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Gödöllő

INDUCING RESISTANCE IN POTATO AND TOBACCO CULTIVARS BY THE COAT PROTEIN GENE OF POTATO VIRUS Y, AND THE FIELD EVALUATION OF THESE CULTIVARS

Main points of the PhD Thesis

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ANTECEDENTS AND OBJECTIVES OF WORK

Potato potyvirus Y (PVY) causes considerable losses in potato and tobacco production world-wide. In Hungary, the virus not only reduces crop production, but also displaced valuable old Hungarian cultivars with excellent traits from agricultural production.

In the 1980s a new PVY strain, which was able to infect the previously resistant potato cultivars, spread in Hungary (Beczner et al., 1984). This virus strain has resistance-breaking ability (Le Romancer-Kerlan, 1992., Van den Heuvel et al., 1994) and causes characteristic necrotic symptoms on the tuber and berry. It was named potato tuber necrotic ringspot virus (PVY^{NTN}). Today it is widespread throughout the world and causes serious yield losses mostly in the cultivated plants of *Solanaceae* family.

It is two decades since genes encoding certain traits can be isolated from any living organism by biotechnological techniques. The selected characteristic can be manifested and inherited in other organisms if it is transferred successfully (Gáborjányi and Balázs, 1984). Producing transgenic plants (e.g. tolerant or resistant varieties) applying such methods is more cost-effective and time-saving than improving plants by traditional breeding practices (Tepfer and Balázs, 1997). Newly emerging strains, biotypes and races of pathogens provide new challenges for specialists continuously.

Plant transformation with constructions containing coat protein genes is the prevailing procedure among virus originated transgenic techniques. This method has been used successfully in several plant-virus combinations.

The objective of our work was to transfer the coat protein gene of potato virus Y (originating from the Hungarian flora) into old susceptible Hungarian potato and tobacco varieties and thus produce resistant transgenic cultivars. One of our aims was to select the best lines by applying molecular analyses and virus infection tests and to examine them under field conditions. We intended to prove that transgenic plants developed by this technology can be used in plant production effectively and safely, reintroducing old cultivars which can only be found in gene banks.

MATERIALS AND METHODS

In our experiment *Solanum tuberosum L. cv* Mindenes (M) and Somogyi Kifli (SK) Hungarian varieties were used. These cultivars had been widespread in Hungary previously but were withdrawn from agricultural production because of their susceptibility to the virus. *In vitro grown* virus-free plantlets of potatoes originating from the gene bank of the Institute of Potato Breeding, University of Veszprém, Georgikon Faculty of Agriculture, Keszthely were used.

Nicotiana tabacum Virgin D (VD), Stamm C2 (SC) and Hevesi 11 (H11) tobacco cultivars were provided by Agrotab Breeding and Seed Production Ltd. in Debrecen - Pallag. Vingin D is a German variety, Stamm C2 is a German breeding line while Hevesi 11 is a Hungarian cultivar whose variety certification was invalidated because of its susceptibility to the virus.

The virus isolate used is a necrotic PVY-H (Hungarian) isolate provided by Dr László Beczner (Plant Protection Research, Budapest). It causes typical necrotic ringspot on the tuber and on the berry and possesses special resistant-breaking feature.

Sterile plant cultures were used for the propagation and transformation of plants.

Plasmid pGAYHCP containing PVY coat protein gene engineered by the Virology work-group of the Agricultural Biotechnology Centre was used for plant transformation (Kollár et al., 1993). PVY-H coat protein gene provided with an AUG translational start codon is located between the 35 S promoter of cauliflower mosaic virus (CaMV) and regulatory NOS terminations within the gene construction based on pGA482 plant expression vector.

Gram- C58C1 (Rif^r) *Agrobacterium tumefaciens* strain was used to transfer the gene construction into the plant genome (Zambryski et al., 1983).

