The Coat Protein Is Required for the Elicitation of the *Capsicum* L\(^2\) Gene-Mediated Resistance Against the Tobamoviruses


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In *Capsicum*, the resistance against tobamoviruses conferred by the L\(^2\) gene is effective against all but one of the known tobamoviruses. Pepper mild mottle virus (PMMoV) is the only virus which escapes its action. To identify the viral factors affecting induction of the hypersensitive reaction (HR) mediated by the *Capsicum* spp. L\(^2\) resistance gene, we have constructed chimeric viral genomes between paprika mild mottle virus (PaMMV) (a virus able to induce the HR) and PMMoV. A hybrid virus with the PaMMV coat protein gene substituted in the PMMoV-S sequences was able to elicit the HR in *Capsicum frutescens* (L\(^2\)L\(^2\)) plants. These data indicate that the sequences that affect induction of the HR mediated by the L\(^2\) resistance gene reside in the coat protein gene. Furthermore, a mutant that codes for a truncated coat protein was able to systemically spread in these plants. Thus, the elicitation of the host response requires the coat protein and not the RNA.

Additional keywords: tobamovirus L\(^2\) resistance gene.

Tobamoviruses are one of the most destructive pathogens of pepper crops. In *Capsicum*, resistance against tobamoviruses is characterized by induction of a hypersensitive reaction (HR), manifested by the appearance of necrotic local lesions at the site of infection. So far, four seemingly allelic resistance genes (L\(^1\)-L\(^4\)) of increased effectiveness at the L locus (Boukema et al. 1980, 1982) have been identified. The viral determinants for inducing the L\(^2\) gene-mediated resistance, we have analyzed here the viral factors inducing the resistance conferred by the L\(^2\) gene. This gene, initially named the L\(^2\) gene (Boukema et al. 1980) was the first plant resistance gene ever identified as a single dominant “Mendelian character” (Holmes 1934). L\(^2\) has been found to be present in certain varieties of *Capsicum frutescens*, such as Tabasco and minimum, being active against all pepper infecting tobamoviruses, with the exception of pepper mild mottle virus (PMMoV). L\(^2\) gene-mediated resistance is manifested through the formation of small necrotic local lesions (NLL).

To identify the viral determinants involved in the elicitation of the L\(^2\) gene mediated resistance, we have constructed chimeric viruses between the S strain of PMMoV (Wetter et al. 1984; Alonso et al. 1989) and paprika mild mottle virus (PaMMV) (Rast 1977; García-Luque et al. 1993). PMMoV-S overcomes the resistance conferred by L\(^2\), and infects these plants in a generalized fashion producing symptoms of mosaic; while PaMMV elicits the HR in L\(^2\) pepper plants. Both viruses belong to the so-called pepper strains of the tobamoviruses, that do not replicate in tomato plants and accumulate to only a low level in some *Nicotiana* species.

The results presented here show that the coat protein gene of PaMMV acts as avirulence factor of the L\(^2\) gene-mediated resistance. Mutations in the coat protein gene leading to the synthesis of a truncated coat protein reveal that a functional coat protein and not the RNA is responsible for the induction of the host response.

**RESULTS**

The viral determinants for inducing the L\(^2\) gene-mediated hypersensitive reaction map to the coat protein gene.

As an initial step to localize the PaMMV sequences involved in the induction of the L\(^2\) gene-mediated hypersensitive reaction, a hybrid virus genome, THG-1, was created. THG-1 contains the 5´-proximal 5348 nt of PMMoV-S fused to the 3´-terminal 1146 nt of PaMMV. The PaMMV region
corresponds to the C-terminal part of the 30K gene, the coat protein (CP) gene and the 3´ noncoding region. In addition, this genomic region contains the promoter elements for the CP subgenomic mRNA and minus-strand RNA synthesis (reviewed in Dawson 1992).

