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Transgenic tobacco plants carrying the non-structural P3 gene of potato virus A

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Abstract

Transgenic tobacco (*Nicotiana tobacum*) plants carrying the gene coding for potato virus A (PVA) non-structural P3 protein were prepared by inoculation with *Agrobacterium tumefaciens*. Seeds from self-pollinated flowers (T_1 generation) were collected. To estimate the effectiveness of vertical transfer of the introduced gene and usefulness of respective plant lines for further experiments, the T_1 generation was characterized by testing its ability to grow in the presence of kanamycin (Km) and by PCR of both neomycin phosphotransferase (nptII) and PVA P3 genes. Eight and ten of 29 lines showed Mendelian segregation of Km-resistant phenotype 3:1 and ≥15:1, respectively, the T_1 of eleven lines showed low Km resistance. Selected PCR-positive lines were tested for the presence of P3 mRNA. In most cases the transgene transcription was dependent on the presence or absence of Km in the plant growth medium. Prepared transgenic plants were furthermore tested for sensitivity to PVA and potato virus Y (PVY) infection. All of them showed identical symptom development as the non-transgenic control plants.

Additional key words: potyvirus, *Agrobacterium tumefaciens*, *Nicotiana tabacum*, PCR, P3 protein, transcription.

Introduction

Potato virus A (PVA) is a member of the genus *Potyviru*s (family *Potyviridae*), the largest and most destructive group of plant viruses. It is widely distributed in potato-growing areas and can decrease the yield of infected potato plants by up to 40 % (Puurand *et al.* 1996). PVA like other members of the genus *Potyvirus* are transmitted non-persistently by aphids, making their spread difficult to control through the use of insecticides (Tomlinson 1987).

The potyviral genome consists of one positive-sense single-stranded RNA molecule. It is translated in the infected cell into a single polyprotein subsequently processed by three virus-encoded proteinases giving rise to up to ten mature polypeptides (Riechmann *et al*. 1992). Although many data are available about possible or definite functions of several potyviral gene products, the real function of the protein P3 remains unknown. Imunocytological studies showed it localized either in the cytoplasm or in the nucleus of infected cells (Rodríguez-Cerezo *et al*. 1993, Langenberg and Zhang 1997). P3 is believed to play a role in virus replication (Merits *et al*. 1999), in cell-to-cell movement (Dougherty and Semler 1993) or as protease cofactor (Riechmann *et al*. 1992). It is not known whether the active form of protein is the P3 alone (Rodríguez-Cerezo and Shaw 1991) or the P3+6K1 complex (Riechmann *et al*. 1995). Because the P3 gene belongs to the least conserved genes among potyviruses (Shukla *et al.* 1989), it is possible that it plays an important role in some highly specific process, *e.g*. in the virus-host interaction pertinent to the host specificity (Jenner *et al*. 2002). The P3 gene has been shown to play a role of a pathogenicity determinant in several virus-host systems (Sáenz *et al*. 2000, Johansen *et al*. 2001, Jenner *et al*. 2003).

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Abbreviations: BAP - benzylaminopurine; CP - coat protein; Km - kanamycin; MES - 2-(N-morpholino)ethanesulfonic acid; MS medium - Murashige and Skoog medium; NAA - α-naphtaleneacetic acid; PDR - pathogen-derived resistance; PVA - potato virus A; PVY - potato virus Y; TVMV - tobacco vein mottling virus.
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Numerous examples of transformation of plants with viral sequences resulting in protection against viral diseases have been reported (pathogen-derived resistance, PDR). Either RNA-mediated or protein-mediated mechanisms can be involved in obtained resistance. Most experiments have been done with introduction of genes for viral structural proteins (coat proteins, CP), however, attempts to apply genes for different non-structural proteins including viral RNA polymerases, proteases or movement proteins have been performed, too (Powell-Abel 1990, Beachy 1997, Goldbach *et al*. 2003).

Transgenic tobacco plants transformed with the tobacco vein mottling virus (TVMV) P3 gene, showed resistance towards most TVMV strains but not towards other potyviruses (Moreno *et al.* 1998). In our work we prepared and analyzed transgenic tobacco plants with introduced P3 gene of PVA.

