

Ribosome Inactivating Protein of barley enhanced resistance to *Rhizoctonia solani* in transgenic potato cultivar ‘Desirée’ in greenhouse conditions

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In the present study, the potato cultivar ‘Desirée’ was transformed via *Agrobacterium tumefaciens* strain LBA4404 containing the plasmid pBIN19 which harbors the Ribosome Inactivating Protein (*rip30*). The potato leaf discs were used as an explant for transformation. The *in vitro* regeneration parameters (percentage of callus regenerated, number of shoots per callus, percentage of regenerated roots and percentage of the transgenic plants) were evaluated. The PCR technique was used for identification of transformed plants. Southern and Western blot analyses were applied for molecular characterization of the transgenic clones. A greenhouse assay was carried out to evaluate the resistance to *Rhizoctonia solani* pathogen of transgenic clones expressing the *rip30* gene. The results revealed that not all the plants developed in selective medium were positive for the corresponding gene using the PCR technique. Southern blot analysis demonstrated that the tested transgenic plants integrated three copies of *rip30* gene into their genome. The expression of the RIP30 protein was confirmed in the leaf extracts of the transgenic clones by Western blot analysis. Resistance evaluation of the transgenic plants in greenhouse conditions showed that disease incidence and severity were reduced for *R. solani*.

Keywords. Potatoes, *Solanum tuberosum*, transgenic plants, *Agrobacterium tumefaciens*, disease resistance, ribosomes, *Rhizoctonia solani*, PCR, greenhouses.

Rip30 améliore la résistance au *Rhizoctonia solani* chez les plantes transgéniques de pomme de terre cultivar ‘Desirée’ en conditions de serre. Dans la présente étude, le cultivar ‘Desirée’ de pomme de terre (*Solanum tuberosum* L.) a été transformé en utilisant la souche LBA4404 d’*Agrobacterium tumefaciens* renfermant le plasmide pBIN19 où est cloné le gène *rip30* (*Ribosome Inactivating Protein*). Des disques de feuilles ont été utilisés comme explants pour la transformation. Les paramètres de régénération *in vitro* qui ont été évalués sont le pourcentage de cals régénérés, le nombre de pousses par cal, le pourcentage d’enracinement des pousses et le pourcentage de plantes transgéniques. La technique PCR a été utilisée pour l’identification des plantes transformées. Les analyses par Southern blot et Western blot ont été utilisées pour la caractérisation moléculaire des transformants. Une inoculation dans des conditions de serre a été réalisée pour évaluer la résistance au pathogène *Rhizoctonia solani* des clones transgéniques exprimant le gène *rip30*. Les résultats de cette étude ont révélé que les plantes développées en milieu sélectif n’ont pas toutes été positives pour le gène correspondant en utilisant la technique de PCR. L’analyse Southern blot a confirmé que les plantes transgéniques ont intégré trois copies du gène *rip30* dans leur génome. L’expression de la protéine RIP30 a été confirmée pour les clones transgéniques par l’analyse Western blot. L’évaluation de la résistance des plantes transgéniques dans des conditions de serre a montré que l’incidence de la maladie et les symptômes ont été réduits pour *R. solani*.

Mots-clés. Pomme de terre, *Solanum tuberosum*, plante transgénique, *Agrobacterium tumefaciens*, résistance aux maladies, ribosome, *Rhizoctonia solani*, PCR, serre.

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important crops worldwide. Unfortunately, commercial

cultivars are particularly susceptible to fungal and bacterial diseases leading to considerable losses in yield and quality of products (Walter et al., 2001; Khan et al., 2008). *Rhizoctonia solani*, the most widely spread

species of *Rhizoctonia*, is a soil borne fungal pathogen. The most common symptom on potato result in two appearances of the disease, namely stem canker and black scurf. These are recognized as necrotic lesions on underground plant parts, and sclerotia covering progeny tubers, respectively (Carling et al., 1986).

Plant disease control based mainly on chemical pesticides is not always effective and is commonly expensive. Furthermore, this treatment involves residual toxicity and could contribute to the selection of fungicide-resistant pathogens. Consequently, constitutive transgenic expression of genes encoding antimicrobial proteins with broad spectrum into commercial cultivars constitutes an interesting approach to reduce losses caused by pathogens.

