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Efficient pathogen-derived resistance induced by integrated potato virus Y coat protein gene in tobacco

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Summary — The coat protein (CP) gene from potato virus Y (Hungarian isolate, PVY-H) was engineered into *Agrobacterium tumefaciens* binary vector for expression in different tobacco lines. Three different *Nicotiana tabacum* breeding lines were transformed and the integration of the CP gene was confirmed by PCR technique using genomic DNA preparations. The transcription and expression of the integrated CP gene was detected by Northern and Western blots. Pathogen-derived resistance was demonstrated by inoculation of the R1 progeny of the transformed lines with purified PVY-H. The efficiency of protection varied between different transgenic plants ranging from almost complete to no protection. Five CP expressing tobacco lines were resistant to challenge infection with PVY-H as indicated by attenuation or absence of symptom development associated with reduction or lack of detectable virus accumulation. Data from Western blots showed that there is no correlation between the level of the expressed CP and the extent of protection. This suggests that the mechanism of the observed resistance is independent of the level of CP accumulation in the transgenic tobacco plants.

potato virus Y / coat protein gene / coat protein-mediated resistance / transgenic plants

Introduction

The first successful demonstration of genetically engineered resistance to a plant virus was reported by Powell *et al* [1] with the integration of the coat protein (CP) gene from tobacco mosaic virus (TMV) into tobacco plants. The resistance provided by expression of a viral coat protein in transgenic plants is a form of the pathogen-derived resistance [2] and was termed CP-mediated resistance [3]. The CP-mediated resistance has been reported for numerous plant viruses from at least nine different plant virus groups (for a review see Beachy *et al* [3] and Gadani *et al* [4]). At present, there are several data regarding the expression of potyvirus CP genes in transgenic plants [5–13]. Various levels of protection against the challenge virus were obtained and the CP-mediated resistance was characterized by a reduced rate of systemic infection, attenuated and delayed appearance of disease symptoms as well as by lower virus titers in the CP expressing plants. The mechanism of the CP-mediated resistance is not fully understood. It may be linked to the inhibition of virus uncoating and/or to the interference with virus movement in the plant [14–18].

Potato virus Y is the type member of the potyviruses, the largest known group of plant viruses [19]. It causes significant yield losses in a number of economically important crops [20]. The potyviruses have flexuous rod-shaped virions and monopartite genomes consisting of a single-stranded messenger-sense RNA of approximately 10 kilobases that contains a 5'-terminal genome linked protein and a poly(A) tract at the 3' end. The viral RNA is translated into a large polyprotein precursor which contains the CP at its C terminus. The polyprotein precursor is co- and post-translationally processed by at least three virus encoded proteases (for a review see Riechmann *et al* [21]).

In this article we report the transformation of three *Nicotiana tabacum* breeding lines with the CP gene of potato virus Y (Hungarian isolate, PVY-H [22]). Our study was aimed at the selection of resistant CP expressing transgenic plant lines. The molecular analysis of PVY-H CP expressing transgenic plants and the results of other research groups working on this phenomenon are discussed.

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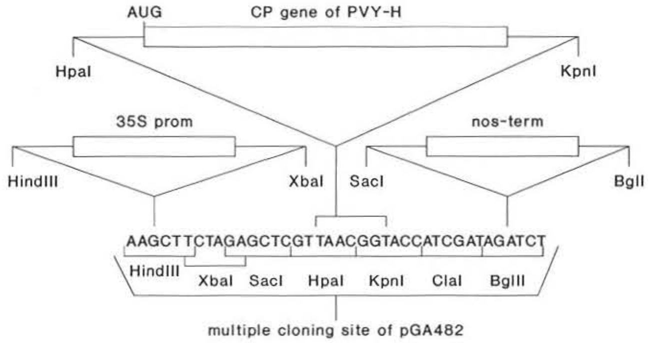

Fig 1. Construction of pGAYHCP. The *Hin*dIII–*Xba*I fragment containing the CaMV 35S promoter and the *Sac*I–*Bgl*I fragment including the nos terminator (both derived from the plasmid pBI221) were inserted into the *Hin*dIII and *Bgl*II restriction sites of pGA482, respectively. The *Hpa*I–*Kpn*I fragment of pYHCP23 carrying the PVY-H CP gene with an inserted AUG translational start codon at its 5' end was ligated into the *Hpa*I–*Kpn*I digested modified plasmid.

