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Enhanced resistance to *Rhizoctonia solani* by combined expression of chitinase and Ribosome Inactivating Protein in transgenic potatoes (*Solanum tuberosum* L.)

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Abstract

Potato (*Solanum tuberosum* L.) is susceptible to many fungal pathogens including *Rhizoctonia solani*. In the present study, the potato cultivar Desirée was transformed via *Agrobacterium tumefaciens* strain GV3101 containing the binary plasmid pGJ132 harboring both the chitinase (*chiA*) and *rip30* genes. The potato leaf disc was used as an explant for transformation. PCR, Southern blot and Western blot were used for characterization of the transgenic plants. In this study it was shown that not all the plants developed in selective medium were positive for the corresponding gene using the PCR technique. Southern blot analysis confirmed that transgenic plants integrated 2-3 copies of *chiA* and *rip30* genes respectively into their genome. The expression of the CHIA and RIP30 proteins was confirmed in the leaf extracts of the transgenic clones by Western blot analysis. Transgenic potato plants expressing *rip30* and *chiA* genes showed enhanced resistance to *R. solani* in a greenhouse assay.

Additional key words: fungal resistance; genes; proteins; southern blot; western blot.

Resumen

Resistencia aumentada a *Rhizoctonia solani* por la expresión combinada de quitinasa y proteínas inactivantes de los ribosomas en patatas transgénicas

La patata (*Solanum tuberosum* L.) es susceptible a muchos hongos fitopatógenos, incluyendo *Rhizoctonia solani*. En el presente estudio, se transformó el cultivar de patata 'Desirée' mediante *Agrobacterium tumefaciens*, cepa GV3101, que contiene el plásmido binario pGJ132 que alberga los genes quitinasa (*chiA*) y *rip30*. Se utilizaron discos de hojas como explante para la transformación de plantas. Se utilizaron las técnicas de PCR, Southern y Western blot para la caracterización de las plantas transgénicas. En este estudio se demostró, mediante PCR, que no todas las plantas que se desarrollaron en medio selectivo fueron positivas para el gen correspondiente. El análisis de Southern blot confirmó que las plantas transgénicas integraron en su genoma 2-3 copias de los genes *chiA* y *rip30*. Se llevó a cabo un ensayo de invernadero para evaluar la resistencia a *R. solani* de los clones transgénicos que expresan los transgenes. Las plantas transgénicas que expresan los genes *rip30* y *chiA* mostraron una resistencia completa a *R. solani*.

Palabras clave adicionales: genes; proteínas; resistencia fúngica; southern blot; western blot.

Introduction

Potato (*Solanum tuberosum* L.), one of the most important food crops in the world, is susceptible to diseases caused by several fungi, bacteria and other pathogens, leading to considerable losses in yield and quality of products (Walter *et al*., 2001; Khan *et al*., 2008). *Rhizoctonia solani* is the causal organism of Rhizoctonia disease complex in potato, resulting in two different symptomologies of the disease, namely stems canker and black scurf. These are recognized as necrotic lesions on underground plant parts, and

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Abbreviations used: ADAS (Adams disease assessment scale); PCR (polymerase chain reaction); PR (pathogenesis-related proteins); RIP (ribosome-inactivating protein).

sclerotia covering progeny tubers, respectively (Carling & Leiner, 1986; Agrios, 1997). In severe infections total yield can be highly reduced (Hide *et al*., 1996). Extensive yield losses, from 10% to 30% on marketable size tubers as a result of Rhizoctonia disease have been reported (Read *et al.,* 1989; Lehtonen *et al*., 2008).

Plants are known to have defence systems that involve the production of pathogenesis-related (PR) proteins, such as antimicrobial enzymes; chitinases, glucanases and ribosome-inactivating proteins (RIPs) (Ward *et al*., 1991). Chitinase catalyzes the hydrolysis of β-1,4 linkages of the N-acetyl-D-glucosamine polymer, called chitin. Chitin is a major component of the cell walls of many fungi. In fact, plant chitinases have exhibited antibiotic activity against various fungal pathogen *in vitro* tests by causing lysis of hyphal tips as well as the inhibition of spore germination and germ tube elongation (Chye *et al*., 2005).

RIPs are plant enzymes that have 28 S rRNA *N*-glycosidase activity which, depending on their specificity, can inactivate foreign ribosomes, thereby shutting down protein synthesis. The most common cytosolic type I RIP from the endosperm of cereal grains do not act on plant ribosomes but can affect foreign ribosomes, such as those of fungi (Stirpe *et al*., 1992; Hartley *et al*., 1996).