As a counter-defence, plants have evolved some potent defense mechanisms, including the synthesis of low-molecular weight peptides with antifungal activity (Selitrennikoff, 2001). Ribosome-inactivating proteins (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 does not act on plant ribosomes but can affect foreign ribosomes, such as those of fungi (Stirpe et al., 1992; Hartley et al., 1996).

Some transgenic plants harboring RIP genes have already been produced (Jach et al., 1995; Bieri et al., 2000). Expression of barley seed RIP reduced development of *R. solani* in transgenic tobacco (Logeman et al., 1992) but had little effect on *Blumeria graminis* in transgenic wheat (Bieri et al., 2000). In previous study, the RIP was targeted to the apoplastic space and may exhibit a lower activity against development of the intracellular haustoria of the mildew pathogen (Punja, 2001).

In the present paper, a transformation of the potato cultivar 'Desirée' is carried out for further study of the effectiveness of RIP gene in the defense process against fungal infection of *R. solani*.

2. MATERIALS AND METHODS

2.1. Plant material

Virus free potato tubers of the tetraploid cultivar 'Desirée' were used for transformation. Etiolated sprouts from these tubers were surface-disinfected and single node segments were excised and placed on MS medium (Murashige and Skoog, 1962) supplemented with 100 g·l⁻¹ of inositol, 30 g·l⁻¹ of sucrose, 0.7% (W/V) of 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 during a photoperiod of 16 h under a 3,000 lux light intensity.

2.2. Bacterial growth

The disarmed *Agrobacterium tumefaciens* strain LBA4404, harboring the pBIN19 (13758 bp) was used. The pBIN19, kindly provided by Dr. J.I. Ruiz de Galarreta (NEIKER, Vitoria), contains the *rip30* gene of 1031 bp coding for Ribosome-Inactivating Proteins of barley (*Hordeum vulgare* L.) (Logeman et al., 1992), driven by CFDV promoter (Randles et al., 1992) and containing the *nptII* gene, coding for Neomycin Phosphotransferase II, conferred kanamycin resistance, as a selectable marker of the transgenic plants (Figure 1).

Agrobacterium tumefaciens 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 50 mg·l⁻¹ of kanamycin,

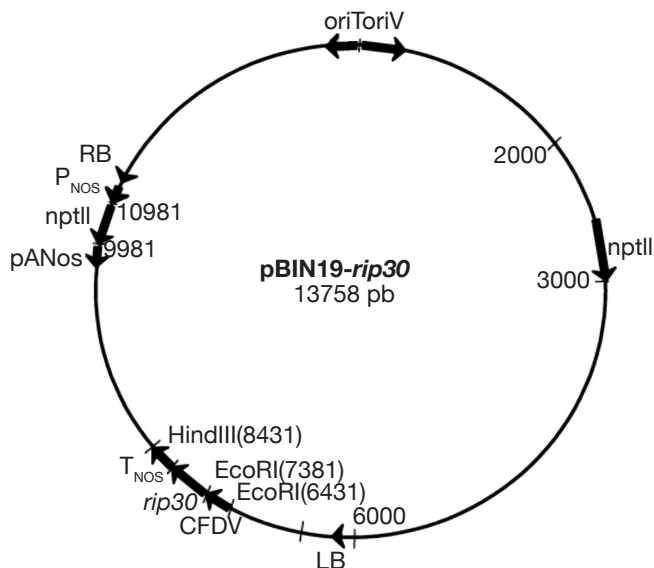


Figure 1. Schematic representation of T-DNA region of pBIN19 (NEIKER, Vitoria) with expected restriction sites — Représentation schématique de la région ADN-T de pBIN19 (NEIKER, Vitoria) avec les sites de restriction.

The binary vector pBIN19-RIP30 contains the kanamycin resistance gene (*nptII*), a Coconut Foliar Decay Virus (CFDV) promoter, and a barley cDNA *rip30*. P_{NOS} and T_{NOS} denote promoter and terminator of the nopaline production gene. RB and LB denote the right and left T-DNA border sequences — Le vecteur binaire pBIN19-RIP30 contient le gène de résistance au kanamycine (*nptII*), le promoteur CFDV (Coconut Foliar Decay Virus), et l'ADNc *rip30* de l'orge. P_{NOS} et T_{NOS} désignent respectivement promoteur et terminateur de la production du gène. RB et LB sont les bords droit et gauche de la séquence d'ADN-T.

