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Influence of a mammalian 2′-5′A oligoadenylate synthetase transgene on tobacco resistance to *Potato virus X* infection

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SUMMARY. The effect of human 2'-5' A oligoadenylate synthethase transgene against *Potato virus X* (PVX) infection in tobacco plants was studied. To the purpose, leaf tissues of cv. Samsun were transformed via *Agrobacterium tumefaciens* LB 4404 carrying a modified PBI121 binary vector deprived of GUS and containing the transgene. Seventy-five percent of R1 derived seeds germinated on kanamycin selective medium, indicating the transgene integration in a single locus. The virus resistance of R1 plantlets was tested by mechanical inoculation with purified suspension of different virus concentration (1, 0.1, and 0.05 mg ml^{-1}). Only plants inoculated with the lowest PVX concentration, showed a reduced virus content (43% that of controls) 20 days after inoculation. This result renders the practical utility of the gene doubtful.

Key words: transgenic plants, resistance, 2'-5'A synthetase.

Introduction

The presence in plants of an antiviral mechanism similar to that of interferon (IFN) 2'-5'An pathway has been hypothesized, since antiviral factors (AVFs), the production of which was stimulated or inhibited by chemicals active also on interferon production in mammalian tissues, were isolated from various virus-infected plants (Faccioli and Capponi, 1983; Faccioli *et al.*,1994). This hypothesis was also supported by the fact that an ATP-polymerizing activity in antivirally active compounds was detected (Faccioli *et al.*, 1994) and that treatments with either ppp2'-5' A3 or 2'-5' A (Faccioli *et al.*,1994) or human interferon-ß (Faccioli and Raiola, 1997), considerably reduced virus replication in plants. However, hybridization experiments with 2'-5' synthetase cDNA probes from humans (clone E18, Benech *et al.*,1985) or rats (clone L3, Coccia *et al.*,1990) have failed to detect 2'-5' synthetase activity in plants (G. Faccioli, unpublished data).

Truve *et al.* (1994) did not detect any 2'-5' A-synthetase activity in healthy or virus-infected plants, but in the latter they did find an activity resembling that of 2'-5'A-dependent ribonuclease. Thus, tobacco and potato plants were transformed with a rat 2'-5'A transgene, obtaining resistance to *Potato virus S* (PVS) and reducing replication of *Potato virus X* (PVX) in tobacco (Truve *et al.*, 1994). However, Ishida *et al.,* (1995) did not find any effect against *Tobacco mosaic virus* (TMV) in tobacco transformed with a human 2'-5'A synthetase gene and other researchers (Ogawa *et al.*, 1996; Mitra *et al.*, 1996) obtained positive results only

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when plants were transformed with both the L RNase gene and the human 2'-5'A synthetase gene, indicating that the two enzymes which are fundamental for the IFN antiviral response in mammals are also necessary for eliciting this type of response in plants.

In the above studies, inoculation tests for checking resistance were done either with virus-infected sap (Mitra *et al*., 1996; Ogawa *et al*.,1996) or with a single virus concentration (Truve *et al*., 1993; 1994). This prompted us to better assess the effect of 2'-5'A oligoadenylate synthetase (2'-5' A-S) gene on PVX infection in transformed tobacco plants by using purified virus suspensions at different concentrations.

Materials and methods

Plant material and virus purification.

Tobacco plants (*Nicotiana tabacum* L., cv. Samsun) were grown in a climatized greenhouse at 22–23°C under natural light supplemented with artificial illumination (3 Klux, Fluora 77R fluorescent tubes). Plants were mechanically inoculated at the four leaf stage with a type X3 strain of PVX isolated from a potato crop near Budrio (Bologna, Italy). Systemically infected leaves were harvested 1 month 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 purified suspensions was evaluated spectrophotometrically using a molar extinction coefficient ($\alpha^{0.1\%}$ at 260 nm) of 2.98.

Gene cloning and plant transformation.