Materials and methods

Virus, RNA isolation, P3 gene amplification: The isolate PVA-LI (Čeřovská and Filigarová 1992) was grown in mechanically inoculated *Nicotiana tabacum* cv. Petit Havana SR1 and total RNA including viral genomic RNA was purified from the leaf tissue using the *RNeasy Plant Mini Kit* (*Qiagen*, Wien, Austria). The P3 gene was amplified by two step reverse transcription PCR using primers
PVA-P3-5 (*AAAAACCATG*GGCACTTCAAATTCTCAGATC),
PVA-P3-3 (*AAAAAAGATC*TTGAAACAGAACCACTTCTGC)
spanning exactly the desired genome region. The primers were designed according to the complete PVA sequence GenBank Acc. No. NC004039 (Puurand *et al.* 1994). Linkers for NcoI and BglII, respectively (shown in italic) were added to their termini for other cloning purposes not relevant and not interfering with presented work. The linker of the sense primer, moreover contained the start codon (underlined) essential for expression of the P3 gene.

Reverse transcription was performed using 200 U of M-MLV reverse transcriptase (*Promega*, Mannheim, Germany) in 0.02 cm^3 reaction mixture. 0.002 cm^3 of cDNA were applied in the PCR using Pfu DNA polymerase (*Promega*): initial denaturation at 94 °C for 5 min was followed by 35 cycles of: 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min. Final elongation at 70 °C for 10 min was performed.

Cloning: The amplimer was excised from the agarose gel, purified by *QIAquick Gel Extraction Kit* (*Qiagen*), blunt ligated in SmaI-digested binary vector pBI121 (*Clontech*, Heidelberg, Germany) and cloned in *E. coli* DH5α. The recombinant plasmid was purified by alkaline lysis method (Sambrook *et al*. 1989) and the insert orientation was estimated by restriction analysis. Selected clone with included sense-oriented P3 gene was controlled by sequencing (*Genomac*, Praha, Czech Republic) and used for transformation of *A. tumefaciens* GV3101 pMP90RK cells according the protocol of Höfgen and Willmitzer (1988).

Plant transformation, regeneration, selection: Selected *A. tumefaciens* clone was cocultivated with leaf discs from germ-free *N. tabacum* cv. Petit Havana SR1 plants as described by Horsch *et al*. (1985). Transformants were selected in shoot-inducing Murashige-Skoog (MS) medium containing 600 mg dm^{-3} 2-(N-morpholino) ethanesulfonic acid (MES), 1 mg dm^{-3} benzylamino-purine (BAP), 0,1 mg dm^{-3} α-naphthaleneacetic acid (NAA), 300 mg dm^{-3} kanamycin sulphate (Km), 500 mg dm^{-3} *Claforan*, 3 % sucrose and 1 % agar. After subsequent three-fold transfer to the fresh selective medium the regenerated plants were finally rooted in pots with sterile soil and grown under controlled conditions (14-h photoperiod, irradiance of 350 μmol m^{-2} s^{-1}, day/night temperature 25/18 °C). The seeds from self-pollinated plants were collected.

Seed germination, molecular analysis of T_1 plants: The obtained seeds were led to germinate aseptically on MS medium with or without Km (100 seeds in each case). The difference in number of seeds germinating in absence and presence of Km was considered as Km-sensitive for segregation ratio estimating.

From the plantlets total nucleic acid was isolated by phenol/chloroform extraction and ethanol precipitation (Sambrook *et al*. 1989). PCR was carried out with each of primer pair nptIIfor/nptIIrev amplifying the gene *nptII* responding for the Km resistance (Beck *et al*. 1982), and PVAP35/PVAP33 amplifying the inserted P3 gene. The PCR cycling conditions for nptII were 35 × (94 ºC/30 s - 60 ºC/30 s - 72 ºC/60 s).

To find out if the P3 gene was transcribed, the total nucleic acids were digested with RNase-free DNase (*Promega*) for 60 min at 37 ºC and RT-PCR and control PCR with PVA P3 primers were performed.

Inoculation experiments: Nine-plant-sets from each transgenic line and from non-transgenic *N. tabacum* control were mechanically inoculated with PVA-LI or PVY^{NTN}. The symptoms were examined daily visually up to 12 weeks after infection. Two and four weeks post inoculation indirect plate-trapped antigen ELISA (Gallo and Matisová 1993) using polyclonal antibodies against PVA (Čeřovská *et al*. 1998) and PVY (Čeřovská 1998) and alkaline phosphatase-labeled anti rabbit antibodies (*Sigma*, St. Louis, USA) was performed.