100 mg·l⁻¹ of streptomycin and 25 mg·l⁻¹ of rifampycin. 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 a MS medium supplemented with 50 mg·l⁻¹ of sucrose, pH 5.6

2.3. Plant transformation

The leaf disc transformation method was used. Five mm leaf discs were cut from *in vitro* plants. Forty explants were used. The explants were infected with 30 ml of *A. tumefaciens* and placed at 25 °C in dark conditions for 1 h. Infected explants were co-cultivated, in the dark, for 2 days on a MS basal medium salts supplemented with 100 g·l⁻¹ of inositol, 30 g·l⁻¹ of sucrose, 0.7% (W/V) of agar, the pH was adjusted to 5.6 and without antibiotics to allow the bacteria to transform leaf cells. Subsequently, the leaf discs were washed with MS basal medium containing 500 mg·l⁻¹ of 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 of zeatin, 20 µg·l⁻¹ of gibberellic acid, 20 µg·l⁻¹ of α-naphthalene acetic acid (α-NAA), 500 mg·l⁻¹ of cefotaxime and 100 mg·l⁻¹ of kanamycin. They were maintained on this medium for 5 weeks at 23 °C during a photoperiod of 16 h for callus induction and shoot regeneration. The resulting shoots of 5 mm tall were regenerated during 4-5 weeks after transformation and were then individually transferred 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 evaluated: percentage of induced callus, percentage of regenerated calli, number of shoots per callus, percentage of regenerated roots and percentage of the transgenic plants.

2.4. PCR amplification

To detect *rip30* gene by PCR, genomic DNA was extracted from leaf material according to Edwards et al. (1991). DNA fragment, containing the *rip30* gene, of 500 bp was amplified by PCR using specific primers. The complete sequence of these primers is as follows:

rip30: 5'CAACCCGGCGCACTTCTC3',

reverse *rip30*: 5'GGCCTTCATCTCATTGCCG3'.

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 Agarose gel electrophoresis 1% (w/v).

2.5. Southern blot analysis

To detect the integration of *rip30* gene in transgenic plants, total DNA was extracted from young leaves. Ten µg of DNA from each sample were digested with EcoRI, separated on a 0.9% agarose gel, transferred to a nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech) and hybridized with the DIG-labeled probe of the *rip30* gene. The fluorescent DNA probe was isolated from the plasmid and digested by HindIII. 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).

2.6. Western blot analysis

To evaluate the expression of the integrated *rip30* gene in the genomic DNA of transgenic plants, the total soluble proteins were extracted from 300 mg of leaf tissue in 100 µg of loading buffer 3X (Laemmli, 1970). The homogenized samples were boiled for 3 min and extracts were centrifuged at 13,000 rpm for 2 min. Then 40 µl of soluble proteins were loaded onto 12% SDS-polyacrylamide gel (SDS-PAGE) according to Laemmli method (1970) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Amersham). Immunodetection was performed using polyclonal anti-serum raised in rabbit against the RIP30 protein and goat-anti-rabbit IgG (Amersham) conjugated to horseradish peroxidase (HRP) as secondary antibody.

2.7. Greenhouse assay of the transgenic plants inoculated with *R. solani*

Seven clones with confirmed insertion of the putative genes were chosen and micropropagated *in vitro*. After 4-5 weeks, five plants for each clone and control were transplanted into individual pots and placed in the greenhouse for further screening for resistance to the fungus *Rhizoctonia solani*. Strain Ci96 was provided by "Servicio de Semillas y Plantas de Vivero de Vitoria, Spain" and 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 inoculum of isolate Ci96 was grown in 250 ml flasks containing 100 g of barley and wheat grains, and 120 ml of distilled water. Each flask was inoculated with 5 plugs of 5 mm diameter of the fungus taken from the margins of a 1 week-old culture of isolate Ci96, grown on PDA medium. Flasks were incubated at 25 °C for 18 days. Inoculum was

added to pots filled with sterilized potting mixture as described by Chand et al. (1982).

