

## Expression of cowpea mosaic virus coat protein precursor in transgenic tobacco plants

D. L. Nida,<sup>1</sup>† J. R. Anjos,<sup>1</sup> G. P. Lomonossoff<sup>2</sup> and S. A. Ghabrial<sup>1\*</sup>

<sup>1</sup>Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40546, U.S.A. and <sup>2</sup>Department of Virus Research, John Innes Institute, John Innes Centre for Plant Science Research, Norwich NR4 7UH, U.K.

Tobacco, *Nicotiana tabacum* L., supports cowpea mosaic virus (CPMV) replication and cell-to-cell movement, and thus may serve as a model system to study coat protein-mediated protection against CPMV. A chimeric gene consisting of the cauliflower mosaic virus 35S promoter, CPMV 60K coat proteins-precursor (CP-P) coding region, and the nopaline synthase polyadenylation signal was transferred to tobacco cv. Burley 21 via the *Agrobacterium tumefaciens* binary vector system. Gene integration and expression in the transgenic tobacco plants were confirmed by Southern and RNA dot blot analyses. Accumulation of CPMV 60K CP-P in transgenic plants, up to 2 µg/g of wet weight tissue, was detected by ELISA and Western blots. The results of Western

blots and immunosorbent electron microscopy further indicated that CPMV CP-P neither undergoes auto-proteolysis to generate the mature viral coat proteins nor assembles into virus-like capsids, suggesting that processing of the CP-P may be required for virus assembly. Because CPMV neither induces symptoms in tobacco nor moves systemically, evaluation of the reactions of the transgenic plants to virus inoculation was based on virus accumulation in the inoculated leaves. Results from such infectivity experiments did not differentiate between CP-P expressers and vector-transformed plants. The transgenic tobacco plants expressing CP-P should provide valuable material for investigating comovirus polyprotein processing and capsid assembly *in vivo*.

### Introduction

Transgenic plants expressing viral genes provide useful systems to investigate host–virus gene product interactions, and in some cases demonstrate a potential for disease control. The expression of tobacco mosaic virus (TMV) coat protein in transgenic tobacco plants has been correlated with a delay in symptom development and virus accumulation after inoculation with TMV (Powell Abel *et al.*, 1986; Nelson *et al.*, 1987). This approach, termed coat protein-mediated protection (Beachy *et al.*, 1990), has been extended to include a number of viruses such as alfalfa mosaic virus (Loesch-Fries *et al.*, 1987; Tumer *et al.*, 1987; van Dun *et al.*, 1987, 1988*a*), tobacco streak virus (van Dun *et al.*, 1988*a*), tobacco rattle virus (van Dun *et al.*, 1987, 1988*b*), cucumber mosaic virus (Cuozzo *et al.*, 1988), potato virus X (Hemenway *et al.*, 1988; Hoekema *et al.*, 1989) and potato virus Y (Lawson *et al.*, 1990).

Coat protein-mediated protection has so far been shown to be applicable to viruses whose capsids contain

a single type of coat protein. This strategy has not been pursued with viruses in the comovirus group whose capsids are made up of two types of coat protein. Cowpea mosaic virus (CPMV), the type member of the comovirus group, has a bipartite genome consisting of two separately encapsidated single-stranded positive sense RNAs, designated M (middle component)-RNA and B (bottom component)-RNA. CPMV expresses its genome via the production of large polyproteins which are proteolytically cleaved into mature viral proteins. M-RNA is translated into two large polyproteins of 95K and 105K with overlapping amino acid sequences. These polyproteins are proteolytically cleaved at similar sites to yield two polypeptides of 58K and 48K, derived from the amino-terminal portions of the polyproteins, together with a 60K polyprotein which represents the precursor to both capsid proteins VP37 and VP23 (Goldbach & van Kammen, 1985). Therefore, it is of interest to determine whether constitutive expression of the coat proteins-precursor (CP-P) of a comovirus in transgenic plants confers protection against virus infection.

To address this question in a plant system that is readily amenable to transformation and regeneration, we chose to transform tobacco with the CPMV 60K CP-P

† Present address: Monsanto Agricultural Company, New Products Division, 700 Chesterfield Village Parkway, Chesterfield, Missouri 63198, U.S.A.

coding region. Although tobacco is not a systemic host for CPMV, it supports virus replication and limited spread in the inoculated leaf (Huber *et al.*, 1977). Thus, tobacco may serve as a model system to study CP-P-mediated protection against CPMV. In this paper, we report the production of transgenic tobacco plants that express CPMV 60K CP-P and describe their response to CPMV infection.

## Methods

**Virus purification.** The SB strain of CPMV (obtained from O. W. Barnett, Clemson University, Clemson, S.C., U.S.A.) was increased in *Vigna unguiculata* Walp. cv. Early Ramshorn. Virus purification was by the method of Ghabrial & Schultz (1983) previously reported for the purification of bean pod mottle virus. Purified virus was quantified by its absorbance at 260 nm assuming  $8.1 A_{260} = 1$  mg nucleoprotein/ml (van Kammen & de Jager, 1978).

