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The use of *pac*1 gene from *Schizosaccaromyces pombe* to protect potato from *Potato virus Y* (PVY) and *Potato virus X* (PVX) infections

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Summary. The effect that *pac*1, a yeast-derived ds-RNA specific RNase gene, has on *Potato virus X* (PVX) and on *Potato virus Y* (PVY) infections in potato was investigated. Tuber disks of cv. Desirée were transformed with LB4404 *Agrobacterium tumefaciens* carrying a modified PBI121 binary vector in which the transgene replaced GUS. Transformed plantlets were tested for possible acquired resistance by inoculation with purified virus suspensions at different concentrations and were ELISA-tested for virus content 40 days later. Plants of two clones had a significantly lower PVX content (66 to 75% that of the control, respectively), when inoculated with a 1 μg ml^{-1} suspension of the virus. One of these clones showed also a reduced PVY accumulation (41% that of the control), when inoculated with a 10 μg ml^{-1} suspension of this virus. Results demonstrated the practical utility of the *pac*1 gene for countering potato virus infections.

Key words: dsRNA-specific RNases, virus resistance.

Introduction

Control of virus infections has been successfully achieved by transferring virus genes (e.g. coat protein [CP] or replicase) into plants, which then acquire pathogen-derived resistance (PDR) (Goldman *et al*., 2003). In such cases, however, the resistance acquired is specific to the parent virus or to closely related virus strains, so that if protection against different viruses is required several transgenes must be used. Another possible drawback of this technique is that it introduces viral sequences into the plant genome, which may lead to heterologous encapsidation, i.e. coating the infecting virus RNA with the transgene product, and thus modifying virus spread and epidemiology (e.g. aphid transmissibility). More recently, non-PDR genes have also been used against viral infections. By affecting specific steps of virus replication, these genes can potentially counter virus replication in general. Among these genes, *pac*1, a type III ds-RNA specific RNase gene derived from *Schyzosaccaromyces pombe* (Iino, 1991; Rotondo and Frendedway, 1996), which degrades ds-replicative forms of ss-RNA viruses both *in vitro* (Ishida *et al*., 1995), and *in vivo*, and which is thought not to interfere with normal plant metabolism. Watanabe *et al*. (1995), for example, reported that the leaves of *pac*1-transgenic tobacco plants showed fewer le-

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sions when challenged with *Tomato mosaic virus* (ToMV), and that tobacco inoculated with *Cucumber mosaic virus* (CMV) and *Potato virus Y* (PVY) showed delayed symptom appearance and lower concentrations of these viruses. Since PVY is the most important virus affecting *Solanum tuberosum* L., and *Potato virus X* (PVX) is also widespread in Italy (Cerato *et al.,* 2002), it was decided to transform potato cv. Desirée with the *pac*1 transgene to find out how it influenced virus replications and hence plant resistance to PVX and Y infection.

Materials and methods

Plant material and virus purification

Tobacco plants (*Nicotiana tabacum* L., cv. Samsun) and potato plants (*Solanum tuberosum* L. cv. Desirée) were grown in a climatised greenhouse at 22–23°C under natural light supplemented with artificial illumination (3 Klux, Fluora 77R fluorescent tubes). Tobacco plants were mechanically inoculated at the four-leaf stage either with a type X3 strain of PVX or a type N strain of PVY isolated from potato crops growing near Budrio (Bologna, Italy). Systemically infected leaves were harvested 20 days (PVX) or 1 month (PVY) later and stored at -25°C for 10–20 days before purification. PVX was purified from tobacco leaves as described by Goodman (1975) and the virus content of the purified suspensions was evaluated spectrophotometrically using a molar extinction coefficient (α0.1% at 260 nm) of 2.98. PVY was purified accordingly to Leiser and Richter (1978) and its concentration evaluated using a molar extinction coefficient of 2.3.

Gene cloning and plant transformation

*Pac*1 gene from *S. pombe* (Accession No. S78982, Rotondo *et al*., 1995), cloned from strain 2740 and inserted in the binary vector PBI 121 was kindly furnished by D. Gallitelli (University of Bari, Italy).

