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Potato Virus Y Coat Protein Gene Induced Resistance in Valuable Potato Cultivars

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The coat protein (CP) gene from a necrotic strain of potato virus Y (PVY) has been engineered into two-old Hungarian cultivars, namely cv. *Mindenes* and *Somogyi kifli.* The integration of the coat protein gene was confirmed by polymerase chain reaction (PCR) using genomic DNA preparations. The transcription and the expression of the integrated CP gene was followed by Northern and Western blot. The pathogene derived resistance was demonstrated by mechanical inoculation of the transformant plants after the transfer of the regenerants into soil. The efficiency of the virus protection varied between different potatoes ranging to complete protection to no protection. All plants were tested in field conditions under the special licence, given the competent authority instructed by the Hungarian gene technology act. In two consecutive years three *Mindenes* independent transformants proved to be highly resistant against two different strains of PVY in provocative experiment.

Keywords: Potato virus Y, coat protein mediated cross protection, valuable potato cultivars.

Several valuable potato cultivars disappeared from today's agriculture due to their extreme susceptibility to different virus diseases. Potato virus Y became to be the most devastating pathogene (Wolf and Horváth, 2000). This could be attributed to the wide-spread of the necrotic strains of PVY in Europe. It is also worth to mention that potato crop is limited to the cultivation only on some cultivars. Old traditionally accepted cultivars were left out from growing, by causing severe loss in the biodiversity of agriculture. Conventional breeding has realised this and initiated the reincarnation of these old cultivars found mostly only in genebanks.

As molecular breeding could give a booster to accelerate potato breeding the pathogene derived resistance could be considered as one of the solutions. Since the first successful demonstration of genetically engineered resistance to a plant virus, hundreds of important plant species were included in this type of exercises (Powell-Abel et al., 1986; Borque et al., 1987; Stark and Beachy, 1989; Fehér et al., 1992; Van der Vlugt et al., 1992; Beachy, 1993; Dinant et al., 1993; Gonsalves and Slightom, 1993; Wilson, 1993; Beachy, 1997; Valkama et al., 2000). The well-known potatoes like *Russet Burbank, Bintje* and *Desiree* were also engineered with viral coat protein genes and proved to be resistant against the superinfecting viruses (Ishida et al., 1989; Kaniewski et

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al., 1990; Lawson et al., 1990; Farinelli et al. 1992; Malnoe et al., 1994). Unfortunately less attempt was made considering valuable other cultivars to involve into this molecular breeding regime. Here we report that other valuable cultivars could be efficiently engineered and make it again profitable for the agriculture including the increase of biodiversity in the agriculture.

Materials and Methods

Virus and gene construct

A necrotic strain of potato virus Y (PVY-H) used (de Bokx and Huttinga, 1981; Beczner et al., 1984; Thole et al., 1993) labelled today as a PVY^{NTN} as a source for construction of the CP gene containing plant expression vector described elsewhere in details (Kollár et al., 1993; Józsa et al., 2002). For challenge infection beside the necrotic strain from where the CP gene originated an ordinary strain $PVY^{O/C}$ was also applied (Wolf and Horváth, 2000).

Plants and transformation

In vitro virus free plantlets of potato *(Solanum tuberosum L.)* cultivars *Mindenes* and *Somogyi kifli* were originated from the plant genebank of Veszprém University Georgikon Agricultural Faculty Institute of Potato Breeding at Keszthely. Overnight culture of the PVY-CP gene containing *Agrobacterium tumefaciens* (C58C1) at 28 °C was used by puting several microliters onto the 5–8 mm size slices from microtubers (Ishida et al., 1989) placed on RB1 media without kanamycine. After two days of incubation in growth room (16 h daylight at 23 °C) tuber slices were immersed into Cefotaxim (500 mg/l) solution for 30 minutes and were placed into RB2 media containing 50 mg kanamycine (Borque et al., 1987). Two-three weeks later small shoots were formed along the edge of the tuber slices. The regenerant shoots were cut of at 1–2 cm size and placed into G80 media for rooting. Plantlets than were transferred into soil and kept in greenhouse conditions.

Molecular analysis of the transformants

DETECTION OF THE INTEGRATION OF THE CP GENE INTO THE POTATO GENOME

Plant DNA extraction was made according to Rubino et al. (1992) while the polymerase chain reaction (PCR) was carried out essentially as described earlier by us in analysing PVY transgenic tobaccos (Józsa et al., 2001). Oligonucleotide primers 5'-CACTTGCTCGAGTATGCTCCACAGC-3' homologous to nucleotides 8776-8800 and 5'-CGTCCGGAGAGACACTACA-3' complementary to nucleotides 9376-9394 chosen from the CP sequences and 3 NTR covering a 618 bp long DNA. The PCR product were analysed by gel electrophoresis and Southern hybridisation with ^{32}P labelled translated CP gene construct as a probe according to Sambrook et al. (1989) manuals.