**Plasmids.** pSPBa1709 was derived from clone M13-3'M4 (Holness *et al.*, 1989) which contained the CPMV 60K CP-P coding region and flanking sequences [M-RNA sequences starting from the *Bam*HI site at position 1504 and continuing through the entire 3' end of the molecule including 17 A residues of the poly(A) tail]. The insert in M13-3'M4 was cloned into *Bam*HI/*Hinc*II-cut M13mp19. To remove most of the sequence upstream of the Met codon which marks the start of the coat protein sequence, M13-3'M4 was cut with *Bam*HI and a 761 bp *Sau*3AT fragment, derived from bases 2067 to 2828 of the CPMV B-RNA sequence, was inserted at the *Bam*HI site. This fragment was used as a 'filler' to allow effective unidirectional deletions to be made by *Bal* 31 exonuclease. It was selected because it restored the *Bam*HI site at one side of the insertion but not the other. The restored *Bam*HI site was the one near the beginning of the CPMV coat protein sequence. The resulting construct was cut with *Bam*HI and digested for various lengths of time with *Bal* 31. After termination of the digestion, the DNA was restricted with *Xma*I, the protruding 5' termini were filled in with Klenow enzyme and dNTPs, and the vector was religated and used to transform *Escherichia coli* strain JM101. A number of plaques were isolated and the extent of the deletion was determined by sequence analysis. One clone, M13Bal-7, had the sequence between nucleotides 1504 and 1524 deleted, thereby removing the two upstream AUG codons which had interfered in previous translation experiments. The replicative form of clone M13Bal-7 was digested with *Eco*RI and *Pst*I, and the DNA fragment was isolated and inserted into the transcription vector pSPT19 (Pharmacia) which had been previously digested with *Eco*RI and *Pst*I. The resulting construct, pSPBa17, in which the coat protein sequence was downstream of an SP6 promoter, was found to have a single base deletion at position 2419 of the M-RNA. This was corrected by replacing the 784 bp *Eco*RV fragment from pSPBa17 with that from a clone which did not have the deletion, thus giving rise to pSPBa1709.

**pMON-CCP60.** An expression vector containing the CPMV 60K CP-P coding region was constructed for tobacco transformation as follows: the CPMV 60K coding sequence, derived from pSPBa1709 as a *Kpn*I/*Pst*I fragment, was inserted into the *Sma*I site of the binary Ti vector pMON530 (Rogers *et al.*, 1987) by dG:dC homopolymeric tailing (Eschenfeldt *et al.*, 1987). The resultant positive-sense construct, as determined by *Hind*III restriction analysis, was designated pMON-CCP60.

**Tobacco transformation.** A freezing and thawing method was employed to transform disarmed *Agrobacterium tumefaciens* Z707 directly with pMON-CCP60 (Holsters *et al.*, 1978). *A. tumefaciens* was

also transformed with pMON530 for the purpose of producing vector-transformed control plants. Sterile leaf discs of *Nicotiana tabacum* cv. Burley 21 were transformed by co-cultivation with *A. tumefaciens* (Horsch *et al.*, 1985). The discs were immersed in the bacterial suspension in Luria broth, blotted on filter paper, and placed on modified Linsmaier and Skoog medium (Kasperbauer & Collins, 1972) for 48 h. Discs were transferred to, and regenerated on, selective modified Linsmaier and Skoog medium containing 500 µg/ml mefoxin and 100 µg/ml kanamycin. Shoots were rooted on R1/2N medium (Nitsch, 1969) containing 500 µg/ml mefoxin and 100 µg/ml kanamycin. Plantlets were potted into sterile Promix B (Premier Brands), and established in a growth chamber at  $25 \pm 5$  °C with a 14 h photoperiod. Once the regenerated plants were established, leaf discs were removed, their surfaces were sterilized, and the discs were placed on selective modified Linsmaier and Skoog medium. Plants capable of forming callus in the presence of kanamycin were further analysed for gene integration and expression. Plants transformed with the CP-P coding sequence were designated KM-1, KM-2, etc. The vector-transformed plants were denoted MON-1, MON-2, etc.

**Southern blots.** Total nucleic acid preparations from regenerated tobacco plants were made by the CTAB procedure as described by Rogers & Bendich (1988). The DNA was doubly digested with *Eco*RI/*Stu*I and subjected to electrophoresis in 0.8% agarose gel. The DNA was transferred onto GeneScreen (New England Nuclear) according to the manufacturer's instructions and hybridized to a <sup>32</sup>P-labelled cDNA oligonucleotide probe of the CPMV 60K coding region (Feinberg & Vogelstein, 1985). The blot was washed, dried and autoradiographed.

**RNA dot blot.** RNA samples (50 µg) extracted from transgenic plants were denatured in 50% formamide and 6% formaldehyde at 60 °C for 10 min. The RNA samples were diluted with an equal volume of distilled water, applied to GeneScreen Plus by use of a dot blot manifold (Schleicher & Schuell) and hybridized with a <sup>32</sup>P-labelled cDNA oligonucleotide probe representing the 60K coding region.