The human 2'-5'A-S gene (clone E18, 48-1; Benech *et al*., 1985) was obtained from a PGEM3 construct (kindly furnished by J. Chebath, Rehovot, Israel) by *EcoR*I overnight digestion at 37°C and made blunt-ended with Klenow polymerase (Sambrook and Russell, 2001). The gene was inserted in the vector PBI221 (Clontech, Palo Alto, CA, USA) deprived of GUS by Sma/ Sac I digestion and made blunt-ended. Proper orientation of the gene with respect to the CaMV 35S promoter was checked by PCR using the primer complementary to the 3' region of the gene and the primer homologous to the 5' region of the promoter, obtaining a product of the expected size (1524 bp). After amplification in *E. coli* NM522 (Pharmacia, Upsala, Sweden), the cassette containing the 2'-5'A-S gene and the Nos terminator was excised from PBI 221 by *Xba* / *EcoR*I digestion and inserted into the corresponding enzymatic sites of the binary vector PBI 121 (Clontech) deprived of the GUS gene. Recombinant plasmid (PBI121-E18) was multiplied in the DH 5a strain of *E. coli* and mobilized into the LB4404 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 1 OD (600 nm wavelenght).

Leaf pieces (1 cm^2) of aseptically grown Samsun tobacco plantlets were floated for 1 min on the bacterial suspension, blotted on sterile filter paper, and placed on a sterile growth medium (4.4 g l^{-1} MS salts, 30 g l^{-1} sucrose, 1 mg l^{-1} benzylaminopurine [BAP] 8 g l^{-1} Phytagar) and 10 ml of a vitamin 100× stock solution (5 mg of nicotinic acid, 5 mg of pyridoxin, 1 mg of thiamine-HCl, 2 mg of glycine in 100 m) adjusted to pH 5.8 with KOH, for 2 days under constant light (3 Klux, Fluora 77R) according to Topping (1998). Explants were then transferred to the same medium, to which 100 mg l^{-1} kanamicin and 500 mg l^{-1} carbenicillin were added, and kept at 26°C with a 16 h light photoperiod. When developing shoots were about 1 cm long, they were transferred to Magenta boxes on a rooting medium where BAP was replaced by 0.02 mg l^{-1} naphtalen acetic acid (NAA) and 1 mg l^{-1} indolacetic acid (IAA). Rooted plantlets at the four-leaf stage were transferred to pots containing a 1:1:1 sterilized mixture of soil, pit and sand, and pots were covered with polyethylene bags for one week to prevent dehydration.

Nucleic acid extraction

DNA was extracted from leaf tissues according to McGarvey and Kaper (1991). Fifty mg of tissue were ground with liquid nitrogen in a mortar, vortexed with 700 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 700 mM NaCl, 10 mM EDTA, O.5% PVP, 0.1% ß-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 acqueous phase was treat-

ed with 1 v isopropanol and 0.1 v 3 M 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 ml TE.

Total RNA extraction for RT-PCR was from 100 mg of fresh tobacco leaf tissue ground in a mortar with liquid nitrogen and the powdered tissue was homogenized with 500 µl of Tri reagent solution (Sigma, St. Louis, MO, USA). One hundred ml of chloroform was added to the slurry, which was left on ice for 10 min and centrifuged at 12.000 *g* for 15 min at 4°C. Isopropanol was added to the acqueous phase (1:1 v), which was left overnight at -20°C. The RNA was then pelleted by centrifuging at 12.000 *g* for 5 min, washed with 70% ethanol, quickly dried, and resuspended in 25 µl of DEPC-treated sterile water.