After 4 months, the crop was harvested and tubers symptoms were assessed. The symptoms evaluation was the appearance of black or brown sclerotia on the tuber surface, using the ADAS scale (Anonymous, 1976), which ranges from 0 to 25% according to the infected surfaces covered by sclerotia.

For the statistical analysis, the ANOVA was performed to evaluate the significance of the differences in the resistance level among the clones. Means were separated using the LSD test ($P < 0.05$). The greenhouse assay was carried out three times into independent assays.

3. RESULTS

3.1. Transformation and characterization of transgenic plants

The results of transformation with the pBIN19 are indicated in **table 1**. Not all the plants growing on the kanamycin-containing medium were positive in the identification of the corresponding gene present on the T-DNA using the PCR technique (**Table 1**). PCR analysis was carried out for all kanamycin resistant plants. This analysis showed that only 47% of the plants, rooted in kanamycin medium, displayed a 500 bp amplification fragment for *rip30* gene. The specific primers did not amplify the corresponding fragments in the untransformed samples (**Figure 2a**).

Southern blot analysis showed that the *rip30* gene was integrated into the genome of the tested transformed plants. In these transformants, three hybridizing bands were detected (**Figure 2b**). Southern blot is given for two selected positive PCR clone, T1 and T2, hybridized with the probes from *rip30*; a three bands hybridization is obtained, indicating that there could be three copies of the *rip30* gene inserted for this transgenic clones. The size of the bands differed, indicating that these transformants have three copies of the *rip30* gene integrated at random sites in the genome. The pattern of integration was variable. No transgene insertion was detected in non-transformed controls.

The Western blot analysis demonstrated that the *rip30* gene is 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 (**Figure 2c**). The control plant non-transformed did not express the RIP30 proteins.

3.2. Resistance evaluation to the virulent isolate of *R. solani* in transgenic plants

The mean symptoms varied between the clones, carrying the *rip30* gene, and ranged from 1.12 to 3.09 (**Table 2**).

Tableau 2. Symptoms of the cv. ‘Desirée’ caused by *Rhizoctonia solani* in the transgenic clones expressing *rip30* gene, tested in greenhouse — *Les symptômes du cultivar ‘Desirée’ causés par Rhizoctonia solani chez les clones transgéniques exprimant le gène rip30, testés sous serre.*

Transgenic clones	¹ Mean symptoms
Control (non-transformed)	8.89 ^e ± 1.09
T1	3.09 ^d ± 0.28
T2	2.66 ^c ± 0.16
T3	2.37 ^b ± 0.17
T4	2.10 ^b ± 0.11
T5	1.42 ^a ± 0.05
T6	1.20 ^a ± 0.03
T7	1.12 ^a ± 0.08
LSD (0.05)	0.6

¹ Symptoms were assessed based on ADAS scale, assigning a value between 0 and 25% according to the infected surface covered by sclerotia. It presents the mean of 25 tubers for each transgenic clone and for the control. The results are the mean of three replicates conducted into three independent assays. Values followed by different letters are significantly different according to the Tukey’s b test ($\alpha \leq 0.05$) — *Les symptômes ont été évalués selon l’échelle ADAS, en attribuant une valeur comprise entre 0 et 25 % selon la surface du tubercule couverte par des sclérotés. Ces valeurs présentent la moyenne de 25 tubercules pour chaque clone transgénique et pour le contrôle. Les résultats sont la moyenne de trois répétitions effectuées dans trois essais indépendants. Les valeurs suivies de lettres différentes sont significativement différentes selon le test b de Tukey ($\alpha \leq 0,05$).*

Table 1. Regeneration from leaf disc explants of the cv. ‘Desirée’ transformed with *Agrobacterium tumefaciens* harboring the pBIN19 and identification of transgenic plants by PCR — *Régénération des explants de disque de feuille du cultivar ‘Desirée’ transformés par Agrobacterium tumefaciens contenant le plasmide pBIN19 et identification des plantes transgéniques par PCR.*

N° of explants	Callus induction (%)	Callus re-generants (%)	N° of calli regenerated	Shoots number per callus	Regenerated roots (%)	Transgenic plants (%)
40	90	70	25.2	8.2	70	47

