

# Replication of Cowpea Mosaic Virus RNA1 or RNA2 Is Specifically Blocked in Transgenic *Nicotiana benthamiana* Plants Expressing the Full-Length Replicase or Movement Protein Genes

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Received 26 October 1994. Accepted 20 January 1995.

*Nicotiana benthamiana* plants were transformed with either the cowpea mosaic virus (CPMV) RNA2-derived movement protein gene or the RNA1-originating replicase gene. For both types of genes, half of the R<sub>2</sub>-generation lines showed complete resistance when challenged with CPMV. Experiments using protoplasts revealed that the resistance operates at the single cell level by specifically preventing replication of the RNA segment, from which the transgene was derived. In both cases, the resistance acts against wild-type strain CPMV-Sb and the very homologous CPMV-S1 and CPMV-S8 strains, but not to other comovirus species including cowpea severe mosaic virus (CPSMV). These data and the inability to detect transgene-encoded proteins, suggest an RNA-mediated nature of the resistance.

*Additional keywords:* comoviruses, plant protection, transformed plants, virus resistance.

Cowpea mosaic virus (CPMV) possesses a genome consisting of two messenger-sense, single-stranded RNA molecules, which are separately encapsidated. Upon translation of both segments, referred to in this paper as RNA1 and RNA2 (formerly B-RNA and M-RNA, respectively), polyproteins are produced from which functional proteins are generated by a defined pathway of proteolytic cleavages. All proteins necessary for viral replication originate from the RNA1-encoded 200-kDa polyprotein. The proteins involved in virus movement and encapsidation are encoded by the smaller RNA2. This RNA segment is translated into two carboxy-coterminal polyproteins as a result of additional initiation at a second in-frame AUG codon. From these two polyproteins beside the two capsid proteins, overlapping 48- and 58-kDa proteins are derived (Vos et al. 1984, Eggen and Van Kammen 1988, Holness et al. 1989). Various CPMV mutants provided genetic evidence that both the capsid proteins and the 48-kDa protein are needed for cell-to-cell movement of the virus, while these proteins are dispensable for virus replication (Wellink and Van Kammen 1989, Kasteel et al. 1993). Electron microscopy studies revealed that for cell-to-cell movement CPMV

generates tubular structures that are presumably penetrating plasmodesmata and that contain virus particles (Van Lent et al. 1990). As similar tubular structures were found on protoplasts that transiently express the RNA2-encoded 48-kDa protein (Wellink et al. 1993), this protein was identified as a movement protein (MP). Though the unique N-terminal domain within the 58-kDa protein is involved in replication of RNA2 (Van Bokhoven et al. 1993a), it has not been excluded that this 58-kDa protein has an additional function in movement in the plant tissue, for instance by interacting with the plasmodesmata or by mediating long-distance movement.

More insight in the functioning of viral proteins in the processes of viral movement and replication can be obtained by studies that involve transgenic plants expressing viral genes, as demonstrated for example with the MP gene of tobacco mosaic virus (TMV) (Wolf et al. 1989; Deom et al. 1990; Ding et al. 1992; reviewed by Wolf and Lucas 1994), sunn-hemp mosaic virus (SHMV) (Deom et al. 1994), alfalfa mosaic virus (AIMV) (Poirson et al. 1993), and cucumber mosaic virus (CMV) (Vaquero et al. 1994) or the replicase protein genes of AIMV and brome mosaic virus (BMV) (Van Dun et al. 1988; Mori et al. 1992). On the other hand, introduction of viral sequences into the genome of a host plant has frequently resulted in pathogen-derived resistance (PDR) (reviewed by Wilson 1993). The first report on PDR described coat protein (CP)-mediated protection against TMV in tobacco plants (Powell Abel et al. 1986). Since then, numerous cases of PDR have been described for viruses of at least 13 different taxonomic groups (Hull and Davies 1992; Kunik et al. 1994). These examples demonstrated that PDR can be accomplished in various ways. Not only CP genes were found to confer resistance to transgenic plants, but also MP genes (Lapidot et al. 1993) and replicase genes (reviewed by Carr and Zaitlin 1993). Even, mutated, or truncated versions of viral genes were shown to be capable of inducing resistance in transgenic plants (Anderson et al. 1992; Braun and Hemenway 1992; Longstaff et al. 1993; Donson et al. 1993; Lapidot et al. 1993; Audy et al. 1994). In some of these cases the resistance was exhibited only against viruses, closely related to the virus from which the transgene originates, while in other cases the resistance was extended to less-related viruses (Donson et al. 1993). It appears that the presence of the

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viral proteins or nucleic acids disturbs the subtle balance in amount, time, and place of viral and host factors needed for a systemic infection of a host plant with a virus. The fact that PDR can be achieved in a variety of ways and can differ considerably in spectrum, implies that distinct molecular mechanisms may underlie different cases of PDR. How exactly the resistance is established remains to be demonstrated.