NORTHERN AND WESTERN BLOT ANALYSIS OF TRANSGENIC POTATOES

Total nucleic acid isolation was essentially performed after White and Kaper (1989) and the samples were separated on formaldehyde gel and than transferred onto Hybond-N membrane. The membranes were hybridised with the nick translated radioactive probe of the CP gene using the standard procedure (Sambrook et al., 1989). The expression of the CP in the transgenic plants was demonstrated by Western hybridisation. The 12% polyacrylamide gel separated protein extract of the plants were transferred onto Hybond-C membrane and the membrane bound proteins were visualised by alkaline phosphatase conjugated PVY CP antibody and its chromogenic substrates (Sambrook et al., 1989).

Plant resistance tests

Plants regenerated from the transformation experiments were subjected to mechanical inoculation using the necrotic strain of PVY at 5 µg/ml concentration. Plants were monitored for a month both by taken leaf disks for dot blot hybridisation and symptom development by visual observation. In field conditions each transformed potato lines after producing enough seed potato were grown in provocative plot (minimum ten of each), where insecticide treatments were omitted. In greenhouse conditions the same potato lines were mechanically inoculated with the two strains of PVY, namely PVY^{NTN} and $PVY^{O/C}$ and also by grafting onto infected tobacco plants. These plants were monitored by double antibody sandwich ELISA.

Enzyme linked immuno assay of the plants (ELISA)

Double antibody sandwich ELISA has been performed according to the procedure of Clark and Adams (1977) using the Loewe Biochemica Gmbh polyclonal antibody detection kit.

Results and Discussion

The coat protein gene of PVY containing plant expression vector which harbours active transcriptional sequences from cauliflower mosaic virus and the nos terminator from *Agrobacterium tumefaciens* was used in C58C1 *Agrobacterium* strain. Sterile micro-tuber slices of the two different potato cultivars were inoculated with the engineered *Agrobacterium* and the kanamycine resistant regenerants were rooted and transferred into soil. Thirty-seven independent transformant lines were obtained from the cultivar *Mindenes* while only four from cultivar *Somogyi kifli.* Kanamycine resistant lines were analysed for the integration of the coat protein gene. All transformants were positive in Southern hybridisation experiments demonstrating the integration of the transgene. A set of *Mindenes* cultivar transformant DNA analysis, shown in *Fig. 1.* Transcription of the transgene was followed by Northern blot analysis (data not shown) while expression of the CP gene was monitored by Western blot. Out of the total forty-one transformed lines only four were negative while the others expressed about the same level of the coat protein. The

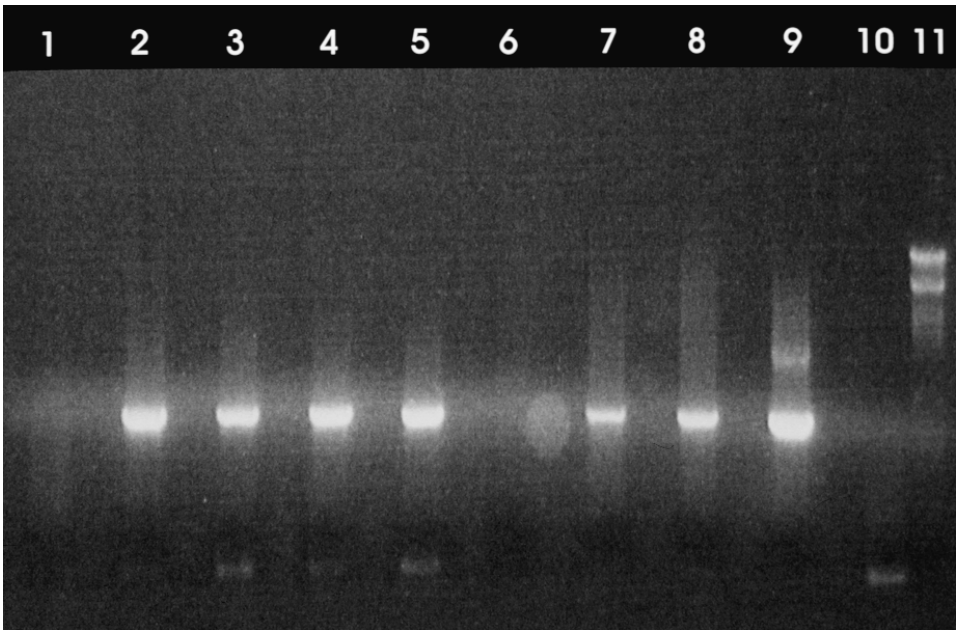

Fig. 1. Agarose gel electrophoresis of PCR product of different transformant *Mindenes* cultivar DNA extracts. Fragments of corresponding to the integrated CP gene were positive in Southern blot analysis. Lane 1 *Mindenes* control, 2–8 lanes M2, M3, M4, M5, M6, M7 and M8 (Lane 9 positive control, lane 10 negative control, lane 11 length marker)

