Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper


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The entire genomic RNA of a Spanish isolate of pepper mild mottle virus (PMMV-S), a resistance-breaking virus in pepper, was cloned and sequenced and shown to be similar to other tobamoviruses in its genomic organization. It consisted of 6357 nucleotides (nt) and contained four open reading frames (ORFs) which encode a 126K protein and a readthrough 183K protein (nt 70 to 4908), a 28K protein (nt 4909 to 5682) and a 17.5K coat protein (nt 5685 to 6158). This is the first tobamovirus in which none of the ORFs overlap. Both its nucleic acid and predicted protein sequences were compared with the previously determined sequences of other tobamoviruses. The variations and similarities found and their relationship with the pathogenicity of this virus are discussed.

Introduction

Pepper mild mottle virus (PMMV) is a member of the tobamovirus group of positive-strand RNA viruses. The complete nucleotide sequence of three other tobamoviruses, tobacco mosaic virus (TMV) (Goelet et al., 1982), tomato mosaic virus (ToