Potato and tobacco cultivars transformation towards potato virus Y resistance

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1. Introduction

Potato virus Y (PVY), the type member of the *Potyvirus* genus in the *Potyviridae* family, is an important pathogen of potato and tobacco in Poland (1). Some potato cultivars extremely resistant to PVY were obtained by introduction of *Ry* resistance gene from *Solanum stoloniferum*, but many economically important Polish potato cultivars are only partially resistant to PVY (2, 3). During the last ten years, a new subgroup of the necrotic PVY strain (PVY^N) has been identified in fields. These isolates overcome resistance to standard necrotic isolates, being more infectious and reaching higher concentrations in plants but inducing milder disease symptoms (2, 3). This group of isolates is spreading in Poland and has been responsible for 80-96% of PVY infections in potato fields during the last three years (4). In 1996, 100% of tobacco test plants, susceptible to PVY, were infected with the necrotic PVY strain (4,5).

The aim of this work is to engineer general resistance to potato virus Y in potato cultivars important for the Polish market, partially resistant to several PVY strains but sensitive to isolates from the new necrotic subgroup (3). To overcome this disadvantage we decided to transform potato plants with modified selected gene from the appropriate PVY isolate. The construct (patent applied for) was devised to be a universal one, inducing resistance in various agriculturally important plants. This universality was attested by induction of virus resistance in tobacco plants.

2. Materials and methods

2.1. Plant material

Tobacco (*Nicotiana tabacum*) plants and a potato (*Solanum tuberosum*) cultivar were selected for transformation.

2.2. Media for potato transformation

MCI medium	**for 1 litre**
Macroelements MS 10x	100 ml
Microelements MS 10x	100 ml (or 1 ml 1000x)
Vitamins Ericksson 1000x	1 ml
Myoinositol (20 mg/ml)	5 ml
Glucose	16 g
Agar (Difco)	8 g
NAA (1 mg/ml)	5 ml
BAP (1 mg/ml)	0.1 ml

GR_2 medium	**for 1 litre**
Macroelements MS 10x	100 ml
Microelements MS 10x	100 ml (or 1 ml 1000x)
Vitamins Ericksson 1000	1 ml
Myoinositol (20 mg/ml)	5 ml
Glucose	16 g
Agar (Difco)	8 g
NAA (1 mg/ml)	0.002 ml
GA_3 (1 mg/ml)	0.002 ml
Zeatin riboside, trans isomer (1 mg/ml)	2 ml

T&L medium	**for 1 litre**
Macroelements M3 10x	100 ml
Microelements MS 10x	100 ml (or 1 ml 1000x)
Vitamins Ericksson 1000x	1 ml
Myoinositol (20 mg/ml)	5 ml
Saccharose	25 g
Agar (Difco)	8 g

Macroelements Tendille and Lecerf (M3) (1974), modified, 10x

	for 1 litre
NH_4NO_3	5.36 g
$Ca(NO_3)_2 \times 4\ H_2O$	4.72 g
$MgSO_4 \times 7\ H_2O$	4.19 g
KH_2PO_4	2.74 g
KCl	3.50 g

2.3. Binary vectors' construction and *A. tumefaciens* transformation

Plasmid, carrying the appropriate fragment of the PVY genomic cDNA, was digested with the restriction enzyme BglII to cut off a 1.2 kb fragment. The fragment was purified from the agarose gel using Glass Milk (Bio 101). Binary vector pROK2 (6) was digested with BamHI and dephosphorylated. The PVY fragment was ligated with the pROK2 plasmid and introduced into competent *E. coli* strain HB101 by electroporation (7). Transformants were grown on LB supplemented with kanamycin (50 μg/ml), screening for plasmids containing the insert was done by growing overnight cultures, submitting them to the alkaline lysis and digestion with XbaI or SalI enzymes. Two clones were isolated — pROKY1 and pROKY2, one containing the insert of the sense orientation, the other in the antisense orientation. High quantities of both plasmids were purified using Midi columns (Qiagen). Electrocompetent *A. tumefaciens* strain C58 pmp90 or LBA 4404 cells were transformed with pROKY1 and pROKY2 binary vectors by electroporation at 1.44 kV (7). Presence of the inserts in kanamycin-resistant colonies was confirmed by alkaline lysis of overnight cultures and subsequent digestion of minipreps by the HindIII restriction enzyme.

2.4. Tobacco leaf disks transformation

20-30 ml LB, supplemented with kanamycin (50 mg/l was inoculated with 0.05-0.1 volume of two nights-old *A. tumefaciens* culture and grown overnight at 28°C. Bacteria were centrifuged for 10 min at 6 krpm and resuspended in 1 ml of liquid MS medium (8). Leaf disks (1 cm/1cm) were cut off from sterile plant material, each disk was wounded several times with a scalpel and put into 20 ml of liquid MS medium. About 100 explants per each experiment were prepared, bacteria were added and disks were incubated with bacteria for 10-20 min. Afterwards, the disks were dried quickly on the sterile paper, put into Petri dishes containing MS medium supplemented with NAA (0.1 mg/l), dishes were sealed with parafilm and incubated 2-3 days in a culture room at 25°C, in darkness. After rinsing with sterile water, the disks were transferred on MS medium containing NAA (0.1 mg/l) and BA (1 mg/l) as well as selective antibiotics — kanamycin (50 μg/ml), cefotaxime (500 μg/ml). That was repeated every two weeks, regenerating shoots were transferred on MS medium supplemented with antibiotics.