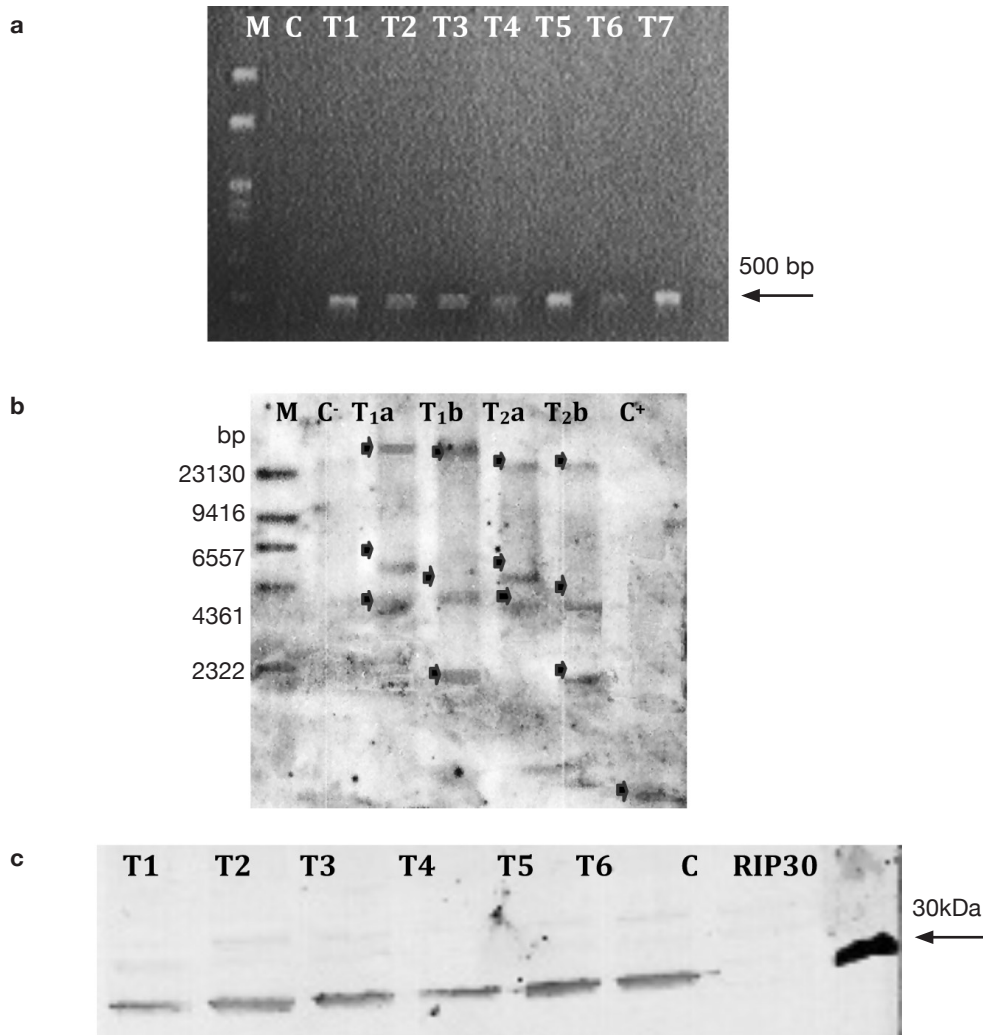


Figure 2.

a: confirmation of the integration of the *rip30* gene in transgenic plants of the cv. 'Desirée' by PCR analysis — *Confirmation de l'intégration du gène rip30 dans les plantes transgéniques du cultivar 'Desirée' par l'analyse PCR.*

M: size marker λ /EcoRI/HindIII — *taille de marqueur λ /EcoRI/HindIII*; C: non-transformed control — *témoin non transformé*; T1-T7: independent transgenic clones showing DNA fragment of *rip30* gene (500pb) — *clones transgéniques indépendants montrant un fragment (500pb) du gène rip30.*

b: Southern blot analysis of the cv. 'Desirée' — *Analyse par Southern blot du cv. Desirée.*