Several microlitres of bacterium culture containing PVY-CP grown overnight at 28 ^{o}C were dropped onto tobacco leaf discs and stem segments of 0,5-1 cm, as well as onto slices of 2-3 mm produced from potato microtubers of 0,5-1 cm (Ishida et al., 1989). Shoots were formed along the edge of leaf discs, stem segments and microtuber slices after four or five weeks. The regenerated shoots were cut off at the size of 1-2 cm and placed into rooting media. Plantlets were then put into soil and grown under greenhouse conditions.

The integration of transgene was examined by PCR (polymerase chain reaction) analysis, then the PCR products were studied by gel electrophoresis and Southern hybridisation (Sambrook et al., 1989). The transcription of RNA was examined by Northern blot analysis, and the expression of CP in transgenic plants was confirmed by Western hybridisation (Sambrook et al., 1989).

Tobacco plants regenerated from the transformation experiments were subjected to mechanical inoculation using cleaned PVY-H at concentrations of 0,5; 5 and 20 μg/ml diluted in 0.1M sodium phosphate buffer (pH 7.2). We inoculated 6 lines of Virgin D and 12-12 lines of Stamm C2 and Hevesi 11. Three plants were used for one virus concentration per each line. Samples from infected plants were taken from the upper leaves on the 5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} days and were evaluated by dot blot hybridization. The development of symptoms was observed visually as well.

Transgenic potato plants were tested under field conditions in accordance with a special licence in co-operation with Dr Sándor Horváth and Dr István Wolf from the University of Veszprém, Georgikon Faculty of Agriculture, Institute of Potato Breeding in Keszthely. 22 Mindenes and 4 Somogyi Kifli transgenic lines were planted in small plots surrounded by corn rows. 20 tubers were placed in a 5-meter-long row, and one or two rows were planted per lines. Virus-free, non-transgenic Mindenes and Somogyi Kifli rows were planted between transgenic rows as controls, while Somogyi Kifli rows inoculated with PVY^{NTN} mechanically were planted to infect the other rows.

The evaluation of resistance was carried out by artificial infections using purified PVY^{NTN} (D-10 isolate) and $PVY^{O/C}$ (Ka-49 isolate) as well. Transgenic Mindenes and Somogyi Kifli plants were infected mechanically using carborundum powder and glass spatula at their 4-leaf-stage. Visual assessment was carried out after 36 days from inoculation, and DAS-ELISA analysis was performed on day 37. Polyclonal antibodies and kits based on antibodies conjugated with alkaline phosphatase used for the analysis were manufactured by Loewe Biochemia Gmbh and Boehringer Mannheim Gmbh.

Virus infection was also studied by *Solanum demissum* A6 biotest. *S. demissum* A6 leaves were removed and infected with the tissue sap of infected transgenic plants on the 37^{th} day of mechanical infection. Four potato plants were used per each transgenic line, and two *S. demissum* A6 leaves were infected per each plant. Removed and infected leaves were

incubated on wet filter-paper in petri-dishes at room temperature. Symptoms of virus infection were examined on day 6 and 7.

On the basis of the results of field experiments three Mindenes lines (M11, M12 and M21) were chosen for grafting. Transgenic potato plants were grafted on tobacco and tomato stocks infected mechanical with PVY^{NTN}, and infected tomato branches were grafted on transgenic potato stocks. Stocks and grafting branches with 8-10 leaves were the same age. In case of tobacco stocks 2 plants were analysed per each transgenic line, while for tomato and potato stocks 3 plants were examined per each line. Virus concentrations were detected by ELISA tests four weeks after grafting.