Materials and methods

Virus and viral RNA purification

PVY-H (Hungarian isolate of potato virus Y) used was a gift from Dr L Beczner (Institute for Plant Protection, Budapest). The virus was propagated in *Nicotiana tabacum* L cv *Xanthi* and purified essentially as described by Leiser and Richter [23]. RNA was isolated from the purified virus sample according to Robaglia *et al* [24].

Construction of the plasmid pGAYHCP

Complementary DNA (cDNA) of the 3' end of PVY-H genomic RNA was synthesized using an oligo(dT) primer with the cDNA Synthesis System Plus Kit (Amersham) according to the supplier's instructions. The cDNA library was cloned into *Sma*I digested and dephosphorylated pUC18 plasmid. The detailed cloning and sequencing strategies of the complete genomic RNA of PVY-H have been described [22]. One clone, pYH20 representing nucleotides from 8568 to 9514 includes the CP gene (nucleotides 8572–9372) and an additional part of the 3' non-coding region of PVY-H. The putative nucleotide positions coding the start and the end of the PVY-H CP gene were determined by the alignment of the 3' terminal nucleotide sequence of PVY-H with several CP coding nucleotide sequences derived from different PVY strains and isolates [24–29]. The *Hin*dIII-*Eco*RI fragment of pYH20 was subcloned into *Hin*dIII-*Eco*RI digested pBluescriptKS+ and an AUG initiation codon was added immediately upstream of the 5' end of the CP gene by site-directed mutagenesis [30]. The *in vitro* mutagenesis was performed by using the Muta-Gen Phagemid Kit (Bio-Rad) following the manufacturer's protocol and the synthetic oligonucleotide 5'-TGTCATTTCC**catg**ttaac*aagcttatcg*-3' comprising of nine nucleotides complementary to the 5' end of the CP gene (in capital letters), a *Nco*I-site (in bold), a *Hpa*I-site (underlined), a *Hin*dIII and *Cla*I-site from the vector cloning site (in italics). The mutated clone, pYHCP23 was sequenced to verify the insertion of the AUG codon (inside of the *Nco*I-site) as well as the nucleotide changes for creating the *Hpa*I-site. The blunt ended *Hin*dIII–*Xba*I fragment from pBI221 [31] containing the cauliflower mosaic virus (CaMV) 35S promoter was introduced into the blunt ended *Hin*dIII-site of the plant expression vector pGA482. The *Agrobacterium tumefaciens* Ti plasmid derivative pGA482 contains a kanamycin resistance marker, the neomycin phosphotransferase II gene, driven by the nopaline synthetase (nos) promoter, as well as a polylinker, which are located within the transferred (T-DNA) region [32]. The *Sac*I–*Bgl*I fragment representing the nopaline synthetase gene termination signal (nos terminator) was removed from the plasmid pBI221, blunt-ended and inserted into the blunt-ended *Bgl*II-site of pGA482. The *Hpa*I–*Kpn*I fragment of pYHCP23 was ligated into the *Hpa*I–*Kpn*I digested, modified pGA482. The plasmid obtained in this manner is pGAYHCP (fig 1).

In vitro transcription and in vitro translation

The pYHCP23 was linearized with *Hin*dIII, extracted with phenol and choroform, precipitated with ethanol and transcribed with T7 RNA polymerase using a T7 transcription system following the manufacturer's (Amersham) instructions. These transcripts were used for *in vitro* translation in rabbit reticulocyte lysate containing ^{35}S-labelled methionine (Amersham) as described by Dougherty and Hiebert [33]. The radiolabelled translation products were analyzed on 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) according to Sambrook *et al* [34] and detected by autoradiography.

Plant transformation

The plasmid containing the CP gene of PVY-H (pGAYHCP) was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating [35]. Leaf discs of three different *Nicotiana tabacum* breeding lines were transformed according to Horsch *et al* [36]. The tobacco breeding lines were provided by Dr Gy Nagy (Tobacco Research Institute, Debrecen/Hungary). Transformed calli were selected on kanamycin containing (300 mg/l) media, and progeny were obtained by germinating the seeds (gathered from the self-fertilized transgenic tobaccos) under sterile conditions in the presence of 150 mg/l kanamycin. The plant expression plasmid pGA482 was used to transform the three breeding lines as mentioned above. Plant lines derived from breeding line 1, 2 and 3 and transformed with the pGAYHCP were designated 1YCP, 2YCP and 3YCP, respectively. Plant lines transformed with the pGA482 were named in the same way (*ie* 1PGA, 2PGA and 3PGA).