THG-1 hybrid genome (Fig. 1) was constructed at the cDNA level by replacing in the full-length cDNA clone pTS the PMMoV-S sequences extending from nucleotide 5348 to 6357 with the corresponding region of PaMMV. A conserved NcoI restriction site in the cDNA sequences of both viruses (nucleotide position 5348 in the PMMoV-S RNA sequence determined in Alonso et al. 1991) was utilized to prepare the hybrid cDNA. The pTHG-1 plasmid was digested with XhoI and transcribed with T7 RNA polymerase. Capped in vitro transcripts were characterized by inoculation in *N. tabacum* L. cv. Xanthi nc, *N. benthamiana* Domin. and *C. frutescens* cv. Tabasco.

Inoculation of THG-1 transcripts in Xanthi nc tobacco produced small necrotic lesions at 2 to 3 days postinoculation (d.p.i.) in all of the inoculated leaves. The lesions were undistinguishable, both in size and appearance rate, from the lesions formed after the infection by PMMoV-S. In contrast to this result, the infection by PaMMV induced formation of visible NLL only in the old leaves of the plant.

In tobacco, the THG-1 transcripts infectivity was about 50% lower than that of the pTS-derived transcripts, as measured by the number of NLL produced in each half-leaf after inoculating with similar amounts of each transcript. The reduced infectivity of the THG-1 transcripts could not be ascribed to the few additional nucleotides of plasmid origin present in the transcripts (5’-CAGAGATC in THG-1, and 5’-CGCG in PMMoV-S), since the number of NLL induced by the chimeric THG-1 virus were also lower when purified virions were used as source of inocula (data not shown). The data thus indicate that in this particular hybrid viral construct, the sequences of PMMoV and PaMMV are not fully compatible. A partly functional THG-1 hybrid 30K protein, or a reduced efficiency of the PMMoV-S-encoded viral polymerase to recognize the PaMMV RNA promoter elements present in the substituted region could account for the observed THG-1 virus diminished infectivity. In *N. benthamiana*, THG-1 virus produced symptoms of leaf curling and stunting at 12 to 15 d.p.i., similar to the symptoms displayed by PMMoV-S-infected plants. These symptoms differed from the PaMMV-induced yellowing of the infected leaves (data not shown).

In Tabasco pepper (L2L2) plants, the in vitro synthesized transcripts from pTHG-1 produced NLL at 3-4 d.p.i. (Fig. 1). The lesions were similar to the ones observed after the inoculation with the parental PaMMV, and distinct from the PMMoV-S-induced symptoms of leaf curling and bright mosaic (Figs. 1 and 2B). By RT-PCR and ELISA assays, neither THG-1 nor PaMMV could be detected in the upper non-inoculated leaves, thus confirming that the hybrid virus was localized to the inoculated leaves (data not shown).

Viral RNA was extracted from virions purified from systemically infected *N. benthamiana* plants and the identity of the viral progeny was tested by RT-PCR, using a pair of primers (corresponding to nucleotide positions 4834 to 4851 and 6321 to 6334 in the PMMoV-S RNA genome) covering the hybrid junction in THG-1. As illustrated in Figure 3A, the HindIII restriction pattern of the cDNA products obtained in

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**Fig. 1.** Pathogenicity of hybrid viruses comprised of sequences derived from PaMMV and PMMoV-S: Open boxes represent PMMoV sequences. Stippled boxes represent sequences from PaMMV. Relevant restriction sites used for constructing the hybrid genomes are indicated. UAA, stop codon introduced into the PaMMV coat protein gene. The symptoms induced in *Capsicum frutescens* L. var. Tabasco plants are described at the right of each construct. S, systemic infection. NLL, necrotic local lesions. A schematic diagram of the tobamoviral genomic organization is shown on top of the diagram. The regions coding for the 126 K and 183K proteins which are part of the polymerase complex, the 30 K protein responsible for the cell-to-cell viral movement and the coat protein are indicated.