In this study we have transgenically expressed both the CPMV 200-kDa replicase and 48-kDa and overlapping 58/48-kDa movement protein genes by transforming *Nicotiana benthamiana* plants. These plants did not allow functional studies of the viral transgenes, but, instead, exhibited a complete resistance when inoculated with CPMV virions or CPMV RNA.

## RESULTS

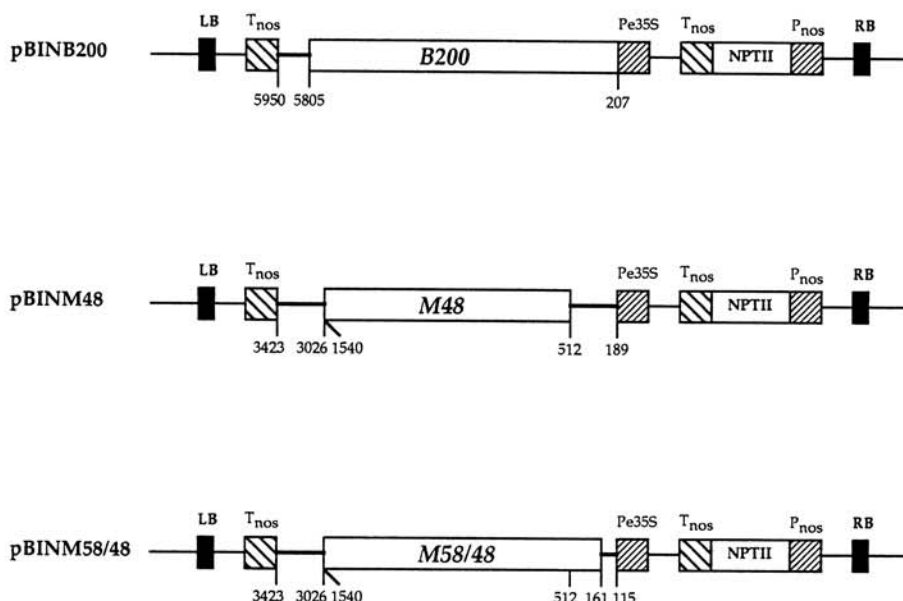
### Three types of transgenic plants.

The binary constructs pBINB200, pBINM48, and pBINM58/48 (Fig. 1) were designed to express the CPMV replicase and MP genes in plants under the control of an enhanced 35S promoter and a NOS terminator. The genes are derived from the plasmids pMB200, pMM48, and pMM58/48, that have previously been shown to express functional proteins upon transient expression in protoplasts (Van Bokhoven et al. 1993b; Wellink et al. 1993). Upon *Agrobacterium tumefaciens*-mediated transformation, kanamycin-resistant shoots (the R<sub>0</sub>-generation plants) were selected. By PCR on DNA and RT-PCR on cDNA preparations of kanamycin resistant plants (Fig. 2), plants that were ex-

pressing the transgenic mRNA were identified and for each construct 10 positive plants were selfed to give R<sub>1</sub>-generation-lines.

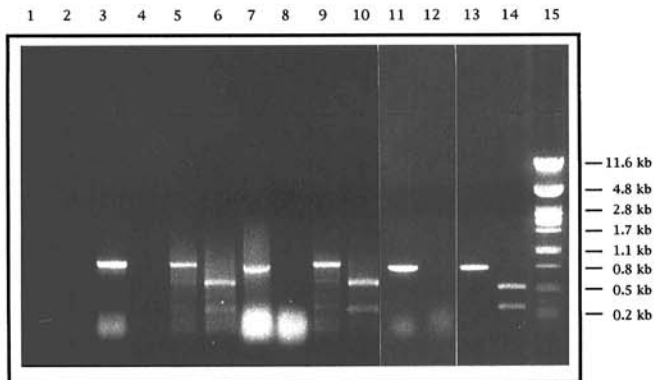
### Transgenic R<sub>1</sub>- and R<sub>2</sub>-generation plants are resistant to CPMV.