Detection of the integrated CP gene in transgenic plant DNA

Plant DNA was extracted essentially as described by Rubino *et al* [37]. Polymerase chain reaction (PCR) was carried out in a Perkin-Elmer thermal cycler using the oligonucleotide primers 5'-CTTCGCAAGACCCTTCCTCT-3' and 5'-ATCGCAAGACCGGCAACAGG-3' chosen from the CaMV 35S promoter and the nos terminator, respectively. 2 µg plant DNA and 500 ng of both primers were used for the amplification, which began with an initial denaturing step (94°C for 4 min) and was followed by 40 reaction cycles containing a melting (94°C for 1 min), an annealing (48°C for 1 min) and a polymerization (72°C for 1 min) step in each cycle. The last polymerization step was extended by 10 min. The PCR products were analyzed by gel electrophoresis and Southern hybridization with ^{32}P-labelled nick-translated pYHCP23 as the probe according to Sambrook *et al* [34].

Nucleic acid analysis of the transgenic plants

For Northern blot analysis the total nucleic acid (TNA) isolation procedure of White and Kaper [38] was followed. The prepared TNA samples were separated on formaldehyde permeated denaturing gel and transferred to Hybond-N membrane (Amersham). For dot blot analysis the RNA preparations denatured with formaldehyde and heat treatment were spotted directly onto the membranes. The membranes were hybridized with ^{32}P-labelled nick-translated pYHCP23 as the probe as described by Sambrook *et al* [34].

Immunological analysis of the transgenic plants

Expression of the CP gene product in transgenic plants was demonstrated by Western blot analysis carried out according to Sambrook *et al* [34]. Total leaf protein was extracted from plant tissue, denatured, fractionated on 12% polyacrylamide gel containing 0.1% SDS and transferred to Hybond-C extra membrane (Amersham). The alkaline phosphatase conjugated anti-PVY CP antibody, and the chromogenic substrates NBT (para-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate para-toluidine salt) (all purchased from Boehringer Mannheim GmbH) were used to detect the membrane-bound protein.

Plant inoculation and plant lines

The purified PVY-H preparation was diluted to 0.5 μg/ml, 5 μg /ml and 20 μg/ml with 0.1 M Na-phosphate buffer (pH 7.2). In three independent experiments the two lower, fully developed leaves of six plants of every tested plant line were inoculated with each PVY-H concentration using carborundum powder as an abrasive with a sterile glass spatula. In the experiments 12 lines of 1 YCP plant line (designated lYCP/1, lYCP/2, ..., 1YCP/12), 12 lines of 2YCP plant line (designated 2YCP/1, 2YCP/2, ..., 2YCP/12) and six lines of 3YCP plant line (designated 3YCP/2, 3YCP/5, 3YCP/7, 3YCP/8, 3YCP/12, 3YCP/42) as well as one line of 1PGA, 2PGA and 3PGA plant lines were inoculated. In addition, in each case 12 non-transformed tobacco plants were inoculated and four plants of each plant line were not inoculated as control plants.

Results and discussion

Molecular cloning, transformation and analysis of plants

From the cDNA library of the clones containing the 3' end of PVY-H the pYH20 was selected, which includes the complete CP gene (800 nucleotides) with an additional 142 nucleotides of the 3' non-coding region of PVY-H. To express the PVY-H CP cDNA in transgenic plants, the CP gene was provided with an AUG translational start codon by site-specific mutagenesis. The resulting pYHCP23 was transcribed *in vitro* and the transcripts were *in vitro* translated in rabbit reticulocyte lysate. The transcripts produced a protein of the expected size confirming that the CP gene could be translated (data not shown). The CP gene containing fragment of pYHCP23 was cloned into the modified pGA482 plasmid which harbours plant transcriptional regulation sequences (*ie* the CaMV 35S promoter and the nos terminator). The resulting pGAYHCP plasmid (fig 1) as well as the original pGA482 plasmid were mobilized into *Agrobacterium tumefaciens* and several lines of kanamycin resistant transgenic YCP (including 1YCP, 2YCP and 3YCP, derived from the three *Nicotiana tabacum* breeding lines) and PGA (including 1PGA, 2PGA and 3PGA) plant lines were obtained *via* the leaf disc transformation procedure. The PGA plant lines carry the neomycin phosphotransferase gene, and the YCP plant lines contain in addition the PVY-H CP gene. The regenerated plants which were transplanted into soil grew vigorously and were normal in appearance.