expression of the protein in the different lines was not significantly different *(Fig. 2).* In the challenge infection two virus strains was used both mechanical inoculation and by graft inoculation in greenhouse conditions. Preliminary screening showed resistance in all cases monitoring the mechanically inoculated plants with 5 μg/ml virus concentration for a month period. No symptom development was detected and dot blot hybridisation was also negative. When higher virus concentration was used for inoculation and graft inoculation was also included and the monitoring lasted for the whole vegetation period different level of resistance was observed ranging from complete resistance to no protection. This was in good agreement between the symptom development and the virus replication, and with the published results of the same kind (Kaniewski et al., 1990; Beachy, 1993; Farinelli and Malnoe, 1993; Beachy, 1997). In two consecutive years in field experiments three *Mindenes* transformants showed high level of resistance namely neither symptoms were observed, nor virus accumulation was detected. All plants were tested regularly with ELISA. In *Table 1* data represents the number of the infected plants from different transformed lines based on visual observations and confirmed by ELISA test in provocative field experiments. As the symptoms and the resistance levels were different in the different transformant lines while more than 90% of the transformant

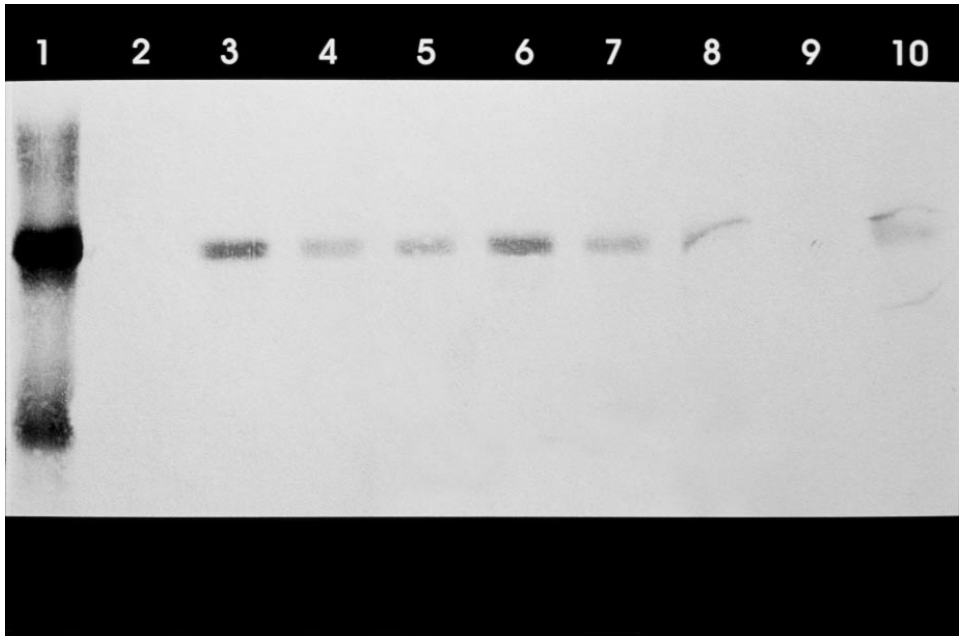

Fig. 2. Western blot analysis. Protein samples prepared from transgenic *Mindenes* lines. Lane 1: Protein extract from infected *Mindenes* plant. Lane 2: Protein extract from healthy *Mindenes* plant. Lanes 3–10: Samples from transformed *Mindens* lines M2, M3, M4, M5, M7, M8, M9 and M10

Table 1

Number of virus-infected plants of different transformed lines in provocative field experiments

Potato lines	No. of Infected plants/grown plants
Somogyi kifli non transformed	40/40
SK-2 CP transformant	37/37
M-3 CP transformant	40/40
M-5 CP transformant	20/30
M-6 CP-transformant	13/13
M-10 CP transformant	39/39
M-11 CP transformant	0/37
M-21 CP transformant	0/40
Mindenes non transformed	38/38
M-12 CP transformant	0/40

Virus infection was monitored both by visual observation and ELISA test during the whole vegetation period

expressed the same level the integrated CP it is plausible that there are no correlation between the resistance level and the expressed coat protein. In short in these transformants the coat protein mediated cross protection has to be independent of the level of CP accumulation. By involving virus susceptible but in other qualities are excellent cultivars in genetic improvement experiments we have demonstrated that forgotten valuable cultivars could be reincarnated. This new genetically engineered *Mindenes* potato cultivar is one of the examples, how biodiversity can be brought back into the agriculture.

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