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QUARTERLY

Expression of the plum pox coat protein gene in transgenic *Nicotiana tabacum* plants*

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Plant expression vector pBI 121 containing the gene encoding coat protein of Plum Pox Virus of the Skierniewice isolate (CP PPV-S) was prepared (clone pCM1). The construct was used for transformation of *Nicotiana tabacum* plants using an *Agrobacterium tumefaciens* based system. About 82% of kanamycin resistant plant lines contained a transgene (the sequence of CP PPV-S) but only 81% of them actively expressed the PPV-S coat protein gene as measured by RT-PCR.

Coat-protein (CP)[1] mediated protection of transgenic plants against viral infection is well documented [1–4]. The mechanism of this protection is much less known. In some cases accumulation of CP was necessary to induce the resistance against viral infection [5], in others the RNA coding for the capsid protein was essential [6, 7] and, accordingly, various protection mechanisms have been proposed. As in the case of other potyviruses, the expression of the Plum Pox Virus (PPV) coat protein in transgenic plants confers resistance to the virus [8]. Aiming at construction of plums tolerant to PPV strains commonly found in Poland we have isolated the PPV-S CP-gene [9]. This work presents the results of introducing the PPV-S CP-gene into tobacco plants used as a model host.

MATERIALS AND METHODS

Construction of plasmids. The cDNA clone, containing a coat protein coding region of plum pox virus was described previously [9]. The clone was named pCPP3.

The plasmid pCM1 contains a *Bam*HI fragment from pCPP3 inserted in the proper orientation in a *Bam*HI site of pBI 121 [10], between the 35S cauliflower mosaic virus promoter and the GUS reporter gene with the nopaline synthase terminator sequences.

Transformation and regeneration of Nicotiana tabacum plants. Leaf discs of *N. tabacum* were transformed by the Agrobacterium-mediated procedure [11]. Transformation of *Agrobacterium tumefaciens* strain 4404 with pCM1 was

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[1] Abbreviations: BAP, 6-benzylaminopurine; bp, base pair; CP, coat protein; PPV, plum pox virus; GUS, β-glucuronidase; IAA, indole-3-acetic acid; MES, 2[*N*-morpholino] ethanesulfonic acid; nt, nucleotide; PEG, polyethylene glycol; RT-PCR, reverse transcriptase-polymerase chain reaction.

performed by the triparental mating procedure [12].

Calluses were obtained and shoots of *N. tabacum* were regenerated on a medium consisting of B5 salts [13], MES buffer, pH 5.7 (0.5 g/l), NH_4NO_3 (250 mg/l), glucose (20 g/l), adenine (40 mg/l), IAA (0.1 mg/l), BAP (1 mg/l), agar (8 g/l), $AgNO_3$ (20 μM), $Na_2S_2O_3$ (160 μM), carbenicillin (500 mg/l) and kanamycin (50 mg/l). Transformed shoots were subsequently rooted on phytohormone-free medium containing 100 mg/l of carbenicillin [14].

DNA and RNA isolation. Total nucleic acids were isolated by the modified guanidinium thiocyanate method [15].

Leaf material, approx. 100 mg fresh weight, was frozen in liquid nitrogen and ground. Powdered leaf tissue was suspended in 500 μl of the denaturing solution [15], and extracted with phenol-chloroform. Total nucleic acids were precipitated with isopropanol, pelleted, washed twice with 75% (v/v) ethanol, vacuum desiccated and dissolved in 50 μl of diethyl pyrocarbonate (DEPC)-treated water. To purify DNA or RNA, this preparation was digested either with RNase A (Boehringer) or with RNase-free DNase (Promega), respectively.

Detection of the coat protein gene in transformed plants. Transformed tobacco plants were checked for the presence of CP gene by PCR analysis. PCR was performed with PPVCP1 and PPVCP2 as primers [9] to generate the 1 025 bp product (993 nt encoding the CP protein plus extra sequences introduced by the primers) under the following condition: denaturation 94°C for 1 min followed by 1 min at 63°C and 1 min at 72°C; 30 cycles.

In paralell, PCR of the $tRNA^{Tyr}$ gene [16] fragment (130 bp) was performed as a control of quality of the DNA used in the PCR reaction. The following oligonucleotides were designated as primers for amplification of the tRNA gene: P3: ctgctgagatctttaggtcac and P4: ctttctgccaaaacggatccaaagc. The reaction mixture was denatured at 94°C for 1 min, annealed at 57°C for 1 min, and elongated at 72°C for 1 min. The cycle was repeated 30 times.