The R1 progeny of the YCP and PGA plants were selected on kanamycin containing (150 mg/l) RM media and planted into the greenhouse. DNA preparations were extracted from leaves of five R1 YCP plants and one R1 PGA plant as well as from leaves of one non-transformed plant and analyzed with PCR amplification of sequences located between the CaMV 35S promoter and the nos terminator by using oligonucleotide primers chosen from the CaMV 35S promoter and the nos terminator, respectively. The presence of the expected sequence for the PVY-H CP message was evident in the five transgenic YCP plants (as shown in figure 2, lanes 1–5) and was not found in the transgenic PGA plant and in the non-transformed plant (fig 2, lanes 6, 7). A transcript of approximately 1200 nucleotides was detected in the RNA extracts of

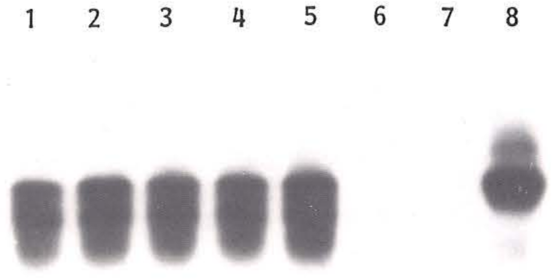

Fig 2. Southern blot analysis. DNA extracts prepared from *Nicotiana tabacum* plants transformed with pGAYHCP (lanes 1–5) or pGA482 (lane 6) were amplified with PCR. Lane 7, PCR product from non-transformed plant; lane 8, product amplified from 1 ng of purified pGAYHCP. The membrane was hybridized with the ^{32}P-labelled pYHCP23 probe.

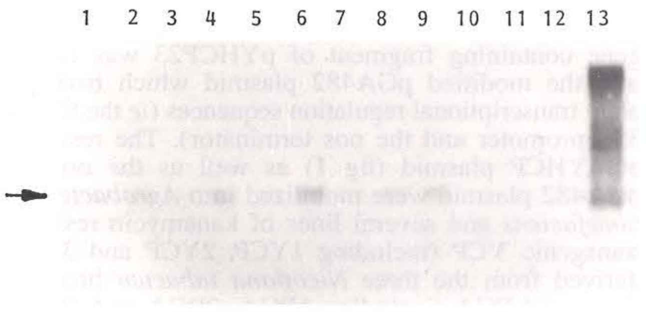

Fig 3. Northern blot analysis. RNA was extracted from transgenic YCP (lanes 1–9) and PGA (lanes 10, 11) plants and from a non-transgenic plant (lane 12). Lane 13, *in vitro* transcript from pYHCP23. Clone pYHCP23 was used as the hybridization probe. The arrow indicates the position of the CP-related transcripts present in the transgenic YCP plants.

the transgenic YCP plants by Northern blot analysis (fig 3, lanes 1–9). The transcript is expected to comprise 942 nucleotides from the PVY-H CP coding sequence and an additional part of the 3' non-coding region completed with the bordering vector sequences and the poly(A) tail. No transcripts were found in samples from the RNA extracts of the PGA and the non-transformed plants (fig 3, lanes 10–12).

The accumulation of the CP in transgenic YCP plants was confirmed by Western blot analysis using polyclonal antisera to PVY CP (fig 4, lanes 2–9). The YCP plants showed generally various levels of CP expression, except for one plant line, which did not contain a detectable amount of CP. In the different transgenic YCP plant lines the maximum CP amount was approximately 0.2 μg protein per g leaf tissue. The CP expression level generally correlated with the CP transcript expression level as determined by the Northern and Western analyses.