The recombinant plasmid (PBI121-pac1) was multiplied in the DH5α strain of *Escherichia coli* and mobilised into the LBA4404 strain of *Agrobacterium tumefaciens* by triparental mating (Ditta *et al.,* 1980), using plasmid PRK 2013 in *E. coli* HB 101 as helper. Recombinant colonies selected on LB plates containing 100 mg l^{-1} kanamycin and 100 mg l^{-1} rifampicin were grown on a rotatory shaker at 28°C in LB liquid medium containing the same level of antibiotics, until the bacterial concentration reached 0.2–0.3 OD (600 nm wavelength). Tuber discs (*Solanum tuberosum*, cv. Desirèe), 1 cm in diameter and 2 to 3 mm thick, were sampled from surface-sterilised tubers with a cork borer and floated on the bacterial suspension for 5 min. Discs were blotted on sterile filter paper and placed on sterile growth medium 3C5ZR (Sheerman *et al*., 1988): 4.3 g l^{-1} MS salts; 30 g l^{-1} sucrose; 8 g l^{-1} agar; 1 mg l^{-1} thiamine-HCl; 0.5 mg l^{-1} nicotinic acid; 0.5 mg l^{-1} pyridoxin-HCl; 1.8 mg l^{-1} zeatine riboside; 0.5 mg l^{-1} indolacetic acid (IAA); 0.4 mg l^{-1} aspartic acid, adjusted to pH 5.8 with KOH for 2 days under constant lighting (3 Klux, Fluora 77R) according to Topping (1998). Explants were transferred to the same growth medium, to which 100 mg l^{-1} kanamicin and 500 mg l^{-1} carbenicillin were added, and maintained at 26°C with a 16 h day. When developing shoots were about 1 cm long, they were transferred to Magenta boxes on a rooting medium (MS 30): 4.3 g l^{-1} MS salts; 30 g l^{-1} sucrose; 8 g l^{-1} agar with 1 mg l^{-1} IAA. Rooted plantlets at the six-leaf stage were transferred to pots containing a 1:1:1 sterilised mixture of soil, pit and sand, and the pots were covered with polyethylene bags for one week to prevent dehydration.

Nucleic acid extraction

DNA was extracted from potato leaf tissues according to McGarvey and Kaper (1991). Fifty to 100 mg of tissue were ground with liquid nitrogen in a mortar, vortexed with 700 μl extraction buffer (50 mM Tris-HCl pH 8.0, 700 mM NaCl, 10 mM EDTA, 0.5% PVP, 0.1% β-mercaptoethanol, 1% CTAB), incubated at 60°C for 45 min, extracted with 1 v chloroform/isoamylic alcohol (24:1), and centrifuged at 15,000 *g* for 5 min. The aqueous phase was treated with 1 v isopropanol and 0.1 v 3M NH_4-acetate and kept for 60 min at -20°C. After centrifugation as above, pellets were washed with 70% ethanol, briefly vacuum-dried, and resuspended in 25 μl TE.

Total RNA extraction, according to Rubino *et al*. (1992) was from 200 mg of leaf tissue which were ground in a mortar with liquid nitrogen, and the powdered tissue was homogenised with 3 ml buffer (0.1 M glycine-OH pH 9.0, containing 100 mM NaCl, 10 mM EDTA, 2% SDS, 1% sodium lauroylsarcosine). An equal volume of phenol-chloroform (1:1, v:v) was added and, after a low speed centrif-

ugation, the aqueous phase was precipitated with ethanol and resuspended in 50 μl of DEPC- treated water.