Ten R<sub>1</sub>-generation lines were chosen and assayed for segregation of kanamycin resistance and resistance against CPMV inoculation. These 10 lines included four lines transformed with the CPMV replicase gene and three lines transformed with each of the MP-gene constructs. The segregation data (Table 1) revealed that in two plants there were single T-DNA insertions (plants BII-9 and 3B) and that eight plants contained two insertions (for instance, plant 76.3), that in some cases were linked (plants BII-3 and 5.4). These integration events were confirmed by Southern blotting of *Bam*HI (for MP gene insertions) or *Xba*I- (for replicase gene insertions) digested genomic DNA and hybridization with either a MP or replicase gene-specific probe (results not shown). For each line, 19 plants were inoculated with plant sap containing CPMV and one plant was inoculated with buffer as a control. Eight days postinoculation, all nontransformed plants displayed characteristic mosaic symptoms, while buffer-inoculated plants remained symptom-free. Interestingly, within some of the transgenic lines (Table 1) there were plants that remained free of symptoms (Fig. 3). These mosaic-free plants were analyzed for CPMV replication in both the inoculated and higher located leaves by ELISA; 12 days post-inoculation these ELISAs were repeated. This resulted in the identification of completely resistant plants in two of the four



**Fig 1.** Diagrammatic representation of the plant transformation vectors pBINB200, pBINM48, and pBINM58/48 which were used to transfer the CPMV 200-kDa replicase and 48-kDa and 58/48-kDa movement protein genes, respectively, to *Nicotiana benthamiana* plants. In plasmid pBINB200, the replicase coding sequence from RNA1 nt 207 to 5805 is present, followed by the RNA1-derived 3' nontranslated-region from nt 5805 to 5950. Plasmid pBINM48 contains as a leader the RNA2-derived sequences from nt 189 to 512, followed by the 48-kDa coding sequences from the AUG codon at position 512 to the stop codon at nt 1540 and a 3' nontranslated-region comprising the RNA2-derived nucleotides 3026 to 3423. Plasmid pBINM58/48 contains as a leader the RNA2-region from nt 115 to 161, succeeded by the 58/48-kDa coding sequences from the AUG codon at position 161 to the stop codon at nt 1540 and followed by a 3' nontranslated-region comprising the RNA2-originating nt 3026 to 3423. LB and RB refer to the left border and right border sequences of the T-DNA of *Agrobacterium tumefaciens*, respectively. T<sub>nos</sub> refers to the nopaline synthetase terminator sequences, P<sub>nos</sub> to the nopaline synthetase promoter sequences, Pe35S to the enhanced CaMV 35S promoter sequences, and NPTII to the kanamycin resistance gene.

pBINB200, two of the three pBINM58/48, and one of the three pBINM48 transgenic lines (Table 1). According to the kanamycin resistance segregation analysis, all these resistant lines contained two insertions of the transgene (Table 1; plant lines C2-6, BII-3, 76.4, 76.3 and 5.4). From all resistant R<sub>1</sub> plants per plant R<sub>2</sub> seeds were harvested and germinated on kanamycin. In this way, resistant R<sub>1</sub> plants giving rise to a homozygous R<sub>2</sub> progeny could be identified for the lines BII-3, 76.4, 76.3, and 5.4 (results not shown). Additionally, this



**Fig. 2.** Agarose gel electrophoresis of the products of the polymerase chain reaction (PCR) reactions on DNA, RNA, or cDNA isolated from nontransformed plants (lanes 1 and 2), or plants transformed with pBINM48 (lanes 3, 4, 5, and 6), pBINM58/48 (lanes 7, 8, 9, and 10) or pBINB200 (lanes 11, 12, 13, and 14). Amplified gene fragments are present in the PCR reactions on preparations of genomic DNA (lanes 3, 7, and 11) and cDNA (lanes 5, 6, 9, 10, 13, and 14). Coding sequences (48 and 58 kDa) were detected by primer combination D<sub>1</sub><sup>\*</sup>-D<sub>2</sub><sup>\*</sup>, which gives rise to a 1-kb fragment, that can be specifically digested with *AccI* into 400- and 600-bp fragments (lanes 6 and 10). Coding sequences (200 kDa) were detected by primer pair T<sub>1</sub>-T<sub>2</sub>, resulting in a 800-bp fragment, which can be specifically digested with *HaeIII* into 300-bp plus 500-bp fragments (lane 14). In genomic DNA preparations of nontransformed plants (lanes 1 and 2) and in total RNA extracts of the transgenic plants (lanes 4, 8, and 12), no amplified fragments can be detected either with primer pair D<sub>1</sub><sup>\*</sup>-D<sub>2</sub><sup>\*</sup> (lanes 1, 4, and 8), or with primer combination T<sub>1</sub>-T<sub>2</sub> (lanes 2 and 12). Lane 15 shows DNA digested with *PstI* as a marker. Especially in lanes 3, 7, and 8 low molecular weight, so-called primer-dimer bands, are visible, which are nonspecific PCR products.

approach revealed that the resistant phenotype was not only displayed by R<sub>1</sub>-plants homozygous for the insertions, but also by heterozygous R<sub>1</sub> plants. Eight R<sub>2</sub> progeny plants of each of the five resistant lines were tested for resistance against CPMV. As expected, plants of all five lines (C2-6, BII-3, 76.4, 76.3, and 5.4) displayed the resistant phenotype (Table 1). However, in line C2-6 beside resistant plants susceptible plants were also present. This segregation for resistance was anticipated since for this line no resistant homozygous R<sub>1</sub> plant could be identified.