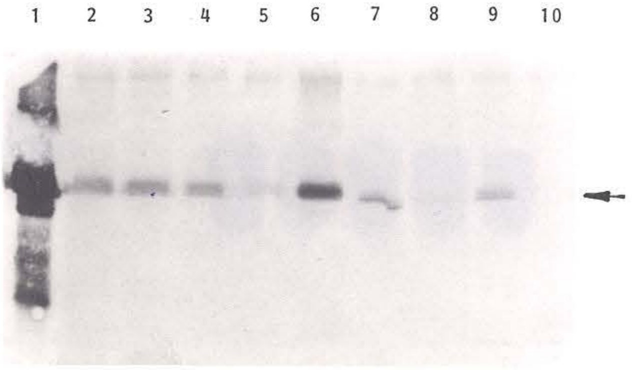

Fig 4. Western blot analysis. Protein samples were prepared from transgenic YCP plants (lanes 2–9) and from a transgenic PGA plant (lane 10). Protein sample from a *Nicotiana tabacum* plant infected with PVY-H was used as the positive control (lane 1). The arrow indicates the position of the CP.

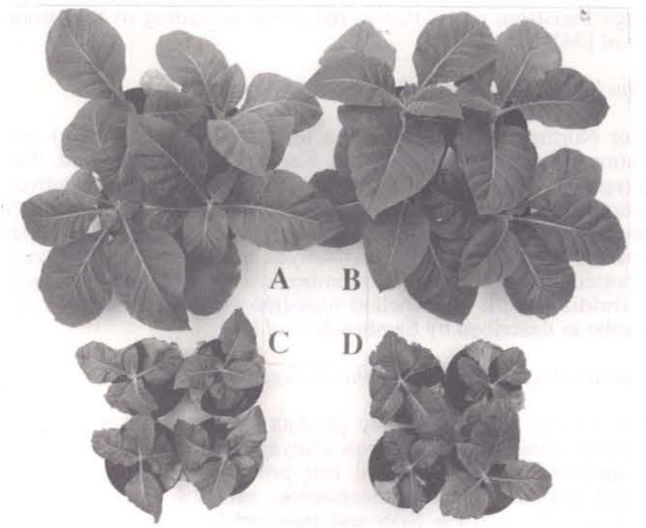

Fig 5. Comparison of symptom development after inoculation with PVY-H. Transgenic YCP/4 plants inoculated with 5 μg/ml PVY-H (B) are similar in appearance as the non-infected control 1YCP/4 plants (A), while transgenic lPGA plants inoculated in the same way (C) show severe disease symptoms as well as the non-transformed, inoculated plants of breeding line 1 (D).

Table I. Susceptibility of the transgenic plants to PVY-H infection. The ratios show the number of diseased plants per number of inoculated plants. The number of plants showing attenuated symptoms is included in the number of diseased plants. In the table we mention only three of the non-resistant plant lines.

	Inoculum concentrations		
Plant lines	*0.5 μg/ml*	*5 μg/ml*	*20 μg/ml*
1YCP/4	0/6	1/6	2/6
1YCP/7	2/6	2/6	0/6
1YCP/8	2/6	1/6	2/6
3YCP/8	3/6	0/6	1/6
3YCP/12	3/6	5/6	4/6
1YCP/3	6/6	6/6	6/6
2YCP/7	6/6	6/6	6/6
3PGA/2	6/6	6/6	6/6

Protection against PVY infection

Non-transformed and kanamycin selected R1 progeny of transformed YCP and PGA tobacco plants were grown in growth chambers with 14 h/23°C light and 10 h/20°C dark cycles. In three independent experiments 12 1YCP lines, 12 2YCP lines and six 3YCP lines were inoculated together with one line of 1PGA, 2PGA or 3PGA as well as with non-transformed tobacco plants as the positive control. The plants were inoculated with 0.5 µg/ml, 5 µg/ml and 20 µg/ml of purified PVY-H. In each experiment non-inoculated plants of the 1YCP, 2YCP or 3YCP plant lines were the negative control plants. The first systemic symptoms appeared on the non-transgenic control plants after 7 days post-inoculation and were manifested as vein clearing on the upper leaves followed by vein necrosis and leaf mottling. In every case the PGA transgenic plant lines responded to the challenge inoculation in a similar manner as the non-transformed control plants (see fig 5C, D). Among the YCP plant lines five lines (1YCP/4, 1YCP/7, 1YCP/8, 3YCP/8 and 3YCP/12) were resistant to PVY-H challenge inoculation, which was indicated by the absence or attenuation of symptom development (fig 5B; table I). However, on plants of the plant line 3YCP/12 mainly attenuated symptoms were observed compared to the positive control plants (table I). The resistance of these lines was not overcome at higher inoculum concentrations (5 µg/ml and 20 µg/ml). The remaining 25 YCP plant lines, for example 1YCP/3, 2YCP/7 (table I) were not protected from PVY-H infection and showed typical wild type symptoms. Significant delay of symptom development in transgenic plants showing viral disease symptoms could not be detected.