**Serology.** The CPMV 60K CP-P was detected in transgenic plants and progeny by either the standard direct double antibody sandwich ELISA as described by Ghabrial & Schultz (1983) or, in some experiments, the indirect ELISA procedure described by Anderson *et al.* (1991) was followed, except that the plant samples were homogenized (1:5 w/v) in PBS pH 7.4, instead of the sodium carbonate buffer pH 9.6.

**Western blots.** Leaf samples from transgenic plants were chopped in 0.75 mM-Tris-HCl pH 6.8 sample buffer (1:1 w/v) containing 10 mM-2-mercaptoethanol and 9 M-urea. The mixture was filtered through 10 µm nylon mesh and the filtrate was designated filtrate 1. The residue was then soaked in 0.5 volume of sample buffer for 15 min, boiled for 15 min and subsequently filtered through 10 µm nylon mesh to give filtrate 2. Concentrated Laemmli loading buffer (Laemmli, 1970) was added to each of filtrates 1 and 2 to a final concentration of 1 ×, boiled for 5 min, and samples were subjected to electrophoresis in 10% polyacrylamide gel. Proteins were electrophoretically transferred onto Immobilon polyvinylidene difluoride membrane (Millipore) with 25 mM-Tris-HCl pH 7.5 containing 190 mM-glycine and 15% methanol (Towbin *et al.*, 1979). The membrane was blocked with BLOTTO, 5% (w/v) Carnation non-fat dry milk and 0.1% sodium azide in TBS (20 mM-Tris-HCl buffer pH 7.5, containing 0.5 M-sodium chloride), washed with TBS-0.05% Tween 20, and incubated with 2 µg/ml CPMV IgG in BLOTTO containing 0.05% Tween 20. The blot was washed, reacted with <sup>125</sup>I-labelled protein A (Burnette, 1981), dried and autoradiographed.

**Immunosorbent electron microscopy (IEM).** In order to determine whether CPMV 60K CP-P produced in transgenic plants may assemble into capsids, clarified leaf extracts from the transgenic plants

expressing CPMV 60K CP-P and the vector-transformed control plants were examined by IEM using an antiserum to CPMV. Purified CPMV and extracts from non-transformed mock- and CPMV-inoculated tobacco plants served as controls.

**Infectivity assays.** In order to assess the susceptibility of transgenic tobacco plants to infection with CPMV, the following experiments were carried out. Transgenic plants grown from seeds of primary transformants shown to express CPMV 60K polyprotein, as determined by ELISA, were used. The upper right-hand quadrant of the Carborundum-dusted youngest nearly fully expanded leaf (leaf three) of transgenic plants in the five-leaf stage was inoculated with 100  $\mu$ l of purified CPMV at a concentration of 1 to 10  $\mu$ g/ml. The virus titres in the inoculated and non-inoculated quadrants of leaves from CPMV 60K expressers and vector control plants were determined by direct ELISA and/or RNA dot blot hybridization using a  $^{32}$ P-labelled oligonucleotide probe derived from cloned cDNA to CPMV B-RNA.

## Results

### Construction and properties of pSPBa1709

Nucleic acid and protein sequencing data have shown that the CPMV 60K CP-P is encoded by residues 1538 to 3298 of the M-RNA sequence (van Wezenbeek *et al.*, 1983). To express the authentic protein, we made use of the fact that it is released from the M-RNA-encoded polyproteins by cleavage at a glutamine-methionine dipeptide (van Wezenbeek *et al.*, 1983). Thus the M-RNA sequence which encodes the 60K protein starts with an AUG codon. Inspection of the sequence around this AUG codon revealed that it appears, fortuitously, to be in a relatively good context (Lutcke *et al.*, 1987) to serve as an initiator for protein synthesis. We therefore produced a clone, pSPBa1709, which contains the sequence of CPMV M-RNA between nucleotides 1525 and the 3' terminus of the RNA [including part of the poly(A) tail] downstream of an SP6 promoter. The clone was designed so that *in vitro* transcription would result in the synthesis of RNA encoding the whole of the 60K sequence and in which the AUG at position 1538 is the 5' proximal AUG.

*In vitro* transcription of *Pst*I-linearized pSPBa1709 with SP6 polymerase resulted in the synthesis of RNA with the expected size of 2.0 kb. Translation of the transcripts in a rabbit reticulocyte lysate system resulted in the synthesis of large amounts of a 60K protein, the mobility of which was identical to that of the 60K CP-P produced by processing the M-RNA-encoded polyproteins (data not shown). This demonstrates that the AUG at position 1538 can act as an initiator of protein synthesis when it is 5' proximal on an RNA. The insert from pSPBa1709 was therefore considered suitable for obtaining 60K expression in transgenic tobacco plants.