PCR amplification and Southern blot hybridisation

DNA (0.5 μg) extracted from transgenic plants was used for PCR in a Perkin-Elmer Cetus Thermal Cycler for 35 cycles adding 2.5 μl of 10× PCR buffer (Boehringer, Mannheim, Germany); 0.1 mM dNTPs; 0.2 μM of each primer of the *pac*1 gene terminal regions: 5'AAAGGATCCATGGGACGGTTTAAGAGGC3' (homologous) and 5'AAAGAGCTCTTAACGGGCAAACTTAGAG3' (complementary), and 1.0 U of Taq DNA polymerase in a total volume of 25 l. Melting, annealing and polymerisation steps were at 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min, with a final extension step of 10 min at 72°C. PCR products were electrophoresed on 1% agarose gel, amplicons were blotted on a nylon membrane (Hybond N+, Amersham Bioscience, Little Chalfont, UK) by capillary transfer (Sambrook and Russell, 2001) and fixed by baking for 2 h at 80°C. DNAs blots were hybridised overnight at 65°C with a *pac*1 digoxigenin probe prepared with Klenow polymerase and DIG DNA labeling mix, according to manufacturer's instructions (Roche, Indianapolis, IN, USA) in a 20 μl reaction. Chemiluminescent detection was according to manufacturer's instructions (BM Chemiluminescence Blotting Substrate, POD, Roche) on Kodak autoradiograph film (Biomax ML, Kodak, Rochester, NY, USA) using a GBX developer and fixer from the same manufacturer.

Northern blot hybridisation

Ten micrograms of total RNA from transgenic plants were mixed with 2.6 vol of denaturing mixtures (7% MOPS 10×, 24% formaldehyde, 69% formamide) and electrophoresed on formaldehyde-permeated, 1.4% agarose gels (Sambrook *et al*., 2001). Northern blots were carried out as above and hybridisation was with a PAC1-^{32}P probe labelled with a "megaprime DNA labelling system" kit (Amersham biosciences) according to Sambrook *et al*. (2001).

Reverse transcription

One microgram of total RNA was treated with 1 U DNase I (Amplif. grade Sigma) in 10 μl total volume containing 1 μl DNase 10× buffer, for 10 min at room temperature. The reaction was stopped with a final 5 mM EDTA solution at 70°C for 10 min. Reverse transcription was carried out using a First strand cDNA synthesis kit for RT-PCR (AMV; Roche), using 1× reaction buffer, 5 mM $MgCl_2$, 1 mM dNTP mix, 2 μl random primers (pdN 6), 50 U RNase inhibitor, 0.8 μg total RNA and 20 U AMV reverse transcriptase in a total volume of 20 μl. The mixture was incubated at 25°C for 10 min, then at 42°C for 1 h. After denaturation at 99°C for 5 min and cooling to 4°C, 5 μl of the mixture was PCR-amplified and electrophoresed in agarose gel.

Plant inoculation and assessment of virus concentration

Forty transgenic plants of each of the two selected clones and 40 control plants were produced by stem cutting propagation. Groups of 20 transformed and 20 untransformed plants were mechanically inoculated at the 12-leaf stage with 2 different concentrations (1 and 0.5 μg ml^{-1}) of PVX purified preparations in 50 mM, pH 7.6 Na-phosphate buffer. An equal number of plants was inoculated with a purified PVY (N) suspension (10 μg ml^{-1}) in 100 mM citrate buffer pH 7.4.

Forty days later the virus content of the four upper expanded leaves of each plant was estimated by DAS-ELISA (Clarks and Adams, 1977) using an anti-PVX polyclonal antiserum and anti-PVY monoclonal antibodies (Bioreba AG, Reinach Switzerland).

The A_{405}nm absorbance values were determined with a Biorad 450 micro plate reader and the mean absorptions of the various lots and their standard errors were statistically worked out. Significant differences between the various lots were calculated with Student's "t" test and the virus concentration was calculated by the correlation between the A_{405} reading and the virus content of purified suspensions of known viral titre. The data were processed using MacIntosh 1.3 Cricket graph software, obtaining for PVX the expression:

$$y= -1.5947+1.0357 \cdot \log(x)$$

and for PVY the expression:

$$y= -2.1968+ 1.050 \cdot \log(x).$$

where y represents the A_{405} reading, and x the virus amount.

