparable to already known values of *Agrobacterium*-mediated transformation in potato and other solanaceous crops [12]. The viability of the transformation strategy adopted is proved by the successful integration of molecules and the stable regeneration of chitinase-producing potato plants. This 7.69% efficiency is reproducible, and combined with clean molecular verification, this system is now a dependable framework of antifungal genes insertion and additional stacking strategies in potato biotechnology.

Schematic representation of the cloning and transformation of *Trichoderma harzianum* chitinase gene into *Solanum tuberosum* cv. Desiree



Schematic representation of the experimental workflow. Stepwise illustration of the cloning and transformation process: (1) amplification of *T. harzianum* chitinase (~1000 bp), (2) insertion into pCAMBIA1301 under CaMV 35S promoter to create the pCAM-trid construct, (3) transformation and validation in *E. coli* and *Agrobacterium tumefaciens* LBA4404, (4) *Agrobacterium*-mediated transformation of *S. tuberosum* cv. Desiree, and (5) molecular confirmation through PCR and dot blot hybridization. Transformation efficiency was 7.69%.

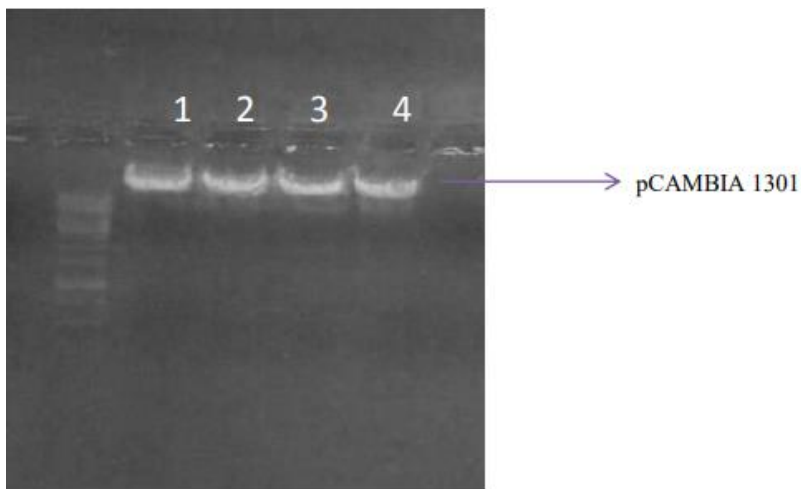


Figure 2. Amplification of *T. harzianum* chitinase gene.

PCR amplification showing a distinct ~1000 bp band corresponding to the chitinase gene using gene-specific primers. Lane M: 1 kb DNA ladder; Lanes 1–6: amplified products; Lane N: negative control (no template DNA).

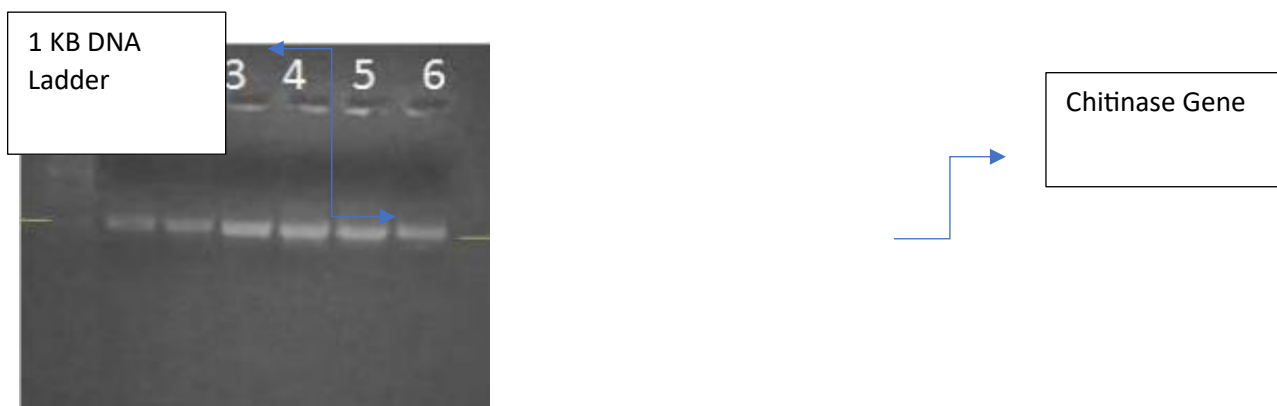


Figure 3. PCR amplification of Chitinase gene along with 1kb DNA ladder. From lane 1-6; Chitinase gene of ~1000bp size