Results and discussion

Totally 38 lines of tobacco plants were regenerated including nine control lines transformed with pBI121 without the PVA P3 gene (Table 1). All transgenic lines manifested Km resistance by growth on the selective medium (T_0 plants) and presence of the P3 gene was demonstrated by PCR in all except control lines (data not shown). The seeds obtained from these plants by self-pollination (the T_1 generation) were tested for their ability to germinate in presence and absence of Km.

Different segregation frequencies (Topping and Lindsey 1997) were recorded in concern with Km-resistant phenotype of the T_1 plants. The frequencies close to theoretical Mendelian segregation ratios were evaluated by the χ^2 test (Table 1). Nine lines showed segregation ratio 3:1 corresponding to the transgene insertion at one locus in the plant DNA, for thirteen lines two or more insertion loci were proposed (segregation ratio ≥15:1). For fourteen lines the Km^r: Km^s ratio was between 3:2 and 1:20 and two lines showed no Km resistance (not significant or not tested by χ^2 test). Such low non-Mendelian segregation ratios could result, *e.g.*, from transgene silencing in T_1. It was indicated by the fact that 11 from these 16 Km-rather-sensitive lines were proved positive for *nptII* gene by PCR. Similarly, Ondřej *et al*. (1999) described decreasing of the hygromycin resistance gene expression in the progeny of resistant transgenic *Arabidopsis thaliana*, while the gene remained detectable by PCR in the sensitive plants. Difficulties with Km selection have been reported by several authors (reviewed by Eu *et al*. 1998).

Table 1. Kanamycin resistance and PCR analysis of T_1 generation of P3 transgenic plants. [a] - groups according the PCR results (see also the text), [b] - control transgenic lines (transformed with pBI121 without P3 gene), [c] - χ^2 tests not significant.

Group[a]	Transgenic line	Phenotype segregation Km^r:Km^s			tested	χ^2	PCR nptII	PCR P3
1	162	63:	2	(31.50)	15:1	1.1169	-	-
	189	18:	1	(18.00)	15:1	0.0315	-	-
	172	49:	35	(1.40)	3:1	12.4444[c]	-	-
	195	61:	39	(1.56)	3:1	10.4533[c]	-	-
	168	34:	66	(0.52)	-	-	-	-
	151	5:	95	(0.05)	-	-	-	-
	157[b]	0:	100	(0.00)	-	-	-	-
2	164	100:	0	-	1:0	-	+	-
	199[b]	100:	0	-	1:0	-	+	-
	194[b]	96:	4	(24.00)	15:1	0.8640	+	-
	197[b]	97:	3	(32.33)	15:1	1.8027	+	-
	182	15:	3	(5.00)	3:1	0.6667	+	-
	154	77:	22	(3.50)	3:1	0.4074	+	-
	192	46:	9	(5.11)	3:1	2.1879	+	-
	155[b]	78:	22	(3.55)	3:1	0.4800	+	-
	203[b]	31:	28	(1.11)	3:1	15.8701[c]	+	-
	209[b]	57:	42	(1.36)	3:1	16.0303[c]	+	-
	204[b]	21:	38	(0.55)	-	-	+	-
	166[b]	16:	84	(0.19)	-	-	+	-
	205	1:	5	(0.20)	-	-	+	-
3	175	19:	1	(19.00)	15:1	0.0533	-	+
4	183	100:	0	-	1:0	-	+	+
	206	100:	0	-	1:0	-	+	+
	207	100:	0	-	1:0	-	+	+
	201	100:	0	-	1:0	-	+	+
	208	97:	3	(32.33)	15:1	1.8027	+	+
	212	96:	4	(24.00)	15:1	0.8640	+	+
	181	69:	21	(3.29)	3:1	0.1333	+	+
	188	37:	12	(3.08)	3:1	0.0068	+	+
	214	81:	19	(4.26)	3:1	1.9200	+	+
	200	78:	22	(3.55)	3:1	0.4800	+	+
	171	77:	21	(3.67)	3:1	0.6667	+	+
	150	19:	39	(0.49)	-	-	+	+
	169	14:	28	(0.50)	-	-	+	+
	178	31:	69	(0.45)	-	-	+	+
	190	4:	67	(0.06)	-	-	+	+
	186	4:	70	(0.06)	-	-	+	+
	167	0:	12	(0.00)	-	-	+	+