**Fig. 2.** Symptomatology of *Capsicum frutescens* cv. Tabasco (L2L2) plants inoculated with PMMoV-S (B), THG-3 (C), PaMMV (D, left half), and THG-3 (D, right half). A, Control mock-inoculated plant.
the amplification of the THG-1 viral progeny was identical to the pattern obtained with the parental transcription clone pTHG-1. The observed HindIII restriction fragments were distinct from those resultant from the amplification of both PaMMV and PMMoV-S RNAs. In all cases, the restriction fragments corresponded to the sizes predicted from the nucleotide sequence.

Additionally, immunological assays were carried out to corroborate the coat protein identity of the chimeric THG-1 virus by using specific anti-PaMMV and anti-PMMoV-S sera, that discriminate PaMMV CP from PMMoV-S CP. Both assays: ELISA tests of the infected tissue and Western blot analysis of purified virus particles confirmed that the coat protein of THG-1 hybrid virus corresponded to that of PaMMV (data not shown).

These results indicate that the viral region responsible for the Capsicum L2 gene-mediated localization of PaMMV maps to the last 1000 nt of the genome.

We have previously described that the tobamoviral coat protein gene is involved in the elicitation of the Capsicum L2 gene-mediated resistance (Berzal-Herranz et al. 1995). Since both Capsicum L2 and L3 resistance genes are likely alleles (Boukema et al. 1980), we considered the possibility that the coat protein was also involved in the Capsicum L2 gene-mediated host response. To test this possibility a chimeric genome, THG-2, was constructed (Figs. 1 and 4), to allow the exact substitution of the PMMoV-S coat protein gene by the corresponding PaMMV region. Since the 30K and CP ORFs do not overlap in the genomes of PaMMV and PMMoV-S, it was possible to manipulate their sequences such that the resultant THG-2 virus would not contain PaMMV sequences other than the coat protein coding region.

In vitro synthesized THG-2 transcripts were directly inoculated onto Tabasco pepper (L2L2) plants. At 3 to 4 d.p.i., NLL appeared that were indistinguishable from those induced by PaMMV (Figs. 1 and 2). In addition, no virus could be detected by RT-PCR or ELISA analysis of the upper non-inoculated leaves of the infected plants (data not shown). To confirm the identity of the THG-2 viral progeny, single Tabasco NLLs were transferred to N. benthamiana plants and after two subsequent passages in this host, virion RNA was extracted from the systemically infected plants. The THG-2 viral RNA was analyzed by RT-PCR amplification and restriction of a cDNA fragment spanning the coat protein gene and the 3′-noncoding region, as described in Materials and Methods. The ClaI digestion of the amplified cDNA products yielded two fragments of 499 and 175 bp from the THG-2 cDNA, leaving uncut the PaMMV and PMMoV-S amplified cDNA products (Fig. 3B). Similarly, EcoRI digestion of the amplified cDNA products yielded the fragments expected from the nucleotide sequences of each virus: 492 and 273 bp in PaMMV, and 401 and 273 bp in THG-2 (Fig. 3B). Western blot analysis of purified virion particles and ELISA test of the infected tissues also showed that THG-2 virus contained the PaMMV CP (data not shown).

These results reveal that the substitution of the PaMMV coat protein gene into the PMMoV-S genome makes that the resistance-breaking PMMoV-S virus becomes localized by the action of the L2 gene.

![Fig. 3. PCR analysis of progeny virion RNAs. cDNA to purified THG-1, THG-2, PaMMV, or PMMoV was synthesized and PCR amplified as described under Materials and Methods. The amplified cDNA products were digested with the restriction enzymes shown on top of the figure. The pTHG-1 lane corresponds to the PCR amplification product of the template plasmid. Numbers on the left show the migration of selected bands from pUC18 digested with SauIII and Hindl used as size standards.](image)

![Fig. 4. Schematic representation of the THG-2 genome organization. Open boxes denote the open reading frames, and horizontal lines represent the noncoding sequences. Open boxes correspond to PMMoVS-S sequences. Stippled boxes correspond to PaMMV sequences. The nucleotide sequences and deduced amino acid sequences surrounding the coat protein gene in PMMoV-S, PaMMV (bold face), and THG-2 hybrid genomes are shown. The ClaI restriction sites used for constructing the hybrid genome is underlined.](image)
Elicitation of the HR is influenced by the coat protein.