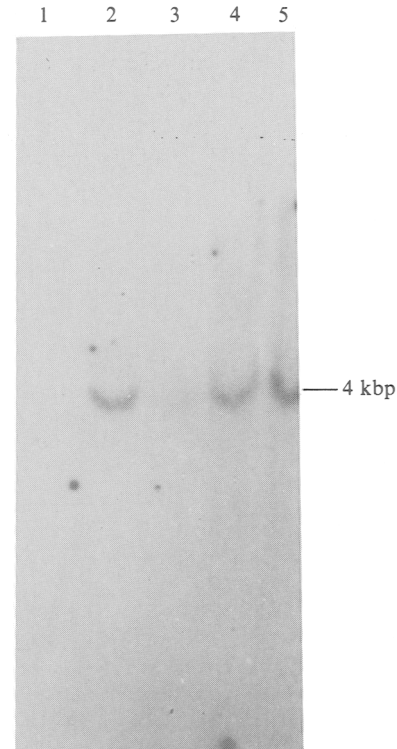


Fig. 1. Autoradiograph of a Southern blot containing *Stu*I/*Eco*RI-digested DNA from four transgenic tobacco plants (KM-6, KM-7, KM-8, and KM-9) transformed with CPMV 60K coding region (lanes 2, 3, 4 and 5) and from the vector-transformed plant, MON-3 (lane 1). The position of a 4 kbp molecular size marker is indicated to the right. The blot was probed with a  $^{32}$ P-labelled CPMV 60K-specific cDNA oligonucleotide probe. Southern blots of DNA from non-transformed plants showed no hybridization signals and were identical to the blot of the vector-transformed sample (data not shown).

### Integration and expression of the chimeric CPMV 60K gene

Of a total of seven kanamycin-resistant tobacco plants, four (KM-6, KM-7, KM-8 and KM-9) were selected for further analysis based on accumulated levels of CP-P, as determined by ELISA. The presence of integrated CPMV 60K CP-P coding region in these transgenic plants was confirmed by Southern blot analysis. The CP-P coding region probe hybridized to a 4 kbp DNA fragment, the expected size for *Stu*I-*Eco*RI digests of DNA from plants transformed with CP-P coding sequence (Fig. 1). No hybridization was detected with DNA from the vector-transformed control plant, MON-3.

Expression of the integrated chimeric genes was analysed by RNA dot blot hybridization. RNA samples from transgenic plants were spotted in duplicate dots and hybridized to the CPMV 60K CP-P probe. Hybridization was evident with plant samples KM-6, KM-7, KM-8 and KM-9 as well as with dilutions of purified CPMV M-

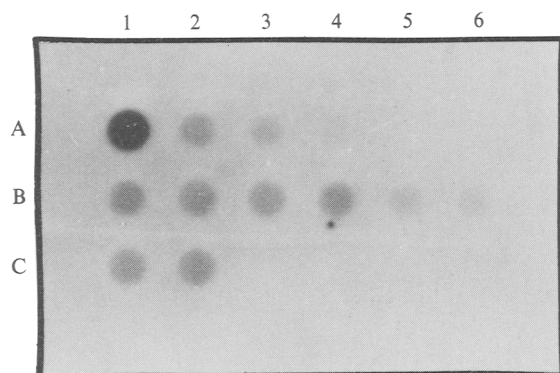


Fig. 2. Autoradiograph of an RNA dot blot containing RNA from four transgenic plants transformed with CPMV 60K coding region (KM-6, KM-7, KM-8 and KM-9) and from a vector-transformed plant (MON-4). The blot was hybridized with a  $^{32}\text{P}$ -labelled CPMV 60K-specific cDNA oligonucleotide probe. Row A: dots 1, 2, 3 and 4 contain 50, 10, 5 and 1 ng of purified CPMV M-RNA, respectively. Row B: RNA from KM-9 (dots 1 and 2), KM-8 (dots 3 and 4) and KM-7 (dots 5 and 6). Row C: RNA from KM-6 (dots 1 and 2) and MON-4 (dots 3 and 4).

RNA (Fig. 2). Variable levels of transcription were detected as indicated by the variation in dot intensity. Approximately 20 ng RNA can be estimated for each of KM-6, KM-8 and KM-9, but only about 5 ng RNA was estimated for KM-7. No hybridization signals were observed with RNA samples from the vector-transformed plant MON-4.

#### Protein expression in transgenic plants

A protein with an estimated  $M_r$  of 60K was detected in Western blots of filtrate 2 samples (see Methods) prepared from the transgenic plant KM-9 using an antiserum to CPMV virions and  $^{125}\text{I}$ -Protein A (Fig. 3, lane 1). No protein bands were observed in such blots with filtrate 1 samples from CP-P-transformed plants or with any of the samples from vector-transformed control

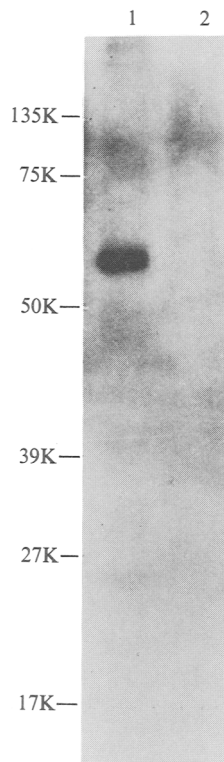


Fig. 3. Accumulation of CPMV 60K coat protein precursor in transgenic tobacco plants. Lanes 1 and 2: protein extracts from plants KM-9 (transformed with CPMV 60K coding region) and MON-3 (a vector-transformed plant), respectively. The Western blot was reacted with an antiserum to CPMV and  $^{125}\text{I}$ -Protein A. Positions of  $M_r$  markers are indicated to the left.