On the other hand, seven lines gave negative result of nptII PCR although they showed at least partial (three of them, however, very high) resistance to Km (Table 1). Possible false negativity of PCR should be taken into consideration. It may be caused by various factors, most probably by different template quality (*e.g.*, different RNA amounts in respective samples, traces of organic inhibitors). We tried to minimize the risk of false negativity by repeated ethanol precipitation and electrophoretic control of DNA samples. DNA from all plant lines migrated as one sharp band in 1 % agarose, no degradation was observed and the RNA content (evaluated visually) was similar in all samples (not shown). Thus we presume other objectives to be responsible for these PCR results. PCR could be negatively affected simply by a minor mutation in one or both primer binding sites without an overall impact on the gene function.

In addition to this discrepancy, the results of nptII and P3 PCR analyses did not correlate each to other in every case (Fig. 1). In summary, four patterns were observed: *1*) Seven lines were negative in PCR for both nptII and P3 genes (one control line and six potential P3 transgenes). *2*) Thirteen lines were positive for the nptII but negative for the P3 gene. Such result should be obtained after transformation with pBI121 without inserted P3 gene. Eight lines falling into this group were really control lines. For the rest (lines 154, 164, 182, 192, 205) some deletion or damage of the P3 gene due to plant genome repair was proposed. Potyviral P3 proteins used to be toxic for bacteria in prokaryotic expression systems (López-Moya and García 2000, Šubr *et al*. 2000). The P3 function in the viral replication cycle is not clear and its biological activity is unknown. It is possible, that in some originally transformed plant lines the P3 gene was eliminated by partial or complete excision before or during gametogenesis and T_1 seeds production. *3*) On the contrary, the line 175 was negative for nptII and positive for the P3 gene. *4*) Finally, 17 lines were positive in PCR

for both tested genes.

No correlation of the plant cloning history (derivation from individual calli) with the classification into above mentioned groups was observed.

Among 29 P3-transgenic lines 18 (62 %) showed Mendelian segregation in T_1 (Table 2) – eight of them monohybrid, for the rest two or more inserted copies of the transgene were proposed. Relatively high rate of lines segregating in non-Mendelian pattern (38 % compared *e.g.* with 22 % described by Ondřej *et al.* 1999) might