To determine if the ability of the PaMMV CP to induce the L\textsuperscript{2} gene-mediated HR resides either in the coat protein or in the RNA itself, a chimeric mutant THG-3 with a frameshift mutation in the coat protein gene was constructed (Fig. 1). Thus, insertion of 4 bp 75 nt downstream of the AUG initiation codon of the PaMMV CP ORF produces a translation frameshift. The putative protein product would be 34 aa long instead of the 161-aa long polypeptide codified by the PaMMV CP ORF comprising the 26 N-terminal amino acids of PaMMV CP and 8 non-PaMMV amino acids at its C-terminus.

The in vitro transcripts generated from the THG-3 plasmids were inoculated directly into \textit{N. tabacum} Xanthi nc and Tabasco pepper plants. In Xanthi nc tobacco, THG-3 mutant induced NLL. However, no NLL were visualized in the inoculated Tabasco pepper leaves. By 15 d.p.i., the THG-3-inoculated Tabasco pepper plants showed an increase of the number of axillary buds relative to the mock-inoculated control plants (Fig. 2C). During the 2-mo period in which plants were inspected, no other visible symptoms were observed. No virion particles were detected when leaf dip preparations were examined by electron microscopy, nor could the viral coat protein be detected in the upper noninoculated leaves extracts by Western blot analysis (data not shown). However, the viral RNA was detected in both inoculated and upper noninoculated leaves at 17 d.p.i. by RT-PCR performed on total RNA (see Materials and Methods).

Nucleotide sequence analysis of the amplified cDNA fragment spanning the coat protein gene showed that the progeny RNA maintained the introduced mutation and there were no other nucleotide changes in this region. These data confirmed that the frameshift coat protein mutant had spread systemically in this host.

To determine the rate of the systemic spread of the coat protein mutant THG-3 vs. PMMoV-S in Tabasco plants, plants were inoculated with total leaf RNA extracted from either THG-3- or PMMoV-S-systemically infected-Tabasco and the presence of the virus was assayed at different d.p.i. by back-inoculation onto Xanthi nc tobacco plants. At 4 d.p.i., NLL were produced after the inoculation of tobacco with sap from either the inoculated or upper noninoculated PMMoV-S infected Tabasco leaves as well as from THG-3 inoculated leaves. The number of NLL induced by THG-3-infected tissue was lower (about 30%) than the ones induced by PMMoV-S. No NLL were visible in tobacco plants when the THG-3-upper noninoculated leaves were used as inoculum. At 7 d.p.i., the inoculation of sap from both THG-3-infected and upper noninoculated Tabasco leaves into tobacco produced NLL, showing that the spread of the coat protein mutant is reduced with respect to PMMoV-S.

Thus, we conclude that the RNA molecule itself is not responsible for the induction of the L\textsuperscript{2} gene-mediated HR and therefore, the PaMMV coat protein is required for the elicitation of HR and restriction of viral spread in L\textsuperscript{2} pepper plants.

DISCUSSION

The construction of chimeric viruses THG-1 and THG-2, containing sequences derived from PMMoV-S and PaMMV, has allowed us to determine that the PaMMV information required for the localization of the viral infection mediated by the pepper L\textsuperscript{2} resistance gene is located within the coat protein gene. The fact that the chimeric mutant THG-3 which codes for a truncated coat protein was able to infect Tabasco (L\textsuperscript{2}L\textsuperscript{2}) pepper systemically excludes the possibility that the RNA molecule itself is the active viral factor involved in the elicitation of the HR, and confirms that the PaMMV coat protein is the avirulence factor of the \textit{Capsicum} L\textsuperscript{2} gene-mediated resistance. Moreover the resistance L\textsuperscript{2}-breaking property of this defective THG-3 isolate shows that the resistance requires production of a virus-encoded protein. That is that resistance breaking is not the result of the suppression of preexisting defense mechanism(s). Instead, it is avirulence which is the positive viral function. This situation is consistent with resistance as manifestation of recognition of the viral elicitor by a host receptor (reviewed in Fraser 1990; Culver et al. 1991; Keen 1992; Staskawicz et al. 1995). Clearly, from the data presented here, the PaMMV CP is a good candidate for being the elicitor molecule of L\textsuperscript{2} resistance.