plants (Fig. 3, lane 2). Examination of extracts from transgenic plants expressing CPMV CP-P (or from the vector-transformed control) by IEM did not reveal the presence of virus-like particles. On the other hand, abundant icosahedral virus particles typical of CPMV were detected in extracts from CPMV-infected non-

Table 1. Effect of extraction and coating buffers on detection of CPMV 60K polyprotein in transgenic plants by indirect ELISA

Buffer*	Dilution	ELISA absorbance value ( $A_{405}$ )†			
		MON-3	KM-6	KM-8	KM-9
PBS (pH 7.4)	1:5	0.033 ± 0.001	0.402 ± 0.027	1.252 ± 0.054	1.141 ± 0.056
	1:50	0.022 ± 0.010	0.252 ± 0.007	0.893 ± 0.064	1.022 ± 0.024
	1:500	0.027 ± 0.018	0.204 ± 0.049	0.707 ± 0.032	0.599 ± 0.039
Carbonate (pH 9.6)	1:5	0.043 ± 0.008	0.088 ± 0.016	0.432 ± 0.067	0.317 ± 0.010
	1:50	0.022 ± 0.001	0.101 ± 0.018	0.544 ± 0.011	0.320 ± 0.005
	1:500	0.018 ± 0.005	0.075 ± 0.006	0.485 ± 0.032	0.201 ± 0.010

\* Buffer used in preparing leaf extracts and coating the polystyrene plates.

† Values are means for three replicates ± standard variation.

Table 2. Replication and cell-to-cell movement of CPMV in inoculated leaves of non-transformed tobacco, as determined by direct ELISA

Dilution* (w/v)	ELISA absorbance value ( $A_{405}$ ) for extract from†		
	Mock-inoculated	Inoculated quadrant‡	Non-inoculated quadrant‡
1:5	0.010 ± 0.016§	1.881 ± 0.171	0.222 ± 0.033
1:50	0.013 ± 0.004	1.845 ± 0.268	0.025 ± 0.016
1:500	0.019 ± 0.032	0.310 ± 0.008	0.021 ± 0.016

\* Samples were tested 1 week post-inoculation; extract preparation and dilutions were made with PBS-polyvinylpyrrolidone-Tween 20. The ELISA plates were read 60 min following substrate addition.

† Samples collected from inoculated regions 24 h post-inoculation gave ELISA absorbance values similar to those obtained with mock-inoculated plants.

‡ The non-inoculated quadrants were on the same half of the leaf as the inoculated quadrants.

§ Values are means of three replicates ± standard deviation. Samples of purified CPMV at concentrations of 1.0, 0.1 and 0.01 µg/ml, which were included in the same ELISA plate, gave readings of >2.0, 0.240 ± 0.068, and 0.044 ± 0.014, respectively.

transformed tobacco and in purified CPMV preparations (data not shown).

The resident CPMV antigen in transgenic tobacco plants was readily detected using a variation of an indirect ELISA method and an antiserum to CPMV virions. The use of PBS in place of carbonate buffer, in the preparation of leaf extracts and the coating of polystyrene plates, significantly enhanced the sensitivity of indirect ELISA in detecting CPMV CP-P (Table 1). The ELISA data indicated that higher levels of CP-P accumulation were detected in the transgenic plants KM-8 and KM-9 than were detected in KM-6 (Table 1).

ELISA testing of seedling progeny from four self-fertilized primary transformants indicated that the progeny segregated at a Mendelian ratio of 3:1 (expressers:non-expressers of CPMV CP-P). The level of CPMV CP-P accumulated in the transgenic plants was estimated, using direct ELISA, to be equivalent to 0.5 to 2.0 µg/g wet weight of tissue. Assuming that soluble proteins constitute 10% of the leaf dry weight and that dry weight is 10% of the wet weight, then CPMV 60K CP-P in transgenic plants represents 0.005 to 0.02% of total soluble proteins.

The 60K principal translation product of *in vitro* transcription of linearized pSPBa1709, as described before, was specifically immunoprecipitated with the same antiserum to CPMV virions used in ELISA (data not shown). Thus, the resident CPMV 60K protein detected in the transgenic plants by Western blots and ELISA is the product of the CP-P coding region.

