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Transformation of *Nicotiana tabacum* cv. Samsun by the Coat Protein Gene of PVY^{NTN}

By

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S u m m a r y

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Potato tuber necrotic ringspot disease, caused by a strain of potato virus Y (PVY^{NTN}) has been reported in many countries in the last decade. It is one of the most harmful potato pathogens shown to have infected potato crops in Slovenia, cv. Igor being the worst affected. The long term aim of our study is to develop virus-resistant transgenic lines of cv. Igor expressing pathogen-derived sequences. The tobacco plants (*Nicotiana tabacum* cv. Samsun) served as a model species for transformation and resistance studies. The coat protein (CP) gene of PVY^{NTN} was cloned into a pUC plasmid and then into a pROK2 plasmid which was mobilised in *Agrobacterium tumefaciens* strain LBA 4404. The presence of CP gene in the regenerated plants was checked by PCR and all primary transformants tested were found to be transgenic. Inoculated plants of the transgenic tobacco lines were tested for resistance to PVY^{NTN} but developed symptoms of PVY^{NTN} and appear to have no virus resistance.

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Introduction

Potato tuber necrotic ringspot disease (PTNRD), caused by $PVY^{NTN,}$ was first found in Hungary (1979) and has since spread throughout Europe (KUS 1994). Viruses from subgroup PVY^{NTN} reduce yield more than other members of PVY^{N} group and are causing circular necrotic rings on the surface of tubers from susceptible potato cultivars. The PTNRD was first noticed in larger scale in Slovenia in 1988 and has subsequently reached epidemic proportions. The selected stock grown in Slovenia practically disappeared from the market and the production of seed potatoes was reduced to less then 100 ha in 1994. It is readily apparent that PTNRD is the most devastating potato disease in the last century in Slovenia (KUS 1994). The loss of many potato cultivars which were bred for growing conditions in Slovenia and suited to Slovenian customers was substantial and there are as yet no suitable substitute cultivars with resistance. Methods of molecular biology offers us a possibility to confer resistance to susceptible cultivars and thus an opportunity to once again grow popular but improved potato cultivars. Presently the most promising method of conferring viral resistance to susceptible cultivars is through the expression of viral genes within the susceptible plant (ALLISON & al. 1997). Many successful introductions of transgenes which confirm resistance to potyviruses have been reported (reviewed by MAITI & HUNT 1997).

Tobacco, *Nicotiana tabacum* was selected as a model system for transformation and resistance studies because it has already been shown that transformation of tobacco with the PVY^{N} CP (coat protein) gene sequence conferred resistance to PVY^{N} (SUDARSONO & al. 1995, SMITH & al. 1994, FARINELLI & MALONE 1993, MCDONALD & al. 1997). With the experience gained from the tobacco study we aim to develop virus-resistant lines of potato cv. Igor expressing pathogen-derived sequences.

Materials and Methods

The PVY^{NTN} Slovenian isolate was isolated from potato cv. Igor and was maintained for inoculation studies in the same cultivar (PETROVIČ & al. 1995).

The cDNA library was made from PVY^{NTN} RNA and CP gene was multiply from the library with PCR. The primers for multiplication of CP sequence were designed on the basis of published sequences of different PVY isolates. To introduce frame-shift (FS) mutation start codon was moved for four nucleotides forward when constructing 5' frame-shift primer. cDNA fragments were cloned into pUC19 or pBluescript II KS(+) (Stratagene) and the sequences were checked. The fragments were than inserted into binary plasmid pROK2 under transcriptional regulation of a CaMV 35S promoter (Fig. 1). This vector was mobilised from *E. coli* into *Agrobacterium tumefaciens* strain LBA 4404 by triparental mating. NPT II gene, confering kanamycin resistance was used as selection marker. Leaf pieces and internodes of *N.tabacum* cv. Samsun were transformed and transformed plants were regenerated essentially as described by HORSCH & al. 1985. Transformed tissue was selected by culturing callus on medium described by BARKER & al. 1993. Primary transformants were micropropagated in node culture and than transferred to soil in green house. The presence of PVY^{NTN} CP gene in primary transformants was confirmed by PCR analysis. The resistance of transgens to PVY^{NTN} was tested by manual and graft inoculation.

Transformed plants were tested with ELISA for presence of PVY^{NTN} coat protein as described by PETROVIČ & al. 1995. Kanamycin resistance in S1 seeds from selfed transformed plants was also analysed as described by BARKER & al. 1993.

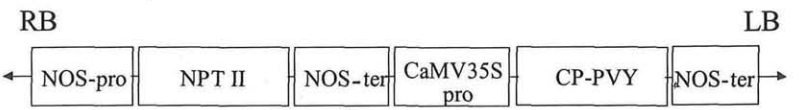

Fig. 1. Construct of the transferred DNA. In frame-shift CP gene construct, only the CP gene sequence was changed as described in text.