RESULTS

Thirty-eight different Virgin D transgenic tobacco lines carrying PVY CP transgene and expressing the CP were produced. Six lines were infected mechanically with purified PVY-H in greenhouse experiments. These lines were the followings: VD1, VD2, VD3, VD4, VD5 and VD6. Infected plants were not found in VD1 and VD5 lines neither by dot blot tests nor visual observations. In the case of the other four lines (VD2, VD3, VD4 and VD6) vein necrosis was observed one month after the inoculation. In these lines virus accumulation was also demonstrated by the data of dot blot analysis. This symptom could only be detected in the plants that were infected by the virus concentration of 5 μg/ml. However, after two months all the symptoms disappeared, and the plants were virus-free again, which were confirmed by visual observations and dot blot tests. These lines showed the phenomenon called 'recovery', which means that the young leaves of plants were free from the virus in spite of the initial infection. Symptoms on the 'recovering' transgenic plants were less serious and appeared later than those on the control plants where virus infection symptoms were detected on the 7^{th} to 10^{th} days.

Up to the present two lines (VD5 and VD17) could be tested for virus resistance under field conditions. During the two-year examination period average natural PVY infection was observed in the fields. All of the susceptible tobacco cultivars were infected, while our transgenic lines were virus-resistant and remained healthy.

In case of Stamm C2 cultivar 55 transgenic lines were produced and all of them were tested by molecular techniques. Coat protein was expressed in 34 lines, however, 8 of these lines showed only a low level of expression. All of the 12 transgenic lines mechanically infected under greenhouse conditions were found to be resistant, which was confirmed by the data of dot blot tests and visual observations. Some of these lines (SC35, SC46, SC47 and SC48) expressed the coat protein at a low level, while the expression of transgene could not be detected in the other lines (SC36, SC39, SC40, SC41, SC43, SC44, SC45 and SC49). Simultaneously, two lines (SC1 and SC4) were tested under field conditions. Coat protein was not expressed in SC1, while SC4 produced the protein. Results were the same as those with Virgin D transgenic lines, i.e. during the two-year examination period and with an average natural PVY infection, both transgenic lines were resistant, while all the susceptible varieties were infected.

Twenty-three different Hevesi 11 transgenic lines were developed, and 17 of them expressed the coat protein. 12 lines were involved in greenhouse experiments. Some did not express the CP (H11-12 and H11-16) but most of them (H11-1, H11-2, H11-3, H11-4, H11-6, H11-7, H11-8, H11-9, H11-10 and H11-11) did. None of the transgenic plants was infected after being inoculated mechanically with purified PVY-H, which were confirmed by dot blot tests and visual observations. Up to now none of these lines was involved in field trials.

From the cultivar Mindenes 37 different transgenic lines were produced, and CP could be detected in 34 lines. We developed 4 Somogyi Kifli transgenic lines and CP expression was demonstrated in 3 lines. None of the 22 Mindenes lines and 3 Somogyi Kifli lines infected mechanically under greenhouse conditions was infected. To confirm these data, different field experiments were conducted. Some of the 22 Mindenes lines and the 4 Somogyi Kifli lines tested in the field expressed the CP at a high level (SK1, SK2, M1, M3, M4, M7, M11, M12, M14, M15, M17, M18, M20, M21, M24, M27 and M33), while others expressed it at a low level (SK3, M2, M5, M8 and M10), and in some lines CP expression could not be detected at all (SK4, M6, M9 and M16). During the two-year examination period several trials were conducted with the same results: Mindenes 11, Mindenes 12 and Mindenes 21 lines proved to be superior owing to their resistance. Our analyses verified that transgene CP was produced in all of these three lines. From the other transgenic plant group examined three prominent lines were found as well: Mindenes 8, Mindenes 9 and Mindenes 15. In M8 the level of CP expression was low, in M9 no expression was detected, while in M15 CP was produced at a high level.

The lines found to be superior in field experiments (M11, M12 and M21) were tested by grafting the next year. Virus concentration was studied in transgenic potato lines grafted on tobacco stocks infected with PVY^{NTN}. Two plants were examined per each transgenic potato lines. ELISA tests showed virus concentration was under the infection limit in each case. The virus concentration in the transgenic potato branches was zero or slightly above than that, while in the stocks it was 10-14 times higher than the threshold value. The results were the same with healthy transgenic potato stocks and infected tomato branches. While the virus concentration in the infected branches was 10-14 times higher than the threshold value, virus could not be detected in the transgenic potato stocks.