Results and discussion

From tuber disks agroinfected with LBA 4404 (carrying PBI121-*pac*1) and grown on kanamicin, 22 shoots were obtained, and 10 derived plantlets were initially selected. The presence of transgenic DNA in PCR-amplified extracts of two transformed plants was clearly visible in the electropherograms as a 1.1 kb band, which was absent in the amplification products of non-transformed plants (Fig. 1A). Positive hybridisation of this band with the digoxigenin-*pac*1 probe confirmed transgene integration in the genome of transformed plants (Fig. 1B). A band of the same size was also present in electropherograms of RT-PCR products of both clones, indicating that the transgene was inserted into a transcriptional-active locus. Northern blot hybridisation of total plant RNAs with the specific ^{32}P-*pac*1 probe showed the presence of a *pac*1 mRNA related band with electrophoretic mobility of about 1.1 kb (data not shown). Hybridisation signals were detectable in both transformed clones (Fig. 3).

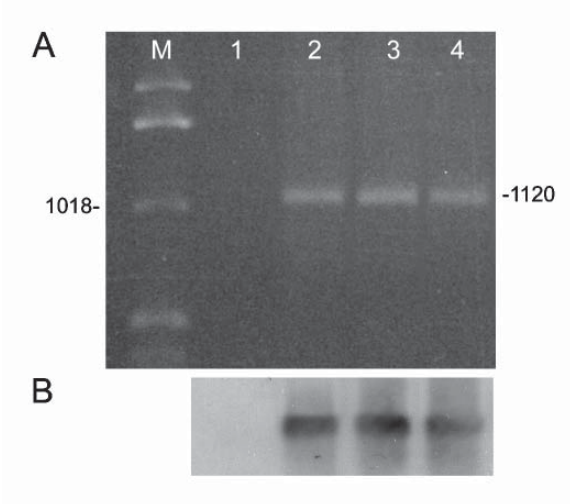

Fig 1. Identification of *pac*1 gene in genomic DNA of potato transformed plants (cv. Desirée). A, 1.2% agarose gel electrophoresis of PCR products: M, DNA molecular weight marker; 1 untransformed potato; 2, 3 potato transformed clones (A and B, respectively); 4 positive control (pBI121-*pac*1). B, hybridization of the above PCR bands with the specific digoxigenin-labelled *pac*1 probe.

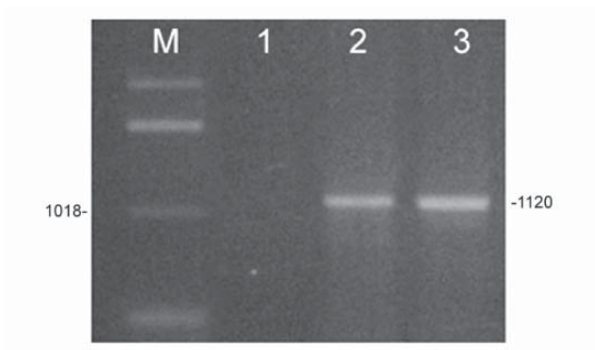

Fig. 2. Agarose gel electrophoresis of RT-PCR products of *pac*1 mRNA isolated from transgenic potato plants: M, DNA molecular weight marker; 1 untransformed potato; 2, 3 transformed clones (A and B, respectively).

ELISA tests carried out on *pac*1 plants 40 days after inoculation, revealed significantly lower amounts of the virus (to 66 and 75% of the control for clones A and B respectively), when plants were inoculated with a 1 μg ml^{-1} PVX purified suspension (Table 1). By contrast, *pac*1-transformed plants did not show any changes in virus concentration when challenged with a 0.5 μg ml^{-1} PVX purified suspension, probably because of insufficient inoculum pressure.

Even better results were obtained against PVY infection, since plants of clone A, inoculated with a 10 μg ml^{-1} suspension of the virus had a much lower virus content (41.3% of the control (Table 1).