#### The resistance does not hold against a heterologous comovirus.

The R<sub>2</sub> progeny of the five resistant lines was also tested for resistance against the CPMV strains S1 and S8 that are, in contrast to the wild-type Sb strain, capable of systematically infecting *V. unguiculata* cv. Early Red (De Jager and Wesseling 1981). In addition, another comovirus species, cowpea severe mosaic virus (CPSMV) (Chen and Bruening 1992a, 1992b), which shares for RNA1 50% and for RNA2 44% nucleotide sequence homology to CPMV, was tested. All lines resistant to CPMV-Sb were found to be resistant to the strains S1 and S8, but not to CPSMV (Table 1). Again, not all plants of line C2-6 exhibited the resistant phenotype.

#### The resistance acts by preventing the replication of the donor-RNA segments.

CMPV could not be detected by ELISA in the resistant lines transformed either with the replicase gene or with the MP gene at 8 or 12 days after inoculation. This result indicates that the lines have complete immunity to CPMV infection. To establish whether the resistance acts at the single cell level, protoplasts of R<sub>2</sub>-generation transgenics were transfected with CPMV RNA. After 18 h of cultivation, replication of RNA1 and RNA2 were separately monitored in immunofluorescence assays by using the two different antisera, α110 and αCPMV, that specifically recognize RNA1 and RNA2 derived proteins, respectively. In the nontransgenic protoplasts both CPMV RNA segments were replicated in 80% of the protoplasts (Table 2). In protoplasts from the MP gene-

**Table 1.** Assessment of segregation of kanamycin resistance in the R<sub>1</sub> progeny and resistance to cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV) in the R<sub>1</sub> and R<sub>2</sub> generations of movement protein gene and replicase gene-transformed *Nicotiana benthamiana* plants

Plant line	Transgene	Kanamycin resistance <sup>a</sup>		Resistance to virus infection <sup>b</sup>				
		R <sub>1</sub> :km <sup>S</sup> :km <sup>R</sup>	Total no.	R <sub>1</sub> + CPMV	R <sub>2</sub> + CPMV	R <sub>2</sub> + S1	R <sub>2</sub> + S8	R <sub>2</sub> + CPSMV
Ben	...	1:0	223	12/12	4/4	4/4	4/4	4/4
C2-4	200	1:14	350	19/19	ND <sup>c</sup>	ND	ND	ND
C2-6	200	1:13	378	12/17	2/8	0/7	1/8	8/8
BII-3	200	1:11	730	10/19	0/7	0/6	0/6	6/6
BII-9	200	1:3	304	19/19	ND	ND	ND	ND
76.4	48	1:14	362	5/18	0/6	0/6	0/6	6/6
3B	48	1:2.5	479	19/19	ND	ND	ND	ND
B4-1	48	1:16	223	19/19	ND	ND	ND	ND
76.3	58/48	1:15	919	7/14	0/4	0/4	0/4	4/4
5.4	58/48	1:10	493	3/16	0/4	0/4	0/4	4/4
4.13	58/48	1:15	200	18/18	ND	ND	ND	ND

<sup>a</sup> Since on one T-DNA insert both a viral gene and a kanamycin resistance gene are present, the number of insertions can be determined by assessment of the segregation ratio for kanamycin resistance in R<sub>1</sub>-generation plants. After germination and cultivation of R<sub>1</sub>-generation plantlets on kanamycin for 2 weeks, the proportions of sensitive and resistant plants were determined.

<sup>b</sup> Resistance is determined as the number of infected plants out of the total number of inoculated plants, as was determined by symptom occurrence and ELISAs. The plants were challenged not only with the wild-type CPMV-Sb strain, but also with the mutant strains S1 and S8.

<sup>c</sup> ND = not determined.

resistant plants RNA1 was still capable of replication but the replication of RNA2 was strongly inhibited; no RNA2-specific proteins were detected in, on average, 99.8% of the protoplasts, while in 0.2% of the protoplasts fluorescence signals at wild-type infection level were observed. However, in the replicase-resistant protoplasts no replication of any CPMV RNA at all was found, except for line C2-6, which showed a reduced percentage of immunofluorescing protoplasts with both antisera (Table 2). The incomplete resistance in line C2-6 is attributed to the presence of protoplasts derived from susceptible plants; the  $R_2$ -progeny of this line were segregating for resistance to CPMV infection (Table 1). Since replication of RNA2 is dependent on replication of RNA1, inhibition of replication of RNA1 prevents the synthesis of RNA2 specific proteins. No differences were observed in the percentages of fluorescing protoplasts between the two different CPMV RNA inoculum concentrations that were used.