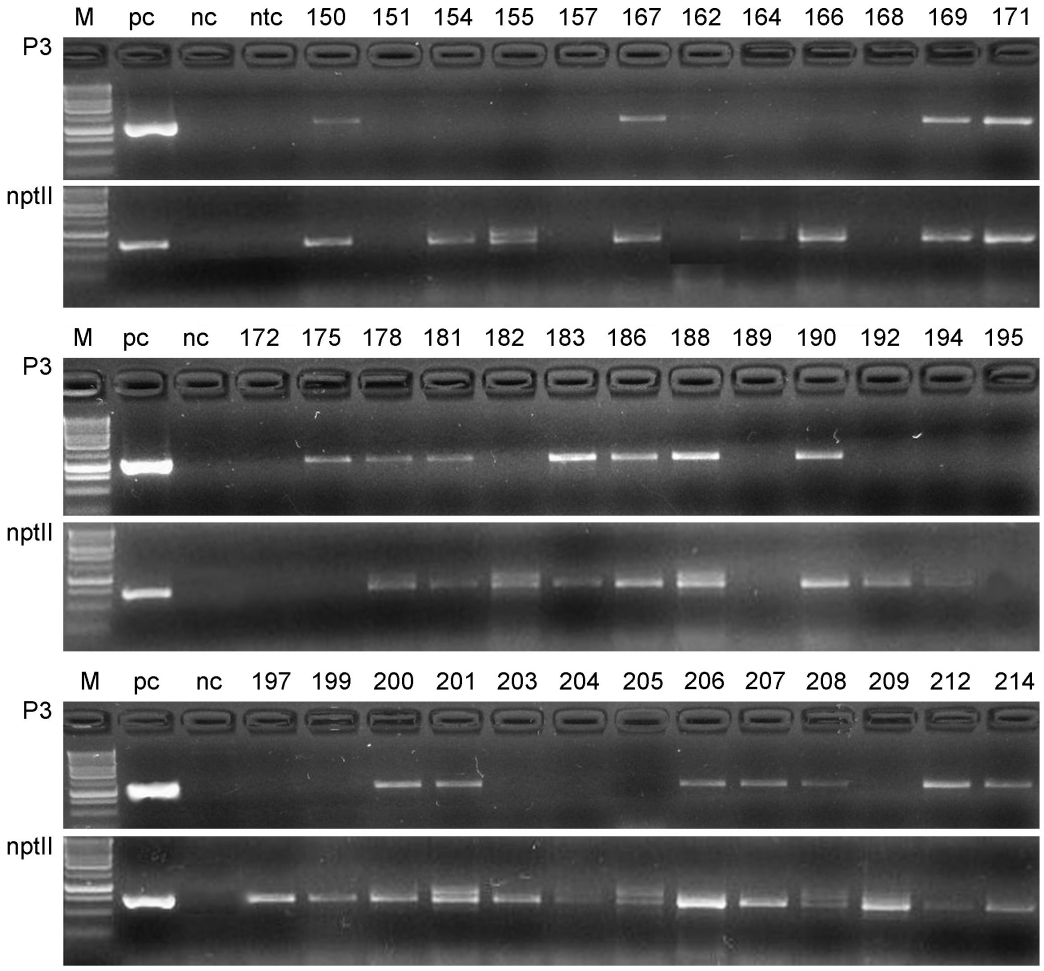

Fig. 1. PCR detection of the PVA P3 and nptII genes in DNA from transgenic tobacco lines. M - BenchTop 1 kb DNA ladder (Promega), pc - positive PCR control (P3 cloned in pBI121 used as template), nc - negative PCR control (water instead of template), ntc - nontransgenic control (DNA from SR1 tobacco plant).

Table 2. Summary of Km^r phenotype segregation in T_1 plants.

Segregation ratio	Number of lines P3-transgenic	control
≥ 15:1	10	3
3:1	8	1
< 3:1	10	4
0:1	1	1
Total	29	9

reflect potential toxicity of the P3 protein and tendency of the P3 gene to be eliminated from the T_0 genome resulting in low amount of transgene T_1 seeds. Júdová *et al.* (2004) proved the transgene (coding for honeybee royal jelly MRJP1 protein) in only 50 % of tobacco plant lines, however, they tested restricted number of them (4 lines).

For further analysis we selected the Km-resistant lines positive in both PCR (group 4 in Table 1). Twelve of them were tested for the presence of P3 mRNA by RT-PCR after DNase treatment. Plants grown in presence

and absence of Km were separately screened. Interestingly, these two sets gave no identical results (Table 3). The P3 mRNA was proved only in plantlets grown on Km plates for transgenic lines 188 and 207. On the contrary, only plants grown without Km showed the P3 transcription in the lines 171, 178, 206 and 214. Both sets of plants were positive for P3 mRNA in the line 183. P3 transcription was not detected in the lines 181, 186, 200, 201 and 208. PCR without reverse transcription from all DNase-treated samples gave negative results. As the presence of antibiotics serve as general stress for the plants, we only can speculate that some stress-dependent regulation events may be responsible for these transcription asymmetries. There are several genes known to be active under stress conditions (Hare *et al*. 1997, Czarnecka-Verner *et al.* 2000, Tasseva *et al*. 2004). On the other hand one cannot exclude that some other genes are completely silenced under stress. The transgene incorporation downstream such regulatory elements could result in its co-regulation.

Table 3. Transcription of the P3 gene in selected transgenic lines in presence and absence of Km

Transgenic line	P3 mRNA Km+	Km-
188	+	-
207	+	-
183	+	+
171	-	+
178	-	+
206	-	+
214	-	+
181	-	-
186	-	-
200	-	-
201	-	-
208	-	-

The results of Moreno (1998) indicated that P3 gene could induce potyvirus-resistance in transgenic plants. Unfortunately, none of our transgenic lines showed any level of resistance or tolerance (including the "recovery effect") against PVA as well as against PVY, strain NTN, as evaluated by symptom recording and by ELISA. Although no resistant plants were obtained, prepared monohybrid transgenic lines will be subjected to further research with respect to possible expression of P3 protein as potential tool to study its function *in planta*.

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