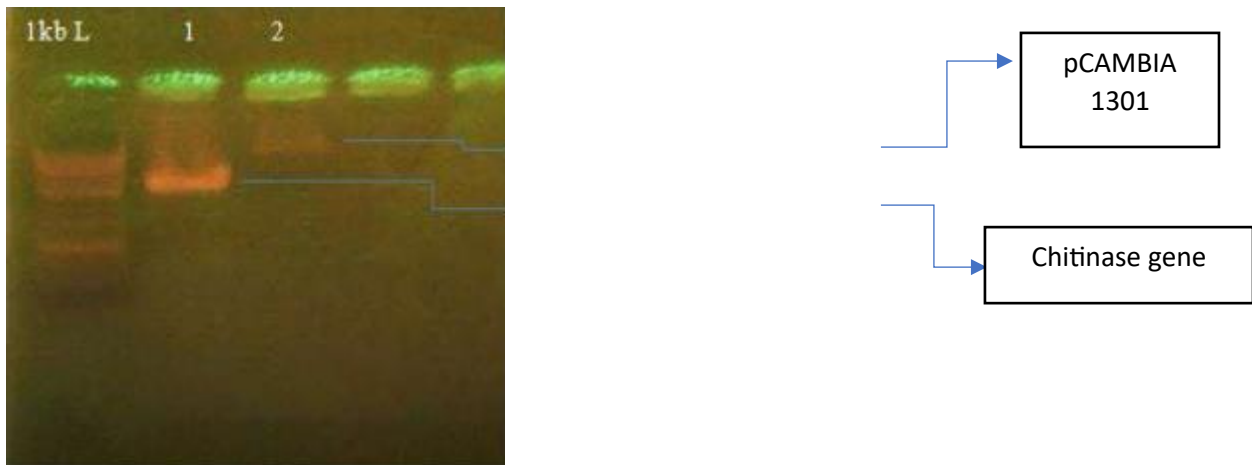


Figure 4. Restriction digestion of pCambia1301 vector and chitinase insert.

Agarose gel electrophoresis showing digestion with *NcoI* and *BglII*. Lane 1: digested chitinase insert (~1 kb); Lane 2: linearized pCambia1301 vector (~13 kb); Lane M: 1 kb DNA ladder.



(A)



(B)

Figure 4. Confirmation of recombinant *E. coli* clones.

(A) Growth of kanamycin- and tetracycline-resistant colonies on LB agar.
(B) Agarose gel showing plasmid DNA from selected colonies. Lanes 1–5: isolated plasmids (~13 kb); Lane M: DNA ladder.

4. Discussion

The current experiment has been effective in cloning, *Agrobacterium*-mediated transformation, and molecular validation of a gene encoding chitinase of *Trichoderma harzianum* in *Solanum tuberosum* cv. Desiree. The obtained transformation efficiency of 7.69 percent matches the reported transformation efficiencies in the *Solanum* species and supports the reproducibility and strength of the implemented methodology [13]. The accuracy of restriction digestion, correct insertion and sequencing verification of the construct (pCAM-trid) all verified the integrity of the structural features of the recombinant construct (pCAM-trid) before transformation into plants, thus ensuring the high level of reliability of the experiment.

The chitinases are important in plant defense as they hydrolyze β -1, 4-glycosidic bonds of the chitin that is a major constituent of the cell walls of fungi [10]. *T. harzianum* chitinase gene (KY290959) is a glycosyl hydrolase family-18 gene, which has been identified to be potent in antifungal action as well as inhibiting sporulation and hyphae development of pathogenic fungi in the phytoplankton [14]. The expression in transgenic plants of this fungus chitinase has been shown to confer this type of broad-spectrum resistance against pathogenic fungi in rice [15], peanut [16], and wheat [10]. Transgene molecular confirmation using PCR and dot-blot hybridization was used in this experiment to confirm stable genomic integration, indicating that transformed potato lines will likely express greater resistance against the major fungal pathogens, including *Alternaria solani*, *Fusarium oxysporum*, and *Rhizoctonia solani*, three of the most devastating agents that cause worldwide losses in potato yield [17].