Analysis of the coat protein gene transcripts in transgenic N. tabacum. The transcript of CP PPV-S was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) using PPVCP1 [9] and PPVCP3 (cgccagatacgcgtttgatttttac, complementary to nucleotides 774–798 of the PPV CP gene) as primers to generate 235 bp fragment. The reaction was performed in 10 μl mixture containing: 10 mM Tris/HCl, pH 9 (at 25°C), 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM dNTPs, 0.5 mM PPVCP1, 0.2 mM PPVCP2, 1 U RNasin (Promega), 1.2 U AMV reverse transcriptase, 1 U Taq DNA polymerase. After denaturation of total RNA (about 50 ng in 2 μl), all reagents were combined together and the reaction mixture was incubated at 42°C for 45 min followed by 5 min at 94°C (1 cycle), and then 1 min at 94°C followed by 1 min at 50°C, 1 min at 72°C (2 cycles), and finally 1 min at 94°C followed by 1 min at 55°C, and 1 min at 72°C (30 cycles).

Additionally, the purity of RNA probes was checked by control RT-PCR reactions. The samples of RNA were digested with RNase A, and RT-PCR reactions were performed as described above. Lack of any product indicated the absence of contaminating DNA.

Analysis of transgenic plants for GUS expression. Selected transgenic plant lines were analysed for GUS expression using the method described by Jefferson [10].

RESULTS AND DISCUSSION

Nicotiana tabacum was transformed with a cDNA fragment of the PPV-S CP region, which was inserted in a proper orientation in the plant expression vector pBI 121 (Fig. 1). The orientation of *Bam*HI insert in the pCM1 was established by restriction enzyme analysis of recombinant plasmid.

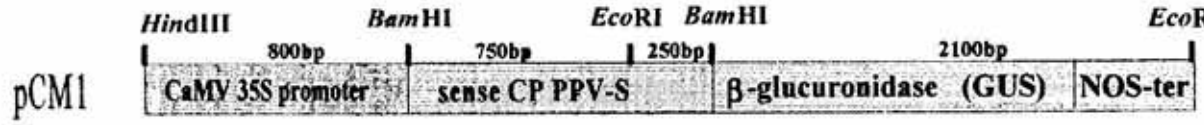

Fig. 1. Orientation of BamHI insert in the pCM1 clone.
Schematic presentation of ligation products of pBI 121 with CP PPV-S sequences.

The PCR reaction indicated that 32 of the 39 transformed lines of *N. tabacum* plants carried PPV-S CP sequences (Fig. 2). The PCR of the 130 bp fragment of the $tRNA^{Tyr}$ gene [16] was performed to check the quality of DNA used for detection of CP sequences in plant lines. In all lines we had appropriate control products (not shown). The obtained R_0 lines of transformed *N. tabacum* were designated from 0-CM1-01 to 0-CM1-41 (lines 0-CM1-26 and 30 were lost) (see Fig. 2). In all transgenic plant lines we observed almost the same content of CP sequences except in lines 0-CM1-01, 04, 27, 29 and 32 which had no detectable CP sequences.

The relative amounts of transcripts from CP genes were determined by RT-PCR reactions (Fig. 3). To check the possible contamination of RNA probes with DNA, control reactions were performed. No probes contained DNA impurities (not shown). In the majority of transgenic plant lines the content of the CP transcript was about the same and rather high. In contrast, the plant lines: 0-CM1-14, 16, 23, 25, 35, and 40 had no detectable transcript, although the content of the CP DNA was the same as in other lines. Plant lines 0-CM1-5, 11, 17, 21, 24, 34, and 38 accumulated less of transcripts than other lines. Obviously, no transcript was detected in the plant lines where PCR showed a lack of the PPV-S CP gene sequences.

It is not clear why the same plants apparently containing the whole PPV-S CP gene did not produce the transcript. One could speculate that there occured a deletion of the sequences located left to the PPV-S CP gene, as it is well known that the left end of T-DNA is transferred to plants less precisely than its right region [17], but all transgenic tobacco lines used for the present investigation were grown on kanamycin containing medium which should have prevented this deletion. Thus, evidently, they expressed the NTP II gene, located in the vector plasmid upstream to the CaMV promoter [10].

All transformed tobacco plants were checked for the content of GUS. The glucuronidase content in a control plant transformed with pBI 121 was comparable to its activity in transgenic tobacco described by Jefferson *et al.* [10]. Plants transformed with the recombinant plasmid pCM1 showed practically no GUS activity. Probably the location of PPV-S CP gene immediately upstream to the GUS gene inhibited its translation, the more so that this inhibition could be probably caused by dissociation of ribosomal subunits encountering two stop codons (UAGUAG; introduced immediately be-