The finding that the tobamoviral coat protein acts as the avr factor for both \textit{Capsicum} L\textsuperscript{2} and L\textsuperscript{1} resistance genes is well in accordance with the genetic work earlier presented by Boukema (1982) in the sense that both genes are alleles of the L locus, although at present the possibility cannot be excluded that they could be closely linked genes.

Since all the tobamoviruses that elicit the L\textsuperscript{2} gene-mediated HR also elicit the HR in L\textsuperscript{1} plants, whereas PMMoV elicit the resistance conferred by the L\textsuperscript{1} gene but overcomes the L\textsuperscript{2} gene-mediated resistance, it might be possible that the L resistance genes against the tobamoviruses in \textit{Capsicum} have evolved to recognize a wider spectrum of tobamoviral coat proteins. A similar situation has been described for the \textit{Nicotiana} N\textsuperscript{1} gene-mediated resistance, able to recognize most of the tobamoviral coat proteins (Culver and Dawson 1991). The phenomenon that the coat protein acts as elicitor of the defense response in different plant genera such as \textit{Nicotiana} and \textit{Capsicum}, and possibly in other host systems (Dawson et al. 1988) might indicate the high degree of conservation of plant disease resistance genes, such that the same pathogen avr factor is functional in widely separated host genera (Whalen et al. 1988; Valent et al. 1991; Goulden and Baulcombe 1993).

It is at present unknown which part of the tobamoviral coat protein is involved in the recognition event that triggers the defense response. It could be either the entire coat protein or part of it. However, in both \textit{Nicotiana} N\textsuperscript{1} and \textit{Capsicum} L\textsuperscript{1} gene-mediated resistances, amino acid substitutions that modify the overall coat protein configuration have been implicated in the observed changes in the pathogen virulence towards the host (Dawson et al. 1988; Saito et al. 1989; Culver et al. 1994; Berzel-Herranz et al. 1995). Accordingly, and due to the ability of the L\textsuperscript{2} pepper plants to recognize all but one of the pepper-infesting tobamoviruses, it is possible that several coat protein structural or conformational configurations might be recognized in this host and that subtle changes that alter the configuration of the protein, such as those previously described (Culver and Dawson 1989b; Culver et al. 1994; Berzel-Herranz et al. 1995), make PMMoV to escape the L\textsuperscript{2} gene action.

The ability of the THG-3 mutant coding for a truncated coat protein to induce in Tabasco pepper plants symptoms that were different from those observed in PMMoV-S-infected...
plants (Fig. 2C and B) indicates that the coat protein modu-
lates symptom expression in this particular host-virus system, as
described for other host-virus combinations such as alfalfa
mosaic virus-tobacco (Neeleman et al. 1991), cucumber mos-
aic virus-tobacco (Shintaku et al. 1992), and tobacco mosaic
virus-tobacco (Dawson et al. 1988; Banerjee et al. 1995). In
contrast, the tobamoviral coat protein does not modify the
symptomatology in either Nicotiana sylvestris (Culver and
Dawson 1989a) or Capsicum chinense plants (Berzal-Herranz
et al. 1995), thus indicating that the effect of the viral coat
protein on symptom expression depends specifically upon the
host-pathogen interaction.