Results and Discussion

The suitability of stem internodes, basal and middle parts of leaves for transformation and regeneration was evaluated. Internodes and basal parts of the leaves proved to have higher regeneration rates after transformation than tissue from middle part of the leaves, when growing on media containing kanamycin (Table 1). Overall regeneration efficiency was higher when transforming with *A. tumefaciens* bearing sense (S) construct, comparing with bacteria carrying the frame-shift (FS) construct (Table 1). The presence of the PVY^{NTN} CP gene was confirmed by PCR analysis in four transgenic tobacco lines bearing sense CP gene and one line carrying the frame-shift CP gene (Fig. 2).

Table 1. Regeneration efficiency of different explants on *Nicotiana tobacum* cv. Samsun growing on media with kanamycin after transformation.

	no. of explants (E)	no. of E forming callus (K)	no. of K forming shoots (P)	no. of separated shoots (A)	no. of shoots forming roots (B)	% of rooted plants (B/Ax100%)
S-basal parts of leaves	46	35	29	44	15	34%
S--middle of the leaf	12	8	4	6	1	16.6%
S-internodes	10	9	4	3	1	33.3%
FS-basal parts of leaves	38	33	26	28	5	17.85%
FS-middle of the leaf	10	0	0	0	0	0%
FS-internodes	9	6	6	4	1	25%

All lines were manually inoculated with PVY^{NTN}, and symptoms appeared in transgenic as well as control plants (Fig. 3). Graft inoculation gave the same results. The presence of PVY^{NTN} in inoculated plants was confirmed by ELISA. This was possible since CP was not detected in transgenic lines bearing PVY^{NTN} CP sense construct with ELISA, which is consistent with the observation of other authors (SUDARSONO & al. 1995, MCDONALD & al. 1997).

Fig. 2. PCR analysis of transformed tobacco lines. Lines from 1S-4S are bearing sense CP gene construct and 1FS line is bearing frame-shift CP gene construct. P is a positive control of PCR reaction (800 bp) and C is PCR reaction with DNA from control plant.

Fig. 3. PVY^{NTN} symptoms on leaf of tobacco cv. Samsun 10 days after inoculation.

It was evident that in the five tested lines introduction of PVY^{NTN} CP transgenes did not affect the susceptibility to infection with PVY^{NTN}. It was assumed that resistance would be found because other authors have reported that insertion of the PVY CP conferred resistance to susceptible cultivars of tobacco (SUDARSONO & al. 1995, SMITH & al. 1994, FARINELLI & MALONE 1993, MCDONALD & al. 1997). However, we did not test many lines and in the future work it may be necessary to test more transgenic lines and repeat the inoculations in autumn since FARINELLI & MALONE 1993 reported that transgenic tobacco plants which were resistant in first experiment showed only delay in infection when

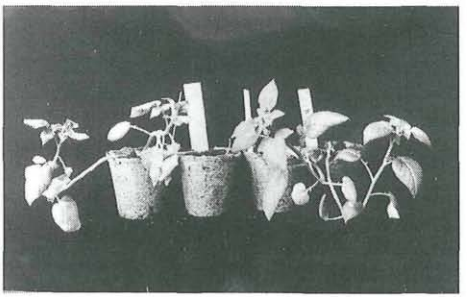

Fig. 4. Potato plants cv. Igor on the right are transformed with CP gene of PVY^{NTN} and are resistant to infection with the same virus isolat. On the left are untransformed control plants with systemic symptoms 14 days after inoculation.

retested in spring. It may also be possible that the Slovenian isolate of PVY^{NTN} is more agresive than other PVY isolates tested and none of the previously mentioned authors worked on tobacco cv. Samsun.

The proportion of kanamycin-resistant and kanamycin-sensitive seedlings in S1 progeny obtained from three transformed lines were determined. Observed segregation ratios predicted that lines 1S and 1FS had one locus containing a functional NPT II gene and line 2S had two loci containing functional NPT II genes (Table 2).

Table 2. Inheritance of kanamycin resistance in S_1 seedling progenies derived from selfing three independent transformants.

Line	Kanamycin sensitivity (resistant/sensitive) Observed	Suggested[a]	Estimated no. of NPT-II genes
1S	148:36	3:1	1
2S	81:6	15:1	2
1FS	138:36	3:1	1

[a] The suggested (theoretical) ratio nearest to the ratio derived from the observed data is given in this column.

The preliminary results on potato cv. Igor transformed with the same constructs showed that three lines out of 19 lines tested are resistant to PVY^{NTN} (Fig. 4) They have no symptoms on the leaves as well as on tubers grown in greenhouse conditions. Further investigations will be made on this lines with special attention focused on the differences of CP sense and frame-shift lines.

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