The traditional approach to managing fungal infections in potato is based mostly on the repeated usage of synthetic fungicides, which are dangerous to the environment, cause resistance in the pathogens, and have adverse effects on the soil and water ecosystems [18]. Production of transgenic crops that contain antifungal genes thus has a lasting and focused substitute. The efficiency of the transformation used in this work is similar or even greater than those previously reported using GUS, Bt, or pathogenicity-related (PR) genes in potato [19], which means that this method can also be used to implement the functional genes in the solanaceous plants [20].

The constitutive CaMV 35S promoter carried in the pCAMBIA1301 vector was probably the reason why transgene expression was stable and uniform and has the potential to give broad-spectrum resistance both in the field and controlled settings. Constitutive overexpression of chitinase can also work in synergy with innate defence mechanisms especially those controlled by PR1 and PR5 genes and increase systemic resistance and reduce pathogen colonization [21]. Such an interlaced response of defense systems resembles the natural resistance mechanisms and improves the general resistance of plants.

In addition to pathogen resistance, the efficient production of chitinase-producing potato lines is a historic advancement toward gene pyramiding, the prudent fusion of several defensive and additional tolerance to stress genes to bring about lasting resistance. The key areas of future research include functional validation of these lines of transgenes using controlled pathogen inoculation tests, quantitative expression profiling of such transgenes under both biotic and abiotic stresses and multi season greenhouse or confined field tests. In addition, fine-tuning of promoters using CRISPR/Cas9 genome editing may allow spatially regulated expression and minimize possible metabolic expenses of constitutive transgene expression.

Altogether, this work proves that the *T. harzianum* chitinase application is possible and effective to enhance the resistance of *S. tuberosum* to fungal diseases genetically. This practice aids eco-friendly farming by decreasing the reliance on chemical fungicides, enhancing the genetic engineering of genetically modified, environmentally safe potato varieties that promote food security and environmental management in the long term.

5. Conclusion and Future Perspectives

This study has been able to develop a consistent and reproducible system of *Agrobacterium*-mediated potato (*Solanum tuberosum* cv. Desiree) transformation as well as to show the maintenance of a stable integration of a 1000 bp *Trichoderma harzianum* chitinase gene (KY290959) under CaMV 35S control. Transgene incorporation was established using molecular confirmation, by PCR, and dot blot hybridisation and the transgene incorporation rate was 7.69; this is within the range of benchmark reports in potato transformation research. The transgenic production of transgenic lines and successful development of the pCAM-trid vector is solid evidence of concept in the implementation of fungal chitinase genes as effective bio-defense against economically important potato pathogen, including *Alternaria solani*, *Fusarium oxysporum*, and *Rhizoctonia solani*. In addition to validation of molecular integration, this study provides a good basis to functional validation of antifungal genes as well as the path to developing long lasting alternatives to chemical fungicides that are not harmful to the environment. The methodology of the study is consistent with the concept of integrated crop-protection models and promotes the objectives of global sustainability to the objectives of enhancing agriculture systems that are resilient to climate changes.

The functional validation of the expressed chitinase gene by means of pathogenicity tests concerning key fungal isolates and transcriptomic and enzymatic profiling to validate constitutive and stress-induced gene expression should be considered the future research. Further, there is a need to carry out field and greenhouse assessments that can determine phenotypic steadiness, yield efficiency and environmental safety. The combination of chitinase and complementary defense gene (gene-stacking) or fine-tuning transgene expression using CRISPR represents equally promising future directions with regard to improving transgene efficacy.

Finally, this study can be seen as a major breakthrough in the way of developing a genotype of *Solanum tuberosum* with a higher level of natural resistance. It emphasizes the possibility of microbial chitinases as renewable bio-technological options to reduce fungal infections in addition to enhancing global food security in terms of safer and more resistant production systems of crops.

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