Virus replication was monitored by taking samples from leaves above the inoculated ones 5, 10, 15 and 20 days post-inoculation. Dot blot analysis of these samples showed a lack of detectable viral RNA in the symptomless plants and a reduced amount of RNA in plants with milder symptoms (fig 6). This indicates that the resistance probably resulted from the inhibition of virus replication, which could cause the attenuation or the lack of the symptom development. No connection could be detected between the expressed CP level and the extent of resistance. Resistance was maintained as long as the tests were terminated after 48 days post-inoculation.

CP-mediated resistance proved to be efficient in many cases including alfalfa mosaic virus (A1MV) [39], cucumber mosaic virus (CMV) [40], potato virus X [41], TMV [1] and tobacco rattle virus [42]. However, conclusive data underlying the mechanism of the CP-mediated resistance are still not available. To date the TMV CP:tobacco combination is the most studied virus CP:host system, in which the requirement of the CP for the protection is confirmed [43]. In this case reasons for the resistance could be the inhibition of uncoating of the challenge virus and its cell-to-cell movement [14–16] as well as the interference with the viral long-distance movement [17]. However, it is apparent that the mechanism of the protection is not the same in different virus:host systems. In the case of TMV, AlMV and some other plant viruses the highest level of CP-mediated resistance correlated with the highest CP expression level [43, 44]. But there are more and more data regarding viral CP expressing plants which do not show this correlation as in the case of CMV [40], potato leaf roll virus [45], PVY [6, 13] and tobacco etch virus (TEV) [9] CP expressing plants. Moreover, Lindbo and Dougherty [9] proved that transgenic plant lines expressing truncated forms of the TEV CP tended to confer greater protection than the full-length CP constructs and that untranslatable TEV CP transcript expressing plants are highly protected against TEV infection [46].

CP-mediated protection may confer broad spectrum resistance as shown in the case of some potyviruses

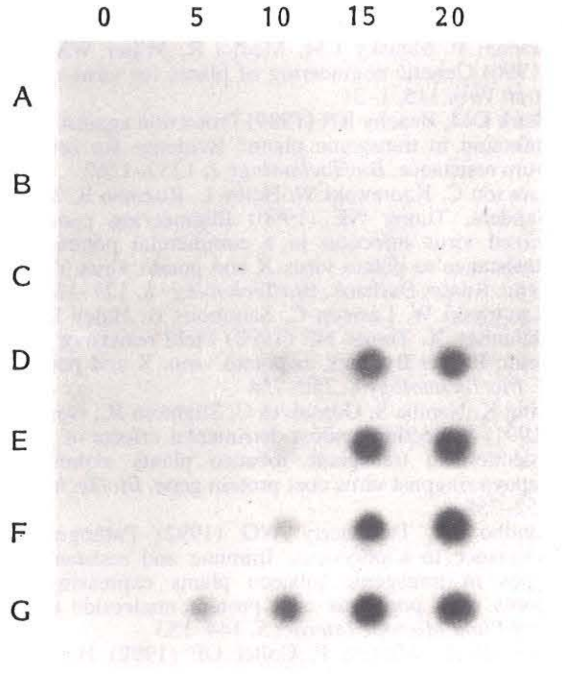

Fig 6. Dot blot analysis. RNA samples from 1YCP/4 (A–C), 1PGA/1 (D, E) and from non-transformed breeding line 1 (F) plants were extracted just before the inoculation with 5 µg/ml PVY-H (first column) and 5, 10, 15 and 20 days post-inoculation (second, third, fourth and fifth column). The numbers above the columns indicate the number of days post-inoculation. Samples in lane G are 0, 5, 15, 50 and 100 ng of purified PVY-H RNA.

[5, 8, 12]. Plants transformed with the CP gene of soybean mosaic virus [5], papaya ringspot virus [8], watermelon mosaic virus II and zucchini yellow mosaic virus [12] were resistant to other potyviruses as for example TEV, PVY and pepper mottle virus but not to TMV or CMV. The results of the field tests with PVY CP transgenic potato plants [7] further encourage us to test more YCP transgenic plants and to investigate whether the five YCP transgenic lines which were resistant to the infection with PVY-H could be protected against other potyviruses and whether they could show field resistance, too.

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