#### Absence of accumulation of transgene-encoded proteins.

All plants tested for resistance expressed transgenic mRNA, as was shown by RT-PCR (Fig 2). Despite several attempts, no accumulation of CPMV specific proteins could be detected in any transgenic  $R_0$  plant; neither in plants generating susceptible lines nor in plants that gave a resistant progeny. Furthermore, accumulation of CPMV MPs was found to be absent in homozygous  $R_2$ -generation plants of the resistant line 5.4. Both the  $\alpha 48$  serum and the  $\alpha 110$  serum, a serum that specifically recognizes RNA1-derived proteins, cross-react with several tobacco proteins, which reduced the specificity of the antisera for the transgene-encoded proteins.

## DISCUSSION

The bipartite nature of the CPMV genome enabled us to show that in *N. benthamiana* plants a resistance against CPMV can be engineered, that is specifically directed against the viral RNA segment from which the transgene is derived. In plants transformed with the RNA1-derived full-length replicase gene, replication of RNA1 and consequently RNA2 was blocked, while in plants expressing the RNA2-originating full-length MP gene, the replication of RNA2, but not of RNA1, was prevented. For replicase genes, resistance obtained by integration of full-length constructs has been

described previously (Audy et al. 1994; Rubino et al. 1993; Braun and Hemenway 1992; Mueller et al. 1994). The results on the 48-kDa and 58/48-kDa transgenics, on the other hand, show for the first time that PDR can be obtained by transformation with a full-length MP gene. Engineered resistance against CPMV has not been described before; transgenic expression of the 60-kDa precursor of the two CPMV capsid proteins of 37 and 23 kDa was shown not to induce PDR (Nida et al. 1992). However, it should be noted that in these experiments only four lines were tested which were selected on high accumulation levels of the CP-precursor protein and that *Nicotiana tabacum*, which is a nonsystemic host of CPMV, was used for transformation.

Resistance was observed in half of the  $R_1$ -generation lines, transformed with each of the three constructs, and occurred as complete immunity to CPMV infection. In all cases, the resistance was retained in the  $R_2$  generation. The resistance was maintained against the CPMV mutants S1 and S8, but it did not hold against the heterologous comovirus CPSMV, which is homologous to CPMV at the nucleotide sequence level in both RNA1 (50%) and RNA2 (44%). This specificity of the resistance implies a rather narrow range of the resistance. Furthermore, the resistance was shown to act at the single cell level as shown by Carr and Zaitlin (1991) for resistance to TMV. This aspect of the resistance was analyzed by immunofluorescence assays on protoplasts transfected with CPMV RNA, using antisera detecting proteins translated from either RNA1 or RNA2. In addition, these experiments revealed that, in all protoplasts, a transgenic replicase gene can reduce the replication of RNA1, and consequently RNA2, to levels that are below detection when using immunofluorescence. However, in protoplasts derived from MP gene-transformed plants, RNA1 replication was still allowed at wild-type infection levels, while RNA2 replication was inhibited. In most protoplasts no RNA2-specific proteins were detected, but up to 0.4% of the protoplasts showed fluorescence signals at a level comparable to wild-type infected protoplasts. The observation, that this leakage of resistance is specific for MP gene-transgenics, suggests that a transgene-specific mechanism underlies this effect. In these experiments the difference between the replicase and the MP gene-transformed plants may be due to replication and translation of RNA1. This process would have taken place in cells transformed with RNA2, but

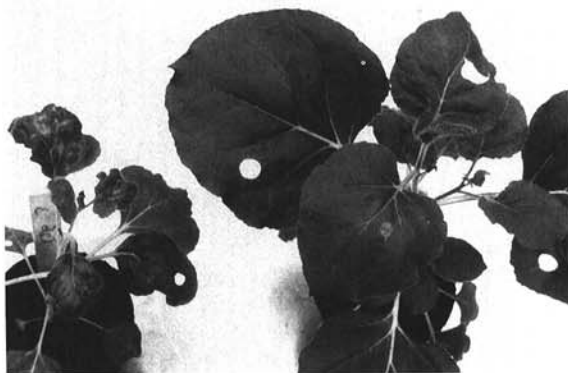


Fig. 3. Within one line, in the segregating  $R_1$ -generation both plants susceptible (left) and resistant (right) to cowpea mosaic virus inoculation can be detected, as shown here for the replicase transformed line C2-6.