Several transgenic plants harbouring PR genes have already been produced, aiming at producing fungal disease-resistant varieties in a variety of crops (Jach *et al*., 1995; Tabei *et al*., 1997; Bieri *et al*., 2000; Datta *et al*., 2001; Jayaraj & Punja, 2007; Xiao *et al*., 2007; Almasia *et al*., 2008; Esfahani *et al*., 2010), including the transgenic tobacco that was engineered with the RIP encoding DNA sequence of barley and exhibits resistance to *R. solani* (Logeman *et al*., 1992). Many of the PR proteins may act synergistically *in vivo* and also enhanced inhibition of fungal growth when tested in combination *in vitro* (Jach *et al*., 1995; Chye *et al*., 2005). Transgenic plants expressing more than one PR protein genes were also developed in potato, rice and tobacco (Zhu *et al*., 1994; Zhu *et al*., 2007; Esfahani *et al*., 2010). Such transgenic plants showed better resistance than those having a single gene.

Transgenesis has proved its value as a method to enhance disease resistance in potato and related species. For example, potato plants transformed with the dermaseptin analog MsrA2 exhibit broad-range resistance to phytopathogenic fungi, including *Cercospora, Fusarium* and *Pythium* (Osusky *et al*., 2005). Also, lysozyme accumulation in transgenic potato plants considerably inhibited growth of several bacterial species, including *Erwinia carotovora* and *Pseudomonas syringae* (Kato *et al*., 1998). Potato plants expressing the AP24, thaumatin-like pathogenesis-related protein, transgene, showed increased resistance to *P. infestans* (Liu *et al*., 1994) and tomato plants transformed with tobacco AP24 and bean chitinase transgenes showed improved resistance to *F. oxysporum* (Ouyang *et al*., 2005).

In the present study, simultaneous integration and expression of the barley *rip30* and the *Serratia marcescens* chitinase (*chiA*) genes was used, and the transgenic potatoes produced were evaluated in greenhouse conditions for their resistance to *R. solani*.

Material and methods

Plant material

Virus free potato tubers of the tetraploid cultivar 'Desirée' was used for transformation. Etiolated sprouts from these tubers were surface disinfected and single-node segments were excised and placed on MS medium (Murashige & Skoog, 1962) supplemented with 100 g L^{-1} inositol, 30 g L^{-1} sucrose, 0.7% (w/v) agar, the pH was adjusted to 5.6. Stems developing from these nodes were propagated *in vitro* by subculturing the top shoots or stem segments including axillary buds every 3-4 weeks. The shoots were grown at 23 °C and a photoperiod of 16 h under 3000 lux light intensity.

Bacterial strain and growth conditions

The disarmed *Agrobacterium tumefaciens* strain GV3101 with the binary vector pGJ132 (16176 bp), kindly provided by Dr. G. Jach (Max Planck Institut für Züchtungsforschung, Cologne, Germany), was used. The T-DNA of pGJ132 contains both *chiA* gene from *Serratia marcescens* (Lund & Dunsmuir, 1992; Howie *et al*., 1994) and *rip30* gene coding for barley (*Hordeum vulgare* L.) RIPs (Logeman *et al*., 1992), both driven by the 35S of *Cauliflower mosaic virus* (CaMV) promoter. The plasmid contains the *nptII* gene coding for neomycin phosphotransferase II that conferred kanamycin resistance, as a selectable marker of the transgenic plants (Fig. 1).