NEW SCIENTIFIC RESULTS

Virus-resistant transgenic lines were produced from three tobacco (Virgin D, Stamm C2 and Hevesi 11) and two potato (Mindenes and Somogyi Kifli) cultivars. These transgenic lines carrying the coat protein gene of PVY-H Hungarian isolate were proved to have a high level of resistance to the virus by molecular analyses and different field examinations.

In the course of the experiment no disadvantageous features were found in the transgenic plants. Quality values in these plants were around the average, however, Mindenes 11, Mindenes 12 and Mindenes 21 demonstrated much higher yield potential with their yields of 38, 26 and 43 t/ha than the Hungarian average (17-19 t/ha).

Our results show that plant breeding and agriculture can profit from the development of resistant transgenic plants produced by transferring viral coat protein genes into susceptible varieties. Similarly to experiments completed abroad, in which PVY-resistant transgenic plants were produced from several potato cultivars (e.g. Russet Burbank, Bintje), it has also become important in Hungary to produce virus-resistant transgenic lines with the use of CP genes originating from the Hungarian isolate. Transgenic plants developed by this technology can be used in agriculture, while our method can be applied in plant breeding, primarily in potato breeding. In this way old varieties, which were withdrawn from cultivation because of their susceptibility to the virus and most of which can only be found in gene banks, can now be reintroduced in the fields, enlarging the number of varieties utilized in agricultural production.

CONCLUSIONS

Among virus-resistant lines we found some lines which produced CP, some lines which produced CP at a low level and others which did not produce CP at all. On the basis of these results we concluded that the resistance to PVY is independent of protein production. These findings coincide with the outcome of other experiments and the characteristic is typical of the *Potyviridae* family. Regner et al. (1992), for example, developed transgenic *N. benthamiana* and *N. clevelandii* plants using the transgene PPV CP. They also came to the conclusion that the presence of protein is not necessary for the manifestation of PPV-resistance. Lindbo and Dougherty (1992) also found RNA-dependent resistance in transgenic tobacco plants carrying the gene TEV CP after the plants were inoculated with TEV. Similarly to our results, several other experiments prove that there is no connection between the extent of resistance and the level of transgene expression in the *Potyviridae* family (van der Vlugt et al., 1992, Kollár et al., 1993, Smith et al., 1995, Hammond and Kamo, 1995).

On the basis of our findings it can be stated that resistance is primarily initiated by the transgene or its transcript. Resistance is probably induced by the phenomenon of gene silencing which is accompanied by low level or undetectable transgene expression. The explanation of the ‘recovery’ of plants in our greenhouse experiments can be gene silencing which is a sequence specific degrading mechanism where a so-called ‘threshold model’ (Smith et al., 1994) manifests in transgenic plants as follows: if the amount of transgenic RNA exceeds a certain threshold level, a citoplasmic RNA degrading mechanism is initiated. As a consequence, nucleic acids originating from the infecting virus, which are homologous to the transgene, are also degraded, and thus virus infection does not occur and plants become resistant to subsequent virus infections as well. According to Goodwin et al. (1996) the ‘recovery’ phenomenon can also be explained by the integration of one or two copies of the transgene in the plant genome, whereas three or more copies are needed for resistance. In case of the ‘recovery’ phenomenon threshold level is reached by the amount of transgene RNA and viral transcripts together, which initiates specific nucleic acid degradation and thus the first symptoms of virus infection are followed by a virus-free state. Due to the initiation the gene silencing, the spreading of singal molecule and the production of specific nucleases plants remain resistant throughout their lives to the viruses having homologous nucleic acids to the transgene or its transcript in the plant.

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