Table 2. Assessment of the percentages of immunofluorescing protoplasts prepared from  $R_2$ -generation transgenic plants, transfected with cowpea mosaic virus (CPMV)-RNA

Plant line	Transgene	Fluorescing protoplasts (% of total) <sup>a</sup>	
		$\alpha 110^b$	$\alpha CPMV^b$
Ben	...	80	80
C2-6	200	5	10
BII-3	200	0	0
76.4	48	80	0
76.3	58/48	65	0.4
5.4	58/48	80	0.2

<sup>a</sup> Protoplasts were transfected with either 1 or 2.5  $\mu$ g of CPMV RNA per million protoplasts. The percentage reflects the average of the two doses, which did not differ significantly (results not shown).

<sup>b</sup> The  $\alpha 110$  antiserum detects RNA1-encoded replicase proteins, while the  $\alpha CPMV$  antiserum detects the capsid proteins, which are encoded by RNA2.



not with RNA1-derived genes. There would consequently have been an excess of replication complex formed that could compete with the resistance-mediating factors for the access to RNA2.

For several cases of PDR, it is known that resistance is mediated either by the transgenic proteins or the transgenic mRNAs. For example, it was shown that a truncated TMV replicase protein can reduce the accumulation of several tobamoviruses (Donson et al. 1993) and that the full-length TMV 54-kDa protein, a putative component of the replicase, and the pea early browning virus equivalent of this protein can confer a resistance at cellular level (Golemboski et al. 1990; Carr et al. 1992; MacFarlane et al. 1992). In these cases the transgenically expressed proteins could compete with factors involved in functioning or formation of the replicase complex or they could interfere with a possible regulatory role of the protein. A role for the transgenic mRNA was assigned in the case of resistance, obtained by the integration of either mutated or full-length replicase genes of PVX (Mueller et al. 1994). Here, resistance coincided with a low mRNA expression level of the transgene. Crossing of resistant, low transgene mRNA expressing plants with susceptible, high mRNA-expressing plants revealed that resistance was dominant over susceptibility and that low transgene mRNA expression was dominant over high expression (Mueller et al. 1994). These results indicate that a low expressed, probably silenced, transgenic mRNA can act in *trans* to suppress the expression of a high expressed transgene or infecting viral RNAs. Since in the CPMV replicase-resistant plants the 200-kDa polyprotein could not be detected, an RNA-based mechanism for this resistance is also assumed.

Lapidot and coworkers (1993) have shown that a truncated, inactive MP of TMV can reduce the spread of several tobamoviruses, a phenomenon not found in transgenic plants expressing the wild-type TMV MP (Holt and Beachy 1991). These results suggest that the truncated MP molecules can preoccupy certain cellular host factors needed for cell-to-cell movement of the virus, making these cellular target sites inaccessible to the MPs of the infecting virus (Lapidot et al. 1993). In contrast, a transgenically expressed CPMV 48-kDa MP gene, that has no function in viral replication (Van Bokhoven et al. 1993a), can specifically inhibit the replication of RNA2 at the cellular level. It is therefore likely that resistance from expression of the CPMV and TMV MP genes operates through different mechanisms, with the CPMV MP resistance being RNA based. It is not known whether in the 58/48 kDa-resistant plants the 58 kDa part is involved in inducing resistance. Since the 58-kDa protein does have a function in replication of RNA2, the protein might have a role in generating resistance, although we were not able to detect the 58-kDa protein in resistant plants.

The rather high percentage of resistant lines as found in our experiments, plus the fact that the transformation plasmids were derived from functionally active expression vectors (Van Bokhoven et al. 1993b; Wellink et al. 1993) make it highly improbable that the resistance is due to a dominant negative effect of the insertion of the sequences as mutated forms, although we can not entirely exclude this. In conclusion, we suppose an RNA-based resistance for all the three types of transgenic plants, although this needs to be tested in more detail.

A model on how RNA-mediated resistance might operate was introduced by Lindbo et al. (1993) and extended by Smith et al. (1994) and Dougherty et al. (1994). They propose that transgene mRNA levels that exceed a certain threshold level activates a cytoplasmic process that targets these mRNAs for inactivation in a sequence specific way. Incoming viral RNAs and/or their replicated progeny will also be affected by this degradation system and thus result in a resistant phenotype. Therefore, RNA-based resistance is inversely correlated with mRNA expression levels, is present at the cellular level (Lindbo and Dougherty 1992), and acts only against very homologous strains. Additionally, it can be induced by a viral infection since the viral RNAs can elevate the mRNA levels beyond the threshold level (Lindbo et al. 1993). Grafting studies revealed that this RNA degradation activity is a programmed cell response which is not induced by a diffusible signalling molecule (Dougherty et al. 1994).

In general it can be said that when full-length viral genes are inserted in plant genomes no predictions either on the occurrence of resistance, or on the ability of performing functional studies, can be made. Much remains to be elucidated of the mechanisms leading to pathogen-derived resistance and the many factors involved in this process.