PCR amplification and Southern blot hybridization

DNA (0.5 mg) extracted from transgenic plants was used for PCR in a Perkin-Elmer Cetus Thermal Cycler for 35 cycles adding 2.5 ml of 10× PCR buffer (Boehringer, Mannheim, Germany); 0.1 mM dNTPs; 0.2 mM of each primer of the E18 gene terminal regions: (nt25) 5'CTCCTGTCAATGATGGATCTC3' (homologous) and 5'5'TAGACATTACCCTCCCATCAG3' (nt1548) (complementary), and 1.0 U of Taq DNA polymerase in a total volume of 25 ml. Melting, annealing and polymerization steps were at 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min respectively, with a final extension step of 10 min at 72°C. PCR products were electrophoresed on 1.4% agarose gel, amplicons were blotted on a nylon membrane (Hybond N+ Amersham, Little Chalfont, UK) by capillary transfer (Sambrook and Russell, 2001) and fixed by baking for 2 h at 80°C. DNAs blots were hybridized overnight at 65°C with a 2'-5'A synthetase digoxigenin probe obtained according to Feinberg and Vogelstein (1983), using Klenow polymerase and DIG DNA labeling mix (Roche) in a 20 ml reaction. Chemiluminescent detection was according to manufacturer's instructions (BM Chemiluminescence Blotting Substrate; POD; Roche, Indianapolis, IN, USA) on Kodak autoradiograph film (Biomax ML) using GBX developer and fixer from the same supplier.

RT-PCR

Before reverse transcription, 1 mg of total RNA was treated with 1 U DNase I (Amplif. grade Sigma) in 10 ml total volume containing 1 ml 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 mg 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 room temperature, 5 µl of the mixture was PCR-amplified and electrophoresed in agarose gel.

Plant inoculation and assessment of virus concentration

Sixty transgenic plants of one clone and 60 control plants were divided into 3 lots of 20 plants each, and inoculated at the four-leaf stage on two fully expanded leaves with three different concentrations of purified PVX preparations in 0.1 M, pH 7.0, Na-phosphate buffer (1, 0.1, and 0.01 mg ml^{-1}). Twenty days later the virus content of three upper expanded leaves of each plant was assayed by DAS-ELISA (Clarks and Adams, 1977) determining A_{405nm} absorbance values with a Biorad 450 microplate reader. The mean absorptions of the various lots and their standard errors were statistically worked out. Significant differences among lots were calculated with Student's T test.

Virus concentration was calculated by the correlation between A_{405} readings and the virus content of purified suspensions of known viral titre and the data were processed using MacIntosh 1.3 Cricket graph software, obtaining the expression:

$$y = -0.84824 + 1.0540 \log (x)$$

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

Results and Discussion

From leaf disks agroinfected with LB 4404 (carrying PBI121-E18) and grown on kanamicin, 30 shoots were obtained and 10 derived plantlets were chosen to verify the presence of the transgene by PCR amplification. Five positive plants were transplanted to pots for seed production and about 130 R_1 seeds per line were germinated on MS medium

containing 200 mg l^{-1} kanamicin. About 75% of the seeds produced viable roots, giving a 3:1 segregation indicative of transgene integration in a single locus. DNA extracted from 5 R_1 plantlets, when PCR-amplified as before, showed permanent transgene integration in the genome. A band of 1.524 kb, corresponding to that of the transgene, was clearly visible in electropherograms of all transformed clones, but not in the controls (Fig. 1A). The positive hybridization with the 2'-5'A-S probe further confirmed the transgene presence in transformed plants (Fig. 1B).

PCR amplifications of reverse-transcribed plant total RNAs clearly revealed a 1.524 kb band in all clones tested, corresponding to 2'-5'A-S transcripts (Fig. 2), and indicating that the relative gene was inserted into a transcriptional-active *locus*. Since 2'-5'A-S integration and expression in the 5 transformed plant lines was very similar, 60 R_2 plants of one line were used to test PVX resistance by inoculation with purified virus preparations at different concentrations.

ELISA tests carried out 20 days post inoculation showed no significant differences in virus content among plants inoculated with the two most concentrated virus inocula (1 and 0.1 μg ml^{-1} of PVX). Only transformed plants inoculated with 0.05 μg ml^{-1} showed a reduced PVX content: 43% that of controls (Table 1). Symptom expression did not differ between the two types of plants. Only a