MATERIALS AND METHODS

Viral sources, purification, and RNA extraction.
The origin of PMMoV-S (pathotype P1.2) has been reported
(Alonso et al. 1989). PaMMV (pathotype P1) corresponds to a
Dutch isolate from pepper described as tobamovirus isolate
P11, and lately renamed PaMMV (Rast 1977; García-Luque et
al. 1993). Viruses were purified and viral RNAs were ex-
tracted as described (Alonso et al. 1991; and García-Luque and

Construction of hybrid viral genomes.
The production of the full-length PMMoV-S cDNA tran-
scription clone pTS and its derivative pTHI-8 was previously
described (Berzal-Herranz et al. 1995). The nucleotide num-
bering is that of Alonso et al. (1991).

DNA templates for different hybrid genomes were con-
structed by substituting the PMMoV-S sequences contained in
plasmid pTS, with the correspondent of PaMMV. Recombi-
nant constructs were tested by restriction enzyme analysis and
partial DNA sequencing.

Two PaMMV cDNA clones: p1C15, spanning from nucleo-
tides 24 to 1137 from the 3’-end, and p1C3 containing the 3’-
terminal 887 nucleotides (García-Luque et al. 1993) were used
to construct the intermediate PaMMV plasmid p1.915. This
clone contains the 3’-terminal 1146 nucleotides and it was
obtained by inserting the 476-bp SmaI-BglII fragment from plasmid
p1C15 into the HindIII-blunt-BglII sites of plasmid p1C3. Hybrid genome THG-1 was created by cloning the
1154 bp Ncol/XbaI-blunt fragment from plasmid p1.915 into
NcoI/EcoRI-blunt-digested plasmid pTS.

To construct pTHG-2, the nucleotide sequences surrounding the
coat protein gene in both PMMoV-S and PaMMV were
mutagenized by PCR. The borders of the PaMMV coat protein
gene were modified by using as template plasmid p1C15 and
the mutagenic oligonucleotides 1CPI and 1CPF as 5’ and 3’
primers. Oligonucleotide 1CPI (5’-GACTACATTTACAC-
TGCCCTTATACTGT) corresponds to the first 14 nt of the
codon of the coat protein gene (underlined) with an added HindIII recog-
nition site (bold). Oligonucleotide 1CPF (5’-GAGTACATTTAC-
ACTTATATACTTG) is complementary to PaMMV nucleotide positions 272 to 287 from the 3’-end, in
which an internal ClaI restriction site (bold) was located next
to the TAA coat protein stop codon (underlined).

The nucleotide sequences upstream the coat protein gene in
PMMoV-S were mutagenized on plasmid pSS2, spanning
from nucleotide 5187-6357 in the PMMoV-S RNA genome
(Berzal-Herranz et al. 1995); by using as 5’ and 3’ primers the
universal M13/pUC reverse sequencing primer, and the oli-
gonucleotides 5’-CCAAAGCTTTAACAAGAAGACTC
complementary to PMMoV-S nucleotide 5665 to 5682 (under-
lined), to which a DraI restriction site (boldface) was added.
The amplified product was doubly restricted with StyI and
Dral, and the 332-bp fragment was gel purified and ligated to
the 507-bp HincII-NsiI fragment obtained after the mutagenic
PCR amplification and restriction of the PaMMV coat protein
gene, above described. Both fragments were cloned together
into the StyI-PstI digested pT3T7 plasmid, generating clone
p1.3.

The coat protein gene 3’ downstream sequences in the
PMMoV-S genome were also modified by PCR using as template
plasmid pSS-2, and the oligonucleotides: 5’-CGAA-
TTCAATTGGTCGATTGCAATAAGTGAACGA, corre-
spanding to PMMoV nucleotide positions 6165 to 6185
(underlined) in which a ClaI (boldface) plus an EcoRI restric-
tion sites were incorporated, and the universal M13/pUC di-
rect sequencing primer as 5’ and 3’ primers, respectively. The
amplified PCR product was restricted with EcoRI, and the
278-bp fragment was cloned into the EcoRI site of plasmid
pT3T7BM. After corroborating the nucleotide sequence, the
270-bp ClaI-EcoRI fragment of the resultant plasmid p2-3’
was ligated to the 823-bp Ncol-ClaI fragment from plasmid
p1.3 and both together to plasmid pTS restricted with NcoI
and EcoRI, producing hybrid genome pTHG-2.