These results demonstrate the effectiveness of

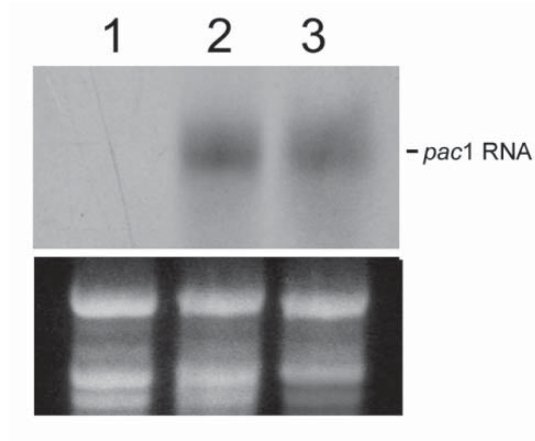

Fig. 3. Northern blot hybridization of total RNA (10 μg) from potato leaf tissues with the specific *pac*1 ^{32}P-labelled probe: 1, untransformed potato; 2, 3 transformed potato clones A and B respectively. Lower panel represents ethidium bromide-stained rRNA as loading control.

Table 1. Virus content of two clones of potato plants transformed with the *pac*1 gene (A and B) and one non-transformed clone 40 days after inoculation with three concentrations of PVX and PVY suspensions, as determined by ELISA tests.

Inoculum concentration		Plant[a]	OD_{405} (M^Em)	Virus amount	
				ng[b]	%
PVX	1 μg ml^{-1}	Transformed A	0.919±0.101[c]	53.5	65
		Transformed B	0.973±0.090[d]	60.3	73
		Non-transformed	1.115±0.073	82.7	100
PVX	0.5 μg ml^{-1}	Transformed A	0.542±0.035	23.1	76.5
		Transformed B	0.579±0.039	25.1	83.1
		Non-transformed	0.662±0.068	30.2	100
PVY	10 μg ml^{-1}	Transformed A	1.021±0.046[c]	232	41.2
		Transformed B	1.520±0.015	693	-
		Nontrasformed	1.425±0.017	563	100

[a] At each concentration 20 transformed and 20 non transformed plants were used.
[b] per g of fresh leaf tissue.
[c] P=0.01
[d] P=0.05

gene coding for a type III yeast ds-RNase in controlling infections by two unrelated RNA viruses in engineered potatoes. The same transgene was also reported to be active in potato against *Potato spindle tuber viroid* (PSTV), of which it reduced the concentration and extent of tuber transmission (Sano *et al*., 1997).

However, in preliminary experiments no positive results were obtained against PVX or PVY (data not shown) when the same transgene was introduced into tobacco plants (cv. Samsun and White Burley). These findings are not in agreement with those by Watanabe *et al*. (1995), who reported a delay in symptom appearance (vein necrosis) and reduced virus accumulation in cv. Xanthi-nc tobacco plants transformed with the transgene in question, and challenged with an unknown concentration of PVY. The effect of other viruses, such as *Cucumber mosaic virus* (CMV), on transgenic tobacco plants is variously reported by different authors. Tepfer did not find any effect on virus replication (personal communication), whereas Watanabe *et al*. (1995) reported that it caused a decrease of virus concentration in plants. These discrepancies may be due to the fact that RNase III type enzymes may also have a role in the host metabolism as suppressors of post transcriptional gene silencing (PTGS) (Lichner *et al*., 2003). It has also been found that the *pac*1 transgene can directly affect plant metabolism, e.g. by silencing an endogenous host nitrate reductase gene (Berthomé *et al*., 2000), thus possibly influencing plant resistance. These multiple roles of *pac*1 protein, in viral dsRNA degradation, plant PTGS suppression and other plant metabolic processes, renders it difficult to elucidate the complex mechanism of action of this protein also in relation to plant species.

Other dsRNA-specific RNases have been used to control virus infections. Langenberg *et al*., (1997), for example, reported induced resistance against viruses with divided genome, but not against the single-genome viruses *Tobacco etch* (TEV) and *Tobacco mosaic* (TMV) viruses, using a bacterial type III RNase gene (*rnc*). Even here, however, interference with some plant phenotypes was observed, in the form of stunting and chlorosis, suggesting the possibility of multiple, unpredictable effects on plant metabolism. It therefore seems that the practical use of this type of genes is limited by various factors, including the plant species to be protected, and needs to be determined on a case-by-case basis until its biological effects are better understood.