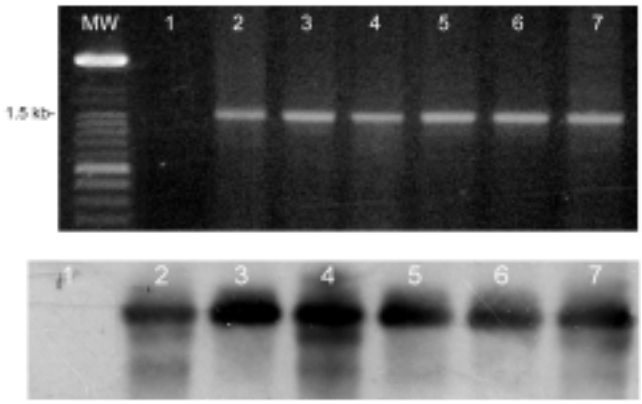

Fig. 1. PCR amplification of the 2'-5'A oligoadenylate synthetase gene from genomic DNA of transformed plants. A, agarose gel electrophoresis of PCR products: MW, DNA molecular weight marker; 1, untrasformed tobacco; 2–6, transformed tobacco plants; 7, positive control (pBI121-E18). B, hybridization of the above PCR bands with the digoxigenin-labelled specific gene probe.

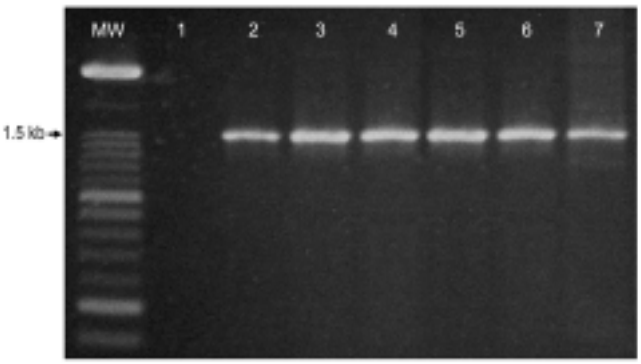

Fig. 2. Agarose gel (1.4%) electrophoresis of RT-PCR products of 2'-5'A synthetase mRNA (clone E18) isolated from transgenic tobacco plants: MW, molecular weight marker; 1, nontransformed control; 2–6, transgenic plants; 7, positive control (pBI121- E18).

Table 1. Virus content of tobacco plants transformed or nontransformed with 2'-5'A synthetase gene inoculated with various PVX concentrations, as determined in ELISA tests.

Inoculum concentration	Plants[a]	$O.D._{405}$ (M ± Em)	Virus amount	
			ng	%
1 μg ml^{-1}	Transformed	2.039±0.050	549	92
	Nontransformed	2.076±0.033	595	100
0.1 μg ml^{-1}	Transformed	1.871±0.066	380	84
	Nontransformed	1.946±0.053	448	100
0.05 μg ml^{-1}	Transformed	0.646±0.087[b]	26	43
	Nontransformed	1.027±0.098[b]	60	100

[a] P=0.01.
[b] At each concentration 20 transformed and 20 nontrasformed plants were used.

slight delay in symptom appearance was observed in transgenic plants inoculated with the lowest PVX concentration.

These results are not in agreement with preliminary tests carried out on few plantlets deriving directly from transformant calli (Faccioli and Raiola, 1998) and do not support the idea that the transgene confers resistance to PVX infection on tobacco as 2'-5' rat synthetase does on potato (Truve *et al*., 1993). Those authors in a following work reported a reduction of PVX infection only in some lines of transgenic tobacco (Truve *et al*., 1994).

Like Truve *et al*. (1993, 1994) we did not determine translational gene expression in plants, for the production of the relative antibody is still under way, but the finding that relatively low virus inocula (1 and 0.1 mg μl^{-1}) did not discriminate between transformed and control plants renders the practical utility of this gene doubtful.

The present results are consistent with Ishida *et al*. (1995) on tobacco carrying human 2'-5A-S, with Ogawa *et al*. (1966) on tobacco transformed with either a human 2'-5A-S or a human L RNase on CMV infection, and with Mitra *et al*. (1966), who established that only the two above genes together confer resistance to TMV, *Alfalfa mosaic virus* (AMV), and *Tobacco etch virus* (TEV) on tobacco.

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