The frameshift coat protein mutant was obtained by filling
the protruding termini of the unique BglII site of clone p1.3.
After digestion with KpnI and NcoI, both fragments: the 415-
bp Ncol-BglII-blunt and the 455-bp BglII-blunt-KpnI were
cloned into the NcoI and KpnI sites of plasmid pT3T7BMB,
resulting in plasmid pBg7. After corroborating that the plasmid
had the desired 4-bp insertion, the 831-bp Ncol-ClaI fragment
was cloned along with the 270-bp ClaI-EcoRI fragment from plasmid
p2-3’ into the NcoI and EcoRI sites of plasmid pTS, resulting in
plasmid pTHG-3.

In vitro synthesis and plant propagation of
viral RNA genomes.

Genomic RNAs were synthesized in vitro transcription of recombinants with T7 RNA polymerase, once they were digested with either XbaI (pTHG-1) or MluI (pTS, pTHG-2, or pTHG-3), and propagated directly in Nicotiana tabacum L. Xanthi nc, Capsicum frutescens L. cv Tabasco (L2’L) and
Nicotiana benthamiana Domin. plants, as previously de-
scribed (Berzal-Herranz et al. 1995).

Single necrotic local lesions from Tabasco pepper plants inoculated with either THG-1 or THG-2 transcripts were used to inoculate N. benthamiana plants to analyze the viral progeny from purified virions.

Infecctivity assays of Tabasco pepper plants inoculated with the different viral RNA genomes were carried out in Xanthi nc
nicotiana plants using sap or total leaf RNA extract as source of
inocula. Total leaf RNA was extracted according to Logemann et al. (1987).

Analysis of viral progenies.
The viral progenies derived from the infectious transcripts
were analyzed by reverse transcriptase-PCR followed by re-
striction enzyme digestion, as described (Tenllado et al. 1994).
The use of oligonucleotides 5’-GTTCGTTTTGGTTATAGA
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PaMMV and PMMoV-S. The ACKNOWLEDGMENTS described (Berzal-Herranz et al. 1995).

Immunosorbent assay (DAS-ELISA) were carried out as described in Berzal-Herranz et al. (1995). Virion particles from PaMMV, PMMoV-S, and THG-2, respectively. Digestion of the amplified fragments with Clal and EcoRI was used to discriminate hybrid virus THG-2 from PaMMV and PMMoV-S. The Clal restriction site was introduced during the construction of hybrid template pTHG-2, and the EcoRI restriction site is present in the PaMMV coat protein gene 516 nt from the 3′ end.

The progeny viral RNA from the mutant coding for a truncated coat protein was analyzed by nuclease acid sequencing through the modified region. Nucleotide sequence was determined on RT-PCR amplified fragments, by using the femtomol sequencing kit (Promega). cDNA was synthesized on total leaf RNA using oligonucleotides 1CPI and 1CPF as primers.

The coat proteins of purified virions were analyzed by Western blot, using specific PaMMV and PMMoV-S antisera, as described in Berzal-Herranz et al. (1995). Virion particles (10 ng) were heated at 100°C for 2 min in SDS-PAGE sample buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.01% Bromphenol blue) and resolved in the discontinuous SDS-PAGE system (Laemmli 1970). The production of the rabbit anti-PaMMV and anti-PMMoV-S sera have been previously reported (Tenllado et al. 1995; Alonso et al. 1991).

Virus detection by RT-PCR and ELISA.

The detection of viral sequences in plants was carried out by RT-PCR on total leaf RNA. cDNA was synthesized using oligonucleotides 1CPI and 1CPF as primers.

The detection and quantification of viral coat proteins in plant extracts by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) were carried out as described (Berzal-Herranz et al. 1995).

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LITERATURE CITED