## MATERIALS AND METHODS

### Plants and viruses.

*Vigna unguiculata* cv. Blackeye (cowpea) was used for the propagation of the CPMV strains Sb, S1, and S8 (kindly provided by C. P. de Jager, Dept. of Virology, WAU) and CPSMV (Chen and Bruening 1992a, 1992b). At the nucleotide level CPSMV RNA1 is 50% and RNA2 is 44% homologous to CPMV RNA1 and RNA2, respectively. Inoculum was freshly prepared from CPMV infected cowpea plants by grinding one leaf in 1.5 ml of 50 mM NaPi-buffer pH 7.2. The CPMV sequences that were used for the construction of the plant transformation vectors, were derived from cDNA clones of strain CPMV-Sb. Strains S1 and S8 are mutants of strain Sb, which are able to systemically infect *V. unguiculata* cv. Early Red, a local lesion host for strain Sb. The mutations are located on RNA1 (De Jager and Wesseling 1981). *Nicotiana benthamiana*, a systemic host for CPMV, was used for the production of transgenic plants.

### Construction of plasmids.

The 200-kDa replicase and the 58/48kDa MP coding sequences are present under the control of an enhanced CaMV 35S promoter and a NOS terminator in the plasmids pMB200 (Van Bokhoven et al. 1993b) and pMM58/48 (Wellink et al. 1993), respectively. In this vector, the 35S promoter is enhanced by duplication of the -352 to -90 region (Kay et al. 1987). The expression cassette of pMB200 was isolated as a *SmaI*-*NotI* fragment and subcloned into *SmaI*-*NotI*-digested *E. coli* BlueScript vector (Stratagene), resulting in plasmid pBSMON200. Plasmid pBINB200 was constructed by cloning the expression cassette of pBSMON200 as two fragments (*SalI*-*BglII* and *BglII*-*HindIII*, respectively) into *SalI*-*HindIII*-digested binary vector pBIN19 (Bevan 1984). The expression cassette of pMM58/48 was subcloned in two fragments (*SmaI*-*XbaI* and *XbaI*-*HindIII*, respectively) in *SmaI*-*HindIII*-digested BlueScript vector which resulted in

plasmid pBSMON58/48. From this plasmid the 48-kDa coding sequence and NOS terminator were isolated as a *Bgl*III–*Sal*I fragment and cloned together with the 35S promoter as a *Hind*III–*Bgl*III fragment into *Sal*I– and *Hind*III-digested pBIN19, giving rise to the plasmid pBINM48. Plasmid pBINM58/48 was constructed similarly as pBINM48, except that not the 48 kDa but the 58/48 kDa coding sequence was used which was present on a 2.1-kb fragment that arose after *Sal*I and partial *Bgl*II digestion of pBSMON58/48.

#### Plant transformation.

Plasmids pBINB200, pBINM48, and pBINM58/48 were conjugated into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Rogers et al. 1986). *Nicotiana benthamiana* leaf explants were transformed as described by Horsch et al. (1985) and shoots were regenerated on MS<sub>20</sub> medium containing (per liter) 0.8 mg of benzylaminopurine and 0.1 mg of 2,4-dichlorophenoxyacetic acid in the presence of 500 mg of cefotaxim and 150 mg of kanamycin. Shoots were excised and rooted on hormone-free medium in the presence of 100 mg of kanamycin per liter. R<sub>0</sub>-generation plantlets were transferred to soil and maintained in the greenhouse, where R<sub>1</sub> generation seeds were harvested. Of this segregating R<sub>1</sub> generation 19 plants were tested for resistance against CPMV. Resistant plants were identified and from each of these plants R<sub>2</sub>-generation seeds were harvested per plant. These seeds were germinated on MS<sub>20</sub>-medium containing 150 mg of kanamycin per liter, and the seedlings were cultivated on the same medium for 2 weeks; the first week the plantlets were kept in the dark and the second week under day-night regime. When possible, homozygous R<sub>2</sub>-generation plants were selected.

#### Nucleic acid analysis.

Prior to the isolation of nucleic acids, it was ensured that the plant material was free of *A. tumefaciens* by cultivation of the transgenic plantlets for at least 2 weeks without cefotaxim.

DNA was isolated from a leaf disk using 100 µl of DNA extraction buffer of 65°C containing 0.14 M Sorbitol, 0.22 M Tris pH 8.0, 0.022 M ethylenediaminetetraacetic acid (EDTA), 0.8 M NaCl, 0.8% CTAB, and 1% sarkosyl. After grinding, 40 µl of chloroform was added and after mixing the samples were incubated for 5 to 30 min at 65°C. From the aqueous phase the DNA was precipitated using isopropanol and subsequently dissolved in 30 µl of water.