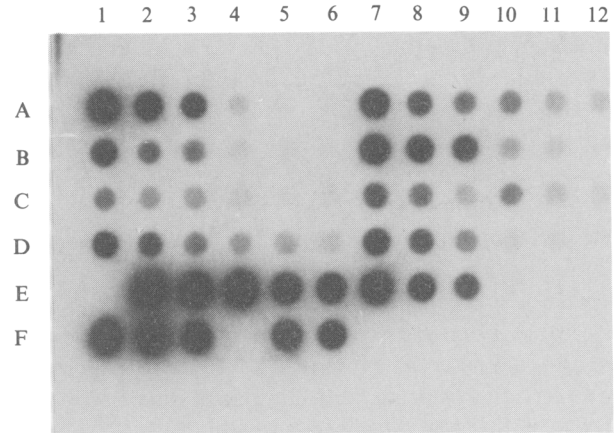


Fig. 4. Dot blot hybridization assay for CPMV B-RNA in total nucleic acid samples from transgenic tobacco transformed with the CPMV 60K coding sequence (progeny from the two primary transformants, KM-6 and KM-9) or from the vector-transformed plant, MON-3. Twofold dilutions of total nucleic acids extracted from 8 mg tissue were made from inoculated and non-inoculated quadrants of the individual leaves, spotted onto GeneScreen Plus membranes and probed with  $^{32}$ P-labelled cloned B-RNA cDNA probe. Unless otherwise specified, samples in columns 1 to 3 and 7 to 9 were from inoculated quadrants; samples in columns 4 to 6 and 10 to 12 were from non-inoculated quadrants. In all cases, the lowest dilution was spotted in the first dot and the highest in the third dot. The samples used were as follows. Row A: 1 to 6, KM-9 (2); 7 to 12, MON-3 (2); row B: 1 to 6, KM-6 (4); 7 to 12, KM-9 (6); row C: 1 to 6, MON-3 (1); 7 to 12, KM-6 (6); row D: 1 to 6, KM-9 (5); 7 to 12, MON-3 (4); row E: 1, mock-inoculated control; 2 to 6, purified B-RNA in amounts of 100, 50, 25, 10 and 1 ng/dot, respectively; 7 to 12, KM-6 (5); row F: 1, 2, 3, 5 and 6, twofold dilutions of total nucleic acids extracted from 4 mg tissue of CPMV-inoculated cowpea.

#### Reaction of transgenic plants to virus infection

Although CPMV neither induces visible symptoms in inoculated tobacco plants nor moves systemically in infected plants, it replicates to a relatively high concentration in the inoculated leaf. One week post-inoculation, CPMV accumulated to approximately 50 and 5 µg/g wet weight tissue in the inoculated and non-inoculated quadrants, respectively, of the individual treated leaves of non-transformed tobacco (Table 2). An inoculum concentration of 10 µg/ml was used in all infectivity assays because preliminary experiments with virus inoculum at 1 and 5 µg/ml did not consistently produce detectable virus levels, within 2 weeks after inoculation, in non-transformed or vector-transformed plants. To evaluate the reactions of the transgenic plants to CPMV inoculation, we relied on dot blot hybridization assays of total nucleic acid preparations from inoculated and non-inoculated quadrants of individual leaves using a  $^{32}$ P-labelled oligonucleotide probe generated from cloned cDNA to CPMV B-RNA. Results from a representative dot blot analysis are shown in Fig. 4. Because of the variability in the virus level in inoculated leaves of

vector-transformed plants as well as among those transformed with the CPMV 60K CP-P sequence, it was not possible to correlate the expression of CP-P with the level of challenge virus replication and movement. Higher virus levels were detected in the non-inoculated quadrants of KM-6(6) and KM-9(5), transgenic progeny considered to be low expressers of CP-P as determined by ELISA prior to inoculation (Fig. 4, row C, dots 10 to 12, and row D, dots 4 to 6, respectively). The ELISA values for extracts from these two plants were less than half those obtained for extracts from the other CP-P expressers (data not shown). The dot blot experiments were repeated 4 times with similar results.

An ELISA was also used to evaluate the response of transgenic plants to CPMV infection. Like those of the dot blot assays, results of ELISA testing of inoculated leaves could not differentiate among CP-P expressers, non-expressers, and vector-transformed controls (data not shown).

## Discussion

The results of Southern blot analysis confirmed the integration of the CPMV 60K CP-P coding region in the genome of transgenic tobacco plants. Data from RNA dot blots, ELISA and Western blotting experiments demonstrated expression of the integrated CP-P gene. The level of CPMV CP-P production is within the range (0.002% to 0.2% of total soluble proteins) reported in transgenic plants transformed with the coat protein genes of viruses whose capsids are composed of a single type of coat protein (Cuozzo *et al.*, 1988; Hemenway *et al.*, 1988; Lawson *et al.*, 1990; Loesch-Fries *et al.*, 1987; van Dun *et al.*, 1987). The results of Western blots and IEM also indicated that CPMV CP-P neither undergoes autoproteolysis to generate the mature viral coat proteins, nor assembles into virus-like capsids. Because purified preparations of CPMV, isolated from cowpea plants, are known to contain empty protein capsids (top component), in addition to the nucleoprotein middle and bottom components, these data suggest that processing of CPMV CP-P may be required for capsid assembly. There could, however, be other explanations. For example, tobacco is not a natural host of CPMV, and consequently the concentration and cellular distribution of CP-P may be different from that in cowpea and unsuitable for empty capsid assembly. Furthermore, it is not known whether encapsidation of viral RNA follows, occurs concomitantly with, or is independent of CP-P processing. A recent report on the detection of empty virus-like particles in transgenic plants expressing the coat protein of arabis mosaic virus, a nepovirus containing a single type of coat protein (Bertioli *et al.*, 1991), is of interest in this regard.