2.5. Potato transformation (according to (9))

Fresh cells of *A. tumefaciens* (one colony in 5 ml of medium) were grown in YEB medium with appropriate antibiotics overnight or for two nights at 28°C. 100 μl of *Agrobacterium* culture was spread on MS medium in Petri dishes. Freshly harvested leaves and internodes were cut with a scalpel, several cuttings were done on each tissue fragment. The explants were put

into Pétri dishes with MS medium and bacteria; dishes were incubated in the darkness for two days at room temperature, in a paper box in a laminar flow cabinet. Then the plant material was transferred on MCI medium with antibiotics: kanamycin (50 μg/ml) and cefotaxime (500 μg/ml). After 10-14 days the plant tissues were transferred on GR_2 medium with both antibiotics, it was repeated every two weeks. Regenerating shoots were moved to T&L medium with both antibiotics.

2.6. Plant DNA isolation for PCR (10)

Potato or tobacco plants, grown *in vitro*, were the source of plant DNA. Leaf disks were macerated in the bottom of the tubes with disposable pestles at room temperature, without buffer, for 15 seconds. 400 μl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added, the samples vortexed for 5 seconds and left at room temperature up to one hour. After centrifugation (5 minutes at 13 krpm), 300 μl of supernatant was transferred to a new tube, mixed with 300 μl of cold isopropanol and left at room temperature for 2 minutes. The samples were centrifuged at 13 krpm for 15 minutes, pellets were vacuum dried and dissolved in 50-100 μl TE. The DNA was stored at 4°C, 2-4 μl of sample was used for a 50 μl PCR reaction.

2.7. PCR amplification

The DNAs were amplified using Taq polymerase (Promega) or PrimeZyme (Biometra). In the standard reaction, 1-4 μl DNA (approximately 20 ng/μl) was added to the mixture containing 25 ng of each appropriate primer (reverse P1 and sense P3), 1x PCR buffer, 2 μl 10 mM dNTPs and 2 units of polymerase, in the final volume of 50 μl. The PCR was done in a thermocycler (Apligene or Biomed) using the following amplification procedure: initial denaturation at 94°C for 4 min, 30 cycles of 30 s at 94°C (denaturation), 30 s at 55-58°C, 30 s at 72°C (elongation) and final 10 min elongation at 72°C. 5-10 μl of PCR sample was analysed in 1% agarose, 1x TBE gel.

2.8. Resistance tests

Transgenic tobacco or potato plants were inoculated with a sap of PVY^N infected tobacco, diluted approximately twofold with tap water. Manual inoculations were done by rubbing the diluted sap onto tobaco or potato leaves previously dusted with carborundum. The virus was detected by DAS-ELISA using monoclonal antibodies against PVY (Bioreba). Disease symptoms were observed visually.

Tobacco lines were micropropagated to have 3-5 copies of each line for the evaluation of resistance in a climatic chamber. After three or four weeks the plants were inoculated. ELISA tests were performed in the second or third week after inoculation, taking as samples well developed leaves.

Transgenic potato clones were micropropagated, transferred to the soil and grown in a greenhouse to produce minitubers. The tubers were collected, presprouted and planted in pots in a greenhouse. Potato plants (ten plants per clone) were inoculated at the emergence of the fourth or fifth leaf. ELISA tests were done twice, in the fifth and seventh week after inoculation. The inoculated and upper leaves (in the fifth week) or only upper leaves (in the seventh week) were collected for test.

3. Results and discussion

3.1. Potato and tobacco transformation

Potato and tobacco were transformed with pROK2 binary plasmids carrying a PVY casette in sense and antisense orientations (plasmids pROKY1 and pROKY2).

About twenty five tobacco and thirty potato lines were regenerated carrying each PVY cDNA insert. Plants were also transformed with plasmids devoid of PVY inserts (pROK2 or pBI121). There was no regeneration in control experiments where plant explants were co-cultivated with non-transformed *A. tumefaciens* on selective media.

Morphologically transgenic tobaccos were identical to the non-transformed plants. The morphology of transgenic potato plants and their tubers is illustrated in Fig.1. Generally, transgenic plants were identical to the parental cultivar. Minitubers of several clones were slightly different from those of non-transformed plants. Final evaluation of these clones awaits further studies.

3.2. Identification of transgene presence by PCR and further molecular analysis

Transgenic lines were analysed by PCR to confirm the presence of transgene. Plant genomic DNA was extracted and an aliquot was submitted to a PCR amplification. Products were run in an agarose gel and identified using standard methods — comparison with the positive control and hybridisation with an appropriate PVY- specific probe. Fifty one out of sixty four potato and forty three out of fifty tobacco plants tested were positive. Approximately 16% of regenerated lines were negative in the PCR reaction, probably because the PVY cDNA was not introduced into their genomes.