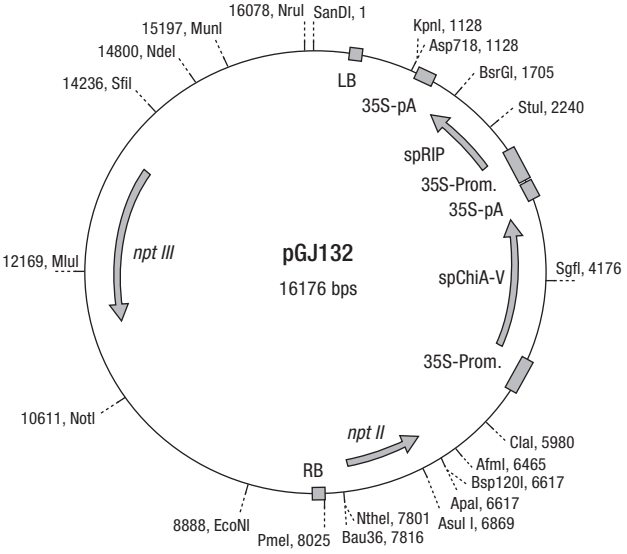

Figure 1. Schematic representation of T-DNA region of pGJ132 (G. Jach, Max Planck Institut für Züchtungsforschung, Cologne, Germany) with expected restriction sites. The binary vector pGJ132-RIP30-CHIA contained the kanamycin resistance gene (*nptII)*, a *Cauliflower mosaic virus* 35S promoter (35S-Prom), and *Serratia marcescens* chitinase cDNA *chiA*, a barley cDNA *rip30*. RB and LB denote the right and left T-DNA border sequences.

A. tumefaciens, GV3101 strain, was grown in Luria-Bertani (LB) medium at 28 °C. For leaf infection, bacteria cells were grown overnight at 28 °C with constant shaking (200 rpm) in YEB medium (Hooykaas *et al*., 1979) supplemented with 100 mg L^{-1} rifampycin, 50 mg L^{-1} of kanamycin, and 100 mg L^{-1} streptomycin. When OD_{600nm} reached 0.8–1, the culture was diluted 1:10 in MS medium. The bacterial cells were collected by centrifugation and resuspended in MS medium supplemented with 50 mg L^{-1} sucrose, pH 5.6.

Plant transformation

The leaf disc transformation method was used. Five mm leaf discs were cut from *in vitro* plants. A total of 40 explants were used. The explants were infected with 30 mL of the *A. tumefaciens* and placed at 25 °C in dark conditions for 1 hour. Infected explants were co-cultivated for 2 days, in the dark, on a MS basal medium without antibiotics to facilitate transformation and supplemented with 100 g L^{-1} of inositol, 30 g L^{-1} sucrose, 0.7% (w/v) agar. The pH was adjusted to 5.6. Subsequently, the leaf discs were washed with MS basal medium containing 500 mg L^{-1} cefotaxime to eliminate bacteria. After drying on sterile filter paper, they were transferred to Petri dishes containing 25 mL of the MS basal medium supplemented with 2 mg L^{-1} zeatin, 20 µg L^{-1} gibberellic acid, 20 µg L^{-1} α-naphthalene acetic acid (α-NAA), 500 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin. They were maintained on this medium for 5 weeks at 23°C and 16 hour photoperiod for shoot regeneration and elongation. The resulting shoots, about 5 mm long, were regenerated during 4-5 weeks after transformation and were then transferred individually to MS medium containing kanamycin (100 mg L^{-1}) and cefotaxime (500 mg L^{-1}) for rooting. Transgenic plants were further transferred to greenhouse conditions.

In this experiment several parameters were recorded: the percentage of explants with induced callus proliferation, the percentage of regenerated calli, the number of shoots per callus and the percentage of transgenic plants.

Molecular characterization of transgenic plants

To detect *rip30* and *chiA* genes by PCR, genomic DNA was extracted from leaf material according to Edwards *et al*. (1991). DNA fragment, containing the *rip*30 or *chiA* genes, of 500 and 700 bp respectively was amplified by PCR using specific primers. The complete sequence of these primers is as follows: *rip30*, 5'CAACCCGGCGCACTTCTC3'; reverse *rip30*, 5'GGCCTTCATCTCATTGCCG3'; forward *chiA*, 5'CTGAAAGAGATTGAAGGCAGCTTC3'; and reverse c*hiA*, 5'CATGGCGGTGCCGACGAC3'. These primers were designed by the software Primer3 (Rozen & Skalestsy, 1999). PCR amplification reactions consisted of an initial denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 1 min, hybridization at 55°C for 1 min, extension at 72 °C for 2 min and a post- extension at 72 °C for 10 min. Taq DNA polymerase was used in all PCR reactions. The resulting PCR products were separated by electrophoresis agarose gel 1% (w/v).

Southern blot analyses were employed to detect the integration of *rip30* and *chiA* in transgenic plants. A total of 10 µg of DNA genomic was extracted from young leaves and digested with *Eco* RI and *Hind* III. The probe of 1936 bp corresponded to the insert from pGJ132, digested with *Stu* I and *Sgf* I, separated on a 0.9% agarose gel, transferred to a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) and hy-

bridized with the DIG-labeled probe of the *rip30* and *chiA* genes. Hybridization, washing and detection were performed using DIG Easy Hyb (hybridization solution) and DIG Wash and Block Buffer set following the supplier's instructions (Boehringer Mannheim).