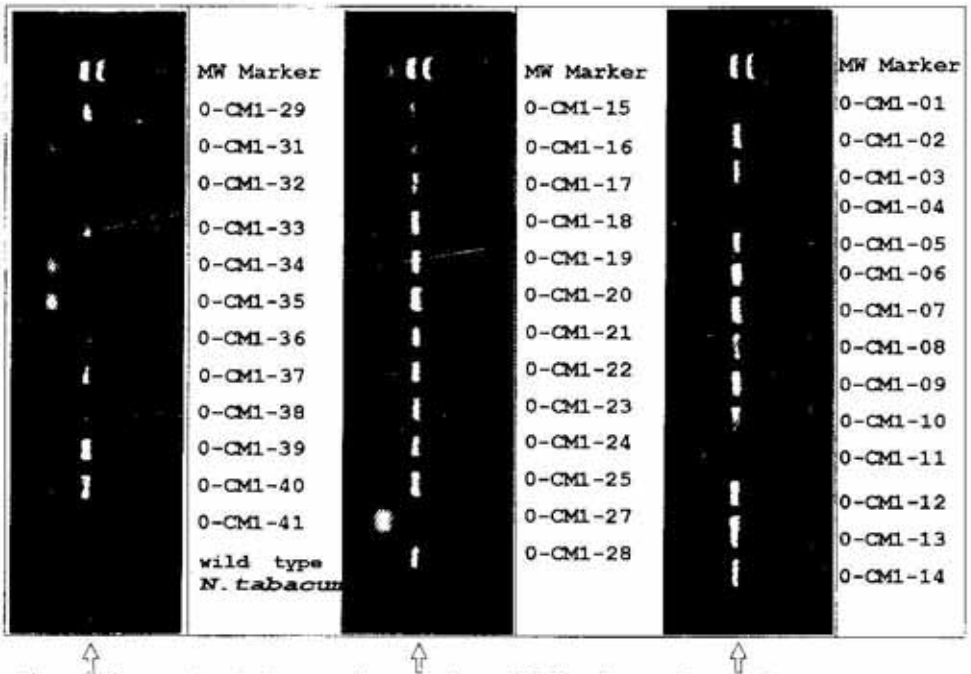

Fig. 2. Detection of the coat protein gene in transformed Nicotiana tabacum lines.
DNAs isolated from transformed plant lines were used for PCR reactions as described in Materials and Methods and electrophoresed on 1% agarose gel. The arrows indicate the 1025 bp bands, PCR products of the CP PPV-S gene. Transgenic plant lines were designated 0-CM1-01–0-CM1-41. The control reaction was performed using the wild type of *N. tabacum* (lane: wild type *N. tabacum*). pBR322 DNA-*Bst*NI digested was used as the relative molecular mass marker (1858, 1058, 929 and 383 bp).

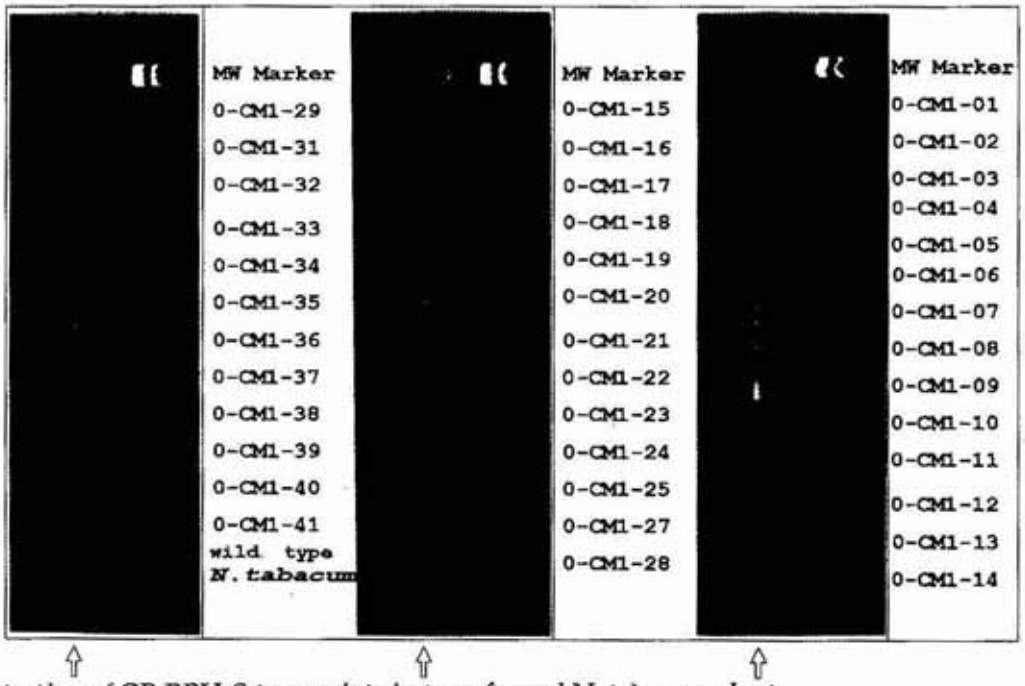

Fig. 3. Detection of CP PPV-S transcripts in transformed N. tabacum plants.
Transcripts of the CP PPV-S gene were detected in transgenic plants by the RT-PCR technique as described in Materials and Methods. RT-PCR products were electrophoresed on 3% agarose gel. As relative molecular mass marker was used pBR322 DNA digested with *Bst*NI (1858, 1058/929, 383 and 121 bp). The arrows indicate 235 bp DNA fragments. Other descriptions as for Fig. 2.

fore AUG codon of the GUS gene sequence) at the end of the CP gene sequence.

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