A modification of an indirect ELISA procedure that involves substituting PBS pH 7.4 for carbonate buffer pH 9.6 [a commonly used buffer for coating the ELISA plates with the initial reactant (Clark *et al.*, 1986)] significantly enhanced the sensitivity of indirect ELISA in detecting CPMV CP-P in transgenic plants. Some viruses dissociate in a high pH buffer such as the carbonate buffer whereas incubation in a neutral pH buffer, i.e. PBS, does not affect particle integrity (Murphy & D'Arcy, 1991). Although we do not know the effect of carbonate buffer on the state of aggregation of CP-P in leaf extracts, the antiserum used in ELISA was raised against CPMV virions and may thus have higher affinity to CP-P in a more aggregated form.

Results of infectivity assays, designed to compare the virus titre in leaves of transgenic tobacco plants inoculated with purified CPMV at a concentration of 10 µg/ml, showed little or no differences between CP-P expressers and vector-transformed plants. Huber *et al.* (1977) reported a longer lag period prior to detectable virus multiplication in tobacco protoplasts inoculated with CPMV compared to cowpea protoplasts. Such a lag period might mask a delay in virus replication resulting from the presence of the endogenous 60K CP-P.

As it is not known how constitutive expression of the coat protein gene confers resistance to viruses whose capsids contain single coat proteins, one cannot predict how efficient the precursor strategy would be. In a recent review (Beachy *et al.*, 1990), the authors advanced the following hypotheses to explain how the resident CP may interfere with some early events in virus infection: (i) the resident CP may inhibit uncoating of incoming virus particles and/or (ii) the CP may bind to the viral RNA thus interfering with gene expression. These hypotheses have been based on several types of experiments involving different RNA viruses with capsids made up of single coat proteins. This raises the interesting question of whether a precursor molecule is capable of performing the same protection-mediated functions as do the mature viral proteins. The conformation of the precursor molecule is expected to be quite distinct from that of the individual mature proteins. Recent X-ray crystallographic studies by Chen *et al.* (1989) have suggested that substantial rearrangements occur in portions of the two capsid proteins of the comovirus BPMV after post-translational cleavage of the precursor. It will be of considerable interest to determine whether differences in conformation between the precursor to the coat proteins and the individual mature viral proteins have a bearing on their distribution in the cell and, in turn, on their abilities to bind to viral RNA and/or to inhibit uncoating of challenge virus particles.

The transgenic tobacco plants expressing CPMV 60K CP-P have provided us with excellent experimental

material to investigate various aspects of the molecular biology of comoviruses. Studies involving electroporation of protoplasts, generated from CP-P expressers, with infectious full-length transcripts from cloned B-RNA cDNA are under way in our laboratory to examine CP-P processing and capsid assembly *in vivo*.

We would like to thank Kathleen Franklin and Thomas Pirone for their comments and the Monsanto Company (St Louis, Missouri, U.S.A.) for the pMON530 plasmid. In the U.K., CPMV was handled under MAFF licence number PHF 49A/63(46). This work was supported in part by a grant from the American Soybean Association (project ASARP-89462) and is published as Journal Series Paper 91-11-110 of the Kentucky Agricultural Experiment Station.