Genomic DNA from transgenic and non-transformed control potato plants was digested with EcoRI enzyme, and hybridized with a DIG-labeled Probe isolated from the plasmids and digested by HindIII — *l'ADN génomique des plantes transgéniques et des plantes non transformées a été digéré avec l'enzyme EcoRI et hybridé avec la sonde DIG-labeled isolée à partir des plasmides et digérée par HindIII*; M: DIG-labeled molecular weight marker λ /HindIII — *marqueur de poids moléculaire λ /HindIII*; C: non-transformed control — *témoin non transformé*; C⁺: the plasmid pBIN19 DNA template as positive control — *le plasmide pBIN19 utilisé comme témoin positif*; T₁a and T₁b: transgenic clones transformed with the pBIN19 and genomic DNA digested with HindIII and EcoRI, respectively — *clones transgéniques transformés avec le vecteur pBIN19 et l'ADN génomique digéré avec HindIII et EcoRI respectivement*; T₂a and T₂b: transgenic clones transformed with the pBIN19 and digested with HindIII and EcoRI respectively — *clones transgéniques transformés avec le pBIN19 et l'ADN génomique digéré avec HindIII et EcoRI respectivement.*

c: Western blot analysis for the expression of RIP30 protein — *Analyse par Western blot de l'expression de la protéine RIP30.*

Protein extracts of young leaves from independent transgenic clones and non-transformed control, resolved in a 12% polyacrylamide gel and subjected to immunoblot analysis using a rabbit polyclonal antiserum — *l'extrait protéique des jeunes feuilles des clones transgéniques et du témoin non transformé, chargé sur gel de polyacrylamide 12 % et soumis à l'analyse immunologique en utilisant un anticorps polyclonal de lapin*; C: non-transformed control — *témoin non transformé*; T1-T5: transgenic independent clones — *clones transgéniques.*

All the tested clones showed lesser symptoms than the non-transgenic control (8.89). The clone T7 showed low symptoms (1.12) revealed by a very mild black scurf symptoms. Statistically, significant differences were observed in symptom severity among the clones, which showed a reduced severity of the symptoms caused by the pathogen. T5, T6 and T7 were the most resistant clones.

4. DISCUSSION

In this study the potato cultivar 'Desirée' was transformed under experimental conditions with *rip30* gene isolated from *Hordeum vulgare* L. The transformation was carried out via *A. tumefaciens*, which has been commonly used for potato transformation and plays a significant role in determining the efficiency of infection (Beaujean et al., 1998; Ducreux et al., 2005; Banerjee et al., 2006).

Not all the plants grown on selective medium with kanamycin were positive in PCR, as reported by Kumar et al. (1995) who observed many escapees despite the selective medium. This could be explained by the fact that some plants could integrate only the gene of resistance to the antibiotic, as the transfer of T-DNA occurs from the right to the left border (Wang et al., 1984). Indeed, in the binary vectors that we used, the *nptII* gene conferring resistance to kanamycin is nearest the right border than *rip30* gene.

The transgenic clones (positive in PCR) were confirmed by Southern blot analysis. This showed integration of three copies of the *rip30* gene into their genome. In previous reports it was shown that the variation in the number of copies is not correlated with the differences in expression level and it is due to the effect of the T-DNA position in different localization of the genome (Deblaere et al., 1987).

The ability of the introduced *rip30* gene to enhance resistance in the transgenic potato plants was studied in greenhouse conditions. The transgenic clones expressing the *rip30* gene were able to reduce symptom severity of *R. solani* in an *in vivo* assay.

These transgenic clones were also evaluated for resistance to *Phytophthora infestans* and no differences were found between the transgenic plants expressing the *rip30* gene and the untransformed control (data not shown). These transgenic clones failed to reduce incidence and severity of *P. infestans*. These results confirmed that the enzyme used in our experiments did not induce resistance to several fungi, and showed a narrow spectrum of antifungal activity. This could be explained by a weak antifungal activity of this enzyme (Lorito et al., 1998).

The research presented here demonstrates a successful transformation of potato plants and the

results revealed that the *rip30* gene was successfully integrated into the genome of transgenic potato and expressed by producing the RIP30 protein. The transgenic clones obtained reduced incidence and severity of *R. solani* but failed to enhance resistance to *P. infestans*.

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