Western blot analysis was used to evaluate the expression of the integrated *rip30* and *chiA* genes in genomic DNA of transgenic plants. Total proteins were extracted from 300 mg of young leaves of potato plant according to Laemmli (1970). The homogenized samples were boiled for 3 min, extracts were centrifuged at 13000 rpm for 2 min, and then 40 μL of soluble proteins were loaded onto 12% SDS-polyacrylamide gel (SDS-PAGE) according to Laemmli (1970) and electroblotted onto a polyvinylidine difluoride (PVDF) membrane (Amersham). Immunodetection was performed using polyclonal anti-serum raised in rabbit against the RIP30 and CHIA proteins and goat-anti-rabbit IgG (Amersham) conjugated to horseradish peroxidise (HRP) as secondary antibody, at 1: 1000 dilution.

Resistance evaluation of the transgenic plants in greenhouse

Five individual transformants were selected. These clones with confirmed insertion of *rip30* and *chiA* genes and untransformed plants were micropropagated *in vitro.* After 4-5 weeks, five plants for each clone and for each control were transplanted into individual pots and placed in the greenhouse for further screening for resistance to the fungus. The *R. solani* strain Ci96, provided by Neiker Institute (Vitoria, Spain) was grown on potato dextrose agar (PDA) medium [20% (w/v) potato, 1.5% (w/v) glucose and 1.5% (w/v) agar] at 25°C with light for 5 days, and subcultured as needed. The inoculation was done as described by Chand & Logan (1983). After 4 months, the crop was harvested and the tuber symptoms were assessed in five tubers per plant. The symptoms evaluated were the appearance of black or brown sclerotia on the tuber surface, using the ADAS (Adams disease assessment scale) (Anonymous, 1976) ranging from 0 to 25% according to the infected surfaces covered by sclerotia. The inoculations were repeated three times for a more effective infection.

For the statistical analysis, an ANOVA was performed to evaluate the significance of the differences in resistance among the clones. Means were separated using the LSD test ($p < 0.05$).

Results

Transformation and characterization of transgenic plants

Transformation with the pGJ132 showed that a total of 70% (28 out of 40) of explants induced callus proliferation. The number of explants with callus regeneration was 17. The total number of shoots was 218. Not all the plants growing on kanamycin-containing medium were positive in the identification of the corresponding gene present on the T-DNA using the PCR technique. Only 65% (141 out of 218) of the plants rooted in kanamycin medium displayed a 500 bp amplification fragment for *rip30* gene and a 700 bp for *chiA* gene. The specific primers did not amplify the corresponding fragments in the untransformed samples (Fig. 2).

Southern blot analysis was performed in 141 positive PCR clones and showed that the *rip*30 and *chiA* genes were integrated into the genome of the tested transformed plants. In these transformants, two and three hybridizing bands were detected for *chiA* and *rip*30 genes respectively. Fig. 3 presents a Southern blot for one selected positive PCR clone (C1) hybridized with the probes for *chiA* and *rip30.* Two bands are obtained for the *chiA* gene (lane C1a), indicating that there could be two copies inserted for this transgenic clone. A digestion with *Hind* III, on the other

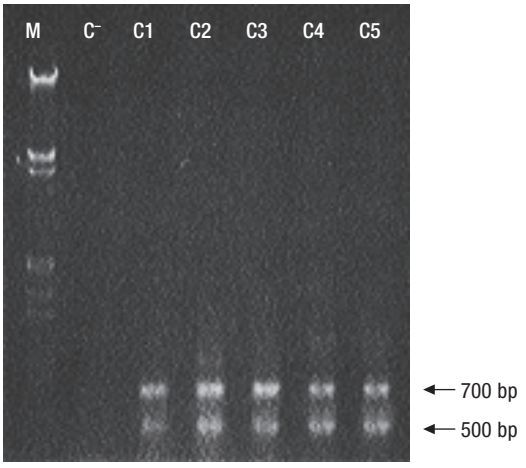

Figure 2. PCR-amplified regions used to confirm the integration of the *rip30* and *chiA* genes in transgenic plants of cv. Desirée. Amplification of *rip30* (500 bp) and *chiA* (700 bp) genes in the transformed potato with the pGJ132. Lane M, size marker. Lane C^-, non-transformed control. Lanes C1–C5, independent transgenic potato clones. Arrows indicate the amplified fragments of *rip30* (500 bp) and *chiA* (700 bp) genes in transgenic clones.