3.3. Resistance analysis

Eighteen pROKY1 and nineteen pROKY2 tobacco lines of T_0 generation were submitted to the resistance tests after the transfer to the soil. Following the inoculation with PVY^N containing sap, disease symptoms have been ob-

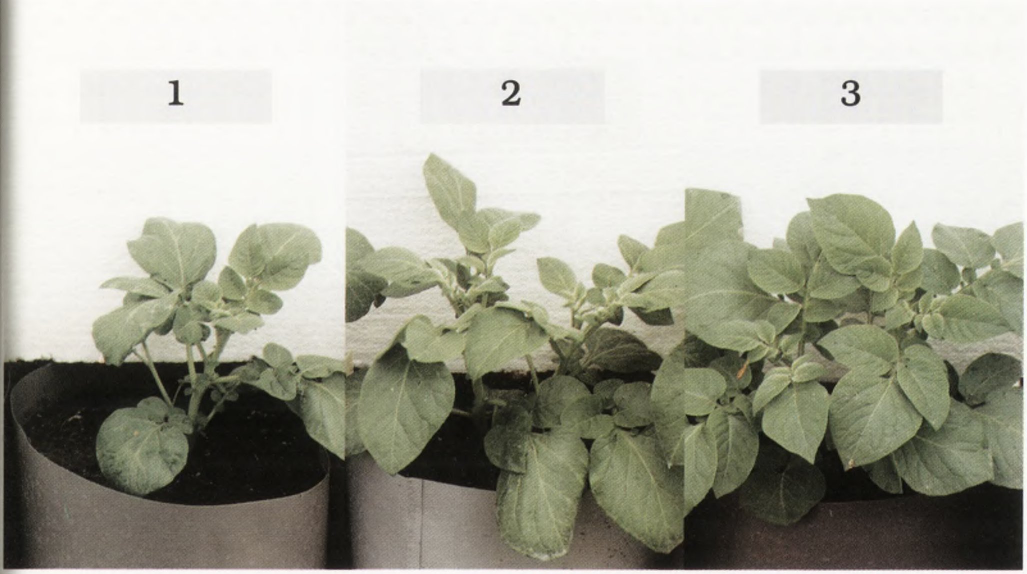

Fig. 1. Morphology of transgenic potato clones. A. General aspect of young plants. B. Mini-ubers: (1) wild-type potato plant, (2) vector-transformed plant, (3) transgenic resistant potato lones.

served for several weeks. In most cases, typical symptoms of PVY^N infection were noticed, consisting of vein clearing and banding or chlorotic mosaic, but very rarely of veinal necrosis. The ELISA tests, done about two weeks after infection, allowed to identify seven plant lines that were partially or totally resistant to PVY (Tab. 1). These plants showed no disease symptoms or very weak ones, reduced to a slight vein clearing. The ELISA values were very low. A "recovery" phenotype was also observed, characterised by a decrease of ELISA values and symptoms disappearance in younger leaves. Four of these tobacco lines contained the antisense RNA expressing transgene.

TABLE 1
PRELIMINARY STUDIES ON RESISTANCE OF TRANSGENIC TOBACCO LINES (TO GENERATION) AND POTATO CLONES TO PNY

Type of construct	Number of tested lines	Number of resistant lines
Tobacco		
pROKY1	18	3
pROKY2	19	4
Potato		
pROKY1	15	2
pROKY2	19	4

Fifteen pROKY1 and nineteen pROKY2 potato clones were tested for resistance to PVY. Symptoms started to develop in the fourth week post inoculation, they were limited to a systemic mosaic. Six clones were symptomless with very low ELISA values, similar to those for non-infected control plants and for inoculated resistant standard plants (Tab. 1). Only two of resistant lines contained the sense RNA expressing transgene.

4. Future prospects

Next generation of preliminary resistant tobacco lines are now submitted to PVY^N resistance tests. Secondary infection on potato transgenic clones will be evaluated. Selected lines and clones will be studied more thoroughly, especially their resistance to other PVY isolates. Molecular analysis of transgenic lines is carried on in order to determine the insert copies number and the level of transcription. The work on transformation of industrial tobacco cultivars has been already started.

New binary vectors carrying PVY^N cDNA fragments were constructed and the work on selection of PVY resistant potato and tobacco lines is carried on. We hope that in a near future we will develop several transgenic lines of economically important cultivars that will be enriched with a new advantageous feature — resistance to infections with necrotic PVY isolates.

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Summary

In recent years a new subgroup of necrotic potato virus Y (PVY) isolates has spread in the Polish fields. To counter this infection, the PVY resistance was introduced into selected potato and tobacco cultivars. Plants were transformed by agroinfection with pROK2 derived binary plasmids, carrying an appropriate fragment of the PVY genome (patent applied for). Kanamycin resistant plants were screened by PCR for the presence of PVY cDNA inserts, positive transformants were tested for virus resistance by inoculation with sap from infected plants, followed by observations of disease symptoms and virus accumulation analysis. Several resistant potato and tobacco clones were identified and their phenotypes were preliminarily characterised.

Key words:
potato virus Y, resistance, transgenic, potato, tobacco.

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