RNA was isolated according to Verwoerd et al. (1989) and purified from contaminating DNA by a DNase treatment. After phenol-chloroform extractions and ethanol precipitation, cDNA was prepared by incubating a mixture of 1 µl (2 µg) of RNA, 1 µl (1 µg) of the appropriate primer (T<sub>2</sub> or D<sub>2</sub><sup>\*</sup> that are, respectively, complementary to the RNA1 nt 1851–1833 or the RNA2 nt 1538–1518) and 8 µl of annealing buffer (250 mM KCl, 10 mM Tris pH 8.3, 1 mM EDTA) for 3 min at 83°C, followed by 30 min of incubation at 42°C. After addition of 15 µl of cDNA buffer (24 mM Tris, pH 8.3, 16 mM MgCl<sub>2</sub>, 8 mM DTT, 0.4 mM dNTPs) and 0.1 µl AMV Reverse Transcriptase (2.5 U/ml, GIBCO), the incubation at 42°C was extended for 90 min.

On 5 µl of either the DNA, RNA, or cDNA preparation, PCR was performed at alternating temperatures of 94°C,

50°C and 72°C for 32 cycles. For the replicase gene primers T<sub>1</sub> and T<sub>2</sub> were used, which are complementary to the RNA1 nt 1019–1039 and 1851–1833 and generate a 800-bp fragment. For the MP gene primer pair D<sub>1</sub><sup>\*</sup> and D<sub>2</sub><sup>\*</sup>, complementary to the RNA2 nt 512–530 and 1538–1518 were used which should result in a 1-kb fragment. For Southern blot analysis, DNA was isolated using an urea buffer (7 M urea, 0.3 M NaCl, 20 mM EDTA, pH 8.0, 50 mM Tris, pH 8.0, 1% sarkosyl) followed by 3 phenol/chloroform extractions, an isopropanol precipitation, again 3 phenol/chloroform extractions and an ethanol precipitation. For each plant, 20 mg of DNA was digested with either *Bam*HI or *Xba*I, for MP or replicase gene transformants respectively. The fragments were separated by electrophoresis on a 1% agarose gel in TAE-buffer (Sambrook et al. 1989), transferred to a nylon membrane (GeneScreen, NEN, Boston) and hybridized with RNA2 or RNA1 specific probes (Sambrook et al. 1989).

#### Plant virus resistance assay.

Approximately 4 to 5 weeks after sowing, two expanded leaves of the transgenic and control *N. benthamiana* plants were sprayed with Carborundum powder and inoculated with plant sap from CPMV-infected cowpea plants. In the segregating R<sub>1</sub> generation, 19 plants per line were tested with CPMV-Sb inoculum, while in the homozygous R<sub>2</sub> generation, eight plants per line were tested using plant sap containing CPMV-Sb, CPMV-S1, CPMV-S8 or CPSMV virions. One week after inoculation, plants were scored for symptoms and analyzed for the presence of virus by ELISA. Both inoculated and higher situated leaves were tested. Two weeks after inoculation symptom-free plants were checked again by ELISA. ELISA was performed as described by Taliansky et al. (1993), with the modification that the plates were coated with plant sap, followed by an incubation with 1000-fold diluted polyclonal antiserum against CPMV particles and an incubation with 2,500-fold diluted goat anti rabbit alkaline phosphatase (GARAP).

#### Protoplast preparation and transfection.

Protoplasts were prepared from fully expanded, smooth leaves of greenhouse-grown R<sub>2</sub>-generation *Nicotiana benthamiana* plants, as described for cowpea by Van Bokhoven et al. (1993b), with the modification that the leaves were subjected to enzyme treatment for 1 h. Protoplast transfection and cultivation were performed as described by Van Bokhoven et al. (1993b), using inoculum concentrations of 1 or 2.5 µg of CPMV RNA per approximately one million protoplasts.

#### Immunofluorescence assay.

Approximately 18 h after transfection, protoplasts were tested for replication of both RNA2 and RNA1, by using the indirect immunofluorescence assay described by Hibi et al. (1975) and Maule et al. (1980). Replication of RNA2 was monitored using antiserum against CPMV particles. Replication of RNA1 was assessed using antiserum against the 110-kDa protein which is a portion of the viral replicase.

#### Protein analysis.

Transgenic plants were fractionated according to Dorssers et al. (1982). Fractions P, F<sub>1</sub>, F<sub>3</sub>, and F<sub>4</sub> were analyzed for proteins by Western blotting assays which were performed as

described by Sambrook et al. (1989) and Van Bokhoven et al. (1990).

## ACKNOWLEDGMENTS

We wish to thank Bert Essenstam for excellent work in the greenhouse, Cees de Jager for providing the CPMV mutants, and Rob Goldbach for valuable discussions and critically reading the manuscript. This work was supported by the Netherlands Foundation of Chemical Research (SON) with financial aid from the Netherlands Organisation for Scientific Research (NWO).

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