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Literature cited

Berthomè R., P.Y. Teycheney, J.P. Renou, Y. Okada and M. Tepfer, 2000. Expression of a yeast RNase III gene in transgenic tobacco silences host nitrite reductase genes. *Plant Molecular Biology* 44, 53–60.

Cerato C., R. Ghedini and P.L. Burzi, 2002. Controllo delle virosi e mantenimento della sanità dei cloni di patata nel miglioramento genetico. *Rivista di Agronomia* 36, 66–68.

Clarks M.F. and A.N. Adams, 1977. Characteristic of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34, 475–483.

Ditta G., S. Stanfield, D. Colbin and D.R. Helinski, 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proceeding of the National Academy of Science* 77, 7347–7351.

Goldbach R., E. Bucher and M. Prins, 2003. Resistance mechanism to plant viruses: an overview. *Virus Research* 92, 207–212.

Goodman R.C., 1975. Reconstitution of Potato Virus X. *Virology* 68, 287–298.

Iino Y., A. Sugimoto and M. Yamamoto, 1991. *S. pombe* pac1, whose overexpression inhibits sexual development, encodes a ribonuclease III-like RNase. *EMBO Journal* 10, 221–226.

Ishida I., T. Ogawa, M. Yoshioca, T. Hori and T. Ohtani, 1995. Production of plants tolerant to multiple viral infections by genetic manipulations. *Acta Horticulturae* 420, 52–57.

Langerberg W.G., L. Zhang, D.L. Court, L. Giunchedi and A. Mitra, 1997. Transgenic tobacco plants expressing the bacterial *rnc* gene resist virus infection. *Molecular Breeding* 3, 391–399.

Leiser R.M. and J. Richter, 1978. Reiningung und elnige Eigenschafteen des Kartoffel-Y-Virus. *Archive Phytopathologische Pflanzenschutz* 14, 337.

Lichner Z., D. Silhavy and J. Burgyan, 2003. Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defences. *Journal of General Virology* 84, 975–980.

McGarvey P. and J.M. Kaper, 1991. A simple and rapid method for screening transgenic plants using the PCR. *Biotechniques* 4, 28–32.

Rotondo G., M. Gillespie and D. Frendewey, 1995. Rescue of the fission yeast snRNA synthesis mutant *snm1* by overexpression of the double-strand-specific Pac1 ribonuclease. *Molecular General Genetics* 247, 698–708.

Rotondo G. and D. Frendewey, 1996. Purification and characterisation of the Pac1 ribonuclease of *Schizosaccharomyces pombe*. *Nucleic Acids Research* 24, 2377–2386.

Rubino L., J. Carrington and M. Russo, 1992. Biologically active Cymbidium Ringspot Virus satellite RNA in transgenic plants suppressed accumulation of diDNA. *Virology* 188, 429–437.

Sambrook J. and D.W. Russell, 2001. *Molecular Cloning: a Laboratory Manual,* 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbour, New York, NY, USA.

Sano T., A. Nagayama, T. Ogawa, I. Ispida and Y. Okada, 1997. Transgenic potato expressing a double-stranded RNA-specific ribonuclease is resistant to potato spindle tuber viroid. *Nature Biotechnology* 15, 1290–1294.

Sheerman S. and Bevan M.W., 1988. A rapid transformation method for *Solanum tuberosum* using binary *A. tumefaciens* vectors. *Plant Cell Reports* 7, 13–16.

Topping J.F., 1998. Tobacco transformation. *Methods in Molecular Biology* 81, 365–372.

Watanabe Y., T. Ogawa, H. Takahashi, I. Ishida, Y. Tajkeuchi, M. Yamamoto and Y. Okada, 1995. Resistance against multiple plant viruses in plants mediated by a double stranded- RNA specific ribonuclease. *FEBS Letters* 372, 165–168.

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