## References

- ANDERSON, J. A., GHABRIAL, S. A. & TAYLOR, N. L. (1991). Natural incidence of peanut stunt virus infection in hybrid populations of *Trifolium ambiguum* × *T. repens*. *Plant Disease* **75**, 156–159.
- BEACHY, R. N., LOESCH-FRIES, S. & TUMER, N. (1990). Coat protein-mediated resistance against virus infection. *Annual Review of Phytopathology* **28**, 451–474.
- BERTIOLI, D. J., HARRIS, R. D., EDWARDS, M. L., COOPER, J. I. & HAWES, W. S. (1991). Transgenic plants and insect cells expressing the coat protein of arabis mosaic virus produce empty virus-like particles. *Journal of General Virology* **72**, 1801–1809.
- BURNETTE, W. N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry* **112**, 195–203.
- CHEN, Z., STAUFFACHER, C., LI, Y., SCHMIDT, T., BOMU, W., KAMER, G., SHANKS, M., LOMONOSOFF, G. & JOHNSON, J. E. (1989). Protein RNA interactions in an icosahedral virus at 3.0 Å resolution. *Science* **245**, 154–159.
- CLARK, M. F., LISTER, R. M. & BAR-JOSEPH, M. (1986). ELISA techniques. *Methods in Enzymology* **118**, 742–766.
- CUOZZO, M., O'CONNELL, K., KANIEWSKI, W., FANG, R.-X., CHUA, N. H. & TUMER, N. E. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology* **6**, 549–557.
- ESCHENFELDT, W. H., PUSKA, R. S. & BERGER, S. L. (1987). Homopolymeric tailing. *Methods in Enzymology* **152**, 337–342.
- FEINBERG, A. P. & VOGELSTEIN, B. (1985). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.
- GHABRIAL, S. A. & SCHULTZ, F. G. (1983). Serological detection of bean pod mottle virus in bean leaf beetles. *Phytopathology* **73**, 480–483.
- GOLDBACH, R. & VAN KAMMEN, A. (1985). Structure, replication and expression of the bipartite genome of cowpea mosaic virus. In *Molecular Plant Virology*, vol. 2, pp. 83–120. Edited by J. W. Davies. Boca Raton: CRC Press.
- HEMENWAY, C., FANG, R.-X., KANIEWSKI, W., CHUA, N. H. & TUMER, N. E. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO Journal* **7**, 1273–1280.
- HOEKEMA, A., HUISMAN, M. J., MOLENDIJK, L., VAN DER ELZEN, P. J. M. & CORNELISSEN, B. J. C. (1989). The genetic engineering of two commercial potato cultivars for resistance to potato virus X. *Bio/Technology* **7**, 273–278.
- HOLNESS, C. L., LOMONOSOFF, G. P., EVANS, D. & MAULE, A. J. (1989). Identification of the initiation codons for translation of cowpea mosaic virus middle component RNA using site-directed mutagenesis of an infectious cDNA clone. *Virology* **172**, 311–320.
- HOLSTERS, M., DEWAELE, D., DEPICKER, A., MESSENS, E., VAN MONTAGU, M. & SCHELL, J. (1978). Transfection and transformation of *Agrobacterium tumefaciens*. *Molecular and General Genetics* **163**, 181–187.
- HORSCH, R. B., FRY, J. E., HOFFMAN, N. C., EICHHOLTZ, D., ROGERS, S. G. & FRALEY, R. T. (1985). A simple and general method for transferring genes into plants. *Science* **227**, 1229–1231.
- HUBER, R., REZELMAN, G., HIBL, T. & VAN KAMMEN, A. (1977). Cowpea mosaic virus infection of protoplasts from Samsun tobacco leaves. *Journal of General Virology* **34**, 315–323.
- KASPERBAUER, M. J. & COLLINS, G. B. (1972). Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. *Crop Science* **12**, 98–101.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAWSON, C., KANIEWSKI, W., HALEY, L., ROZMAN, R., NEWELL, C., SANDERS, P. & TUMER, N. (1990). Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. *Bio/Technology* **7**, 273–278.
- LOESCH-FRIES, L. S., MERLO, D., ZINNEN, T., BURHOP, L., HILL, K., KRAHN, K., JARVIS, N., NELSON, S. & HALK, E. (1987). Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. *EMBO Journal* **6**, 1845–1851.
- LUTCKE, H. A., CHOW, K. C., MICKEL, F. S., MOSS, K. A., KERN, H. F. & SCHEELE, G. A. (1987). Selection of AUG codons differs in plants and animals. *EMBO Journal* **6**, 43–48.
- MURPHY, J. F. & D'ARCY, C. (1991). Influence of ELISA conditions on detection of serological relationships among luteoviruses. *Journal of Virological Methods* **31**, 263–272.
- NELSON, R. S., POWELL ABEL, P. & BEACHY, R. N. (1987). Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. *Virology* **158**, 126–132.
- NITSCH, J. P. (1969). Experimental androgenesis in *Nicotiana*. *Phytomorphology* **19**, 389–404.
- POWELL ABEL, P., NELSON, R. S., DE, B., HOFFMAN, N., ROGERS, S. E., FRALEY, R. T. & BEACHY, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738–743.
- ROGERS, S. G. & BENDICH, A. J. (1988). Extraction of DNA from plant tissues. In *Plant Molecular Biology Manual*, pp. A6 1–10. Edited by S. B. Gelvin & R. A. Schilperoot. Dordrecht: Kluwer Academic Publishers.
- ROGERS, S. G., KLEE, H. J., HORSCH, R. B. & FRALEY, R. T. (1987). Improved vectors for plant transformation: expression cassette vectors and new selectable markers. *Methods in Enzymology* **153**, 253–277.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, U.S.A.* **76**, 4350–4354.
- TUMER, N. E., O'CONNELL, K. M., NELSON, R. S., SANDERS, P. R., BEACHY, R. N., FRALEY, R. T. & SHAH, D. M. (1987). Expression of alfalfa mosaic virus coat protein gene confers cross protection in transgenic tobacco and tomato plants. *EMBO Journal* **6**, 1181–1188.
- VAN DUN, C., BOL, J. F. & VAN VLOTEN-DOTING, L. (1987). Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* **159**, 299–305.
- VAN DUN, C., OVERDUIN, B., VAN VLOTEN-DOTING, L. & BOL, J. F. (1988a). Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross protect against alfalfa mosaic virus infection. *Virology* **164**, 383–389.
- VAN DUN, C. & BOL, J. F. (1988b). Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. *Virology* **167**, 649–652.
- VAN KAMMEN, A. & DE JAGER, C. P. (1978). Cowpea mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 197.
- VAN WEZENBEEK, P., VERVER, J., HARMSSEN, J., VOS, P. & VAN KAMMEN, A. (1983). Primary structure and gene organization of the middle component RNA of cowpea mosaic virus. *EMBO Journal* **2**, 941–946.