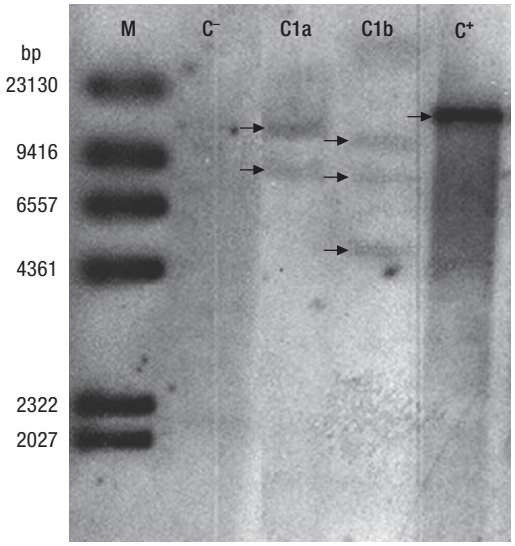

Figure 3. Southern blot analysis of the cv. Desirée. Genomic DNA (10 µg) from transgenic and non-transgenic control potato plants was digested with *Eco*RI and *Hind*III enzymes, and hybridized with a DIG-labeled probe for *chiA* and *rip30* genes. Lane M, DIG-labeled molecular weight marker III. Lane C^-, non-transformed control. Lane C1a and C1b, independent transgenic clone transformed with the pGJ132 and genomic DNA digested with *Eco* RI and *Hind* III, respectively. Lane C^+, the plasmid pGJ132 DNA template as positive control. The arrows indicate the transgenes copy number in the transgenic clone (bands with different locations).

hand, gives three bands of the *rip30* gene (Fig. 3, lane C1b). The size of the bands differed, indicating that this transformant have two and three copies of the *chiA* and *rip*30 genes respectively integrated at random sites in the genome. The pattern of integration was variable. Therefore, 84% (118 out of 141) of the transgenic clones showed two and three copies of the *chiA* and *rip*30 genes integrated, respectively, whereas 23 transgenic clones showed two copies of both genes. No transgene insertion was detected in non-transformed controls.

The Western blot analysis demonstrated that the *rip30* and *chiA* genes are transcribed and translated into proteins in the tested transgenic clones. The 30 kDa peptide of the RIP30 protein was detected as a single band in the total protein extracts from the transgenic plants with varying levels of expression in different clones (Fig. 4a). Also, the 52 kDa peptide of the CHIA protein was expressed at different detectable levels in the tested transgenic plants (Fig. 4b). The control non-transformed plant did not express the RIP30 and CHIA proteins.

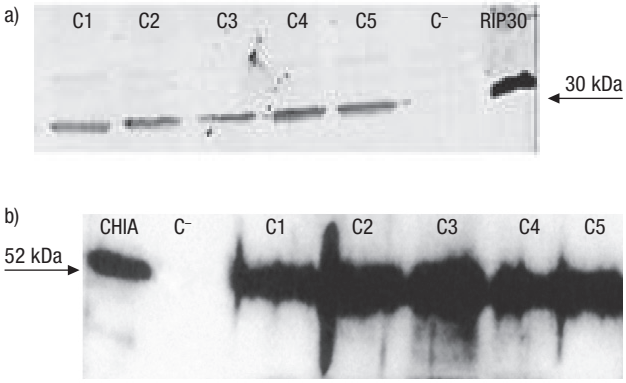

Figure 4. Western blot analysis of RIP30 and CHIA proteins in cv. 'Desirée'. Protein extracts (10 µg) of young leaves from five independent transgenic clones (C1-C5) and non-transformed control (C^-), resolved in a 15% polyacrylamide gel and subjected to immunoblot analysis using antibodies for rabbit polyclonal antiserum. Arrows indicate the 30 kDa band of the RIP30 protein (a) and the 52 kDa band of the CHIA protein (b).

Resistance evaluation to virulent isolate of *R. solani*

The results of greenhouse evaluation of the transgenic clones expressing the *rip30+chiA* genes are presented in Table 1. The clones expressing *chiA* and *rip30* genes showed an enhanced resistance to the pathogen and no symptoms were found in their tubers. The non-transgenic control presented damages of 10% of the surface tuber covered by sclerotia, compared with the high level of protection observed in the transgenic clones.

Table 1. Symptoms caused by *R. solani* in the transgenic clones of potato 'Desirée' expressing *rip30* and *chiA* genes, tested in greenhouse

Transgenic clones	Mean symptoms[1]
Control (non-transformed)	10.00 b
T1	0.00 a
T2	0.00 a
T3	0.00 a
T4	0.02 a
T5	0.03 a

[1] Symptoms were assessed by ADAS scale (Anonymous, 1976), assigning values between 0 and 25% according to the infected surface covered by sclerotia. The mean of a total of 25 tubers for each transgenic clone and for the control is shown. Values followed by different letters are significantly different according to the Tukey's b test ($\alpha \leq 0.05$).

Discussion

In this study, the potato cv. 'Desirée' was transformed with an association of *chiA* and *rip30* genes isolated from *Serratia marcescens* and *Hordeum vulgare* L., respectively. This was attempted because application of multiple resistance genes to plant genetic manipulations could widen the spectrum and increase the level of resistance in many crops (McDowell & Woffenden, 2003; Zhu *et al*., 2007).

Potato leaf discs were used as explants for transformation with *A. tumefaciens*, which has been commonly used for this purpose in potato (Beaujean *et al*., 1998; Ducreux *et al*., 2005; Banerjee *et al*., 2006). In previous studies the potato micro-tuber discs, were found to be effective explants for transformation (Esfahani *et al*., 2010). In the laboratory, however, when micro-tuber discs were used there was a lack of response of some varieties and even a decrease in the regeneration rate (M'hamdi *et al*., 2003). In this study, using leaf disc explants, the regeneration rate was high and the transgenic shoots obtained were morphologically normal.

Differences between the number of plants grown on selective medium with kanamycin and those positive in the PCR assay were noted. Something similar was reported by Kumar *et al*. (1995), who observed many escapees despite the selective medium. This could be explained by the fact that calli are formed by a set of juxtaposed cells and, thus, some of these cells although not transformed, can escape the selective antibiotic and regenerate shoots (M'hamdi *et al*., 2003). Moreover, some plants may integrate only the gene of resistance to the antibiotic but not the transgene, as the transfer of T-DNA occurs from the right to the left border and the actual transgene is not integrated (Wang *et al*., 1984). Indeed, in the binary vectors that we used, the *nptII* gene conferring resistance to kanamycin is nearest to the right border than the *chiA* and *rip30* genes. The transgenic clones (positive in PCR) were confirmed by Southern blot analysis. This showed that the PCR product was the result of T-DNA integration into the genome of transgenic plants. We observed integration of two and three copies of *chiA* and *rip30* genes, respectively, into the genome of 118 clones.

The greenhouse assay was carried out to evaluate resistance to the pathogen in transgenic plants and showed enhanced resistance in transgenic clones expressing the *rip30* and *chiA* genes. The combined expression of these two genes was effective in preventing disease development. The deployment of genetic engineering approaches that involve the expression of two or more antifungal gene products in a specific crop should provide more effective and broad-spectrum disease control (Jach *et al*., 1995; Melchers & Stuiver, 2000; Anand *et al*., 2003; Almasia *et al.,* 2008; Esfahani *et al*., 2010).

A previous study by Esfahani *et al.* (2010) showed that a combination of chitinase (*chit*42) and glucanase (*bgn*13.1) genes isolated from different *Trichoderma* species increased the antifungal activity against *R. solani* AG-3 in transgenic potatoes. Chye *et al*. (2005) reported that transgenic potato plants co-expressing *Brassica juncea* chitinase (B_jCHI_1) and *Hevea brasiliensis* β-1,3-glucanase (H_bGLU) inhibited fungal growth better than extracts from transgenic potato expressing either B_jCHI_1 or H_bGLU, suggesting a synergistic effect. Observations of collapsed epidermal cells in the co-expressing potato roots suggest that these proteins effectively degrade the fungal cell wall, producing elicitors that initiate other defense responses causing epidermal cell collapse that ultimately restricts further fungal penetration.

Our results revealed that the *rip30* and *chiA* genes were successfully integrated into the genome of transgenic potato plants, and expressed as revealed by the production of the RIP30 and CHIA proteins. The expression of these two proteins together demonstrated enhanced resistance to *R. solani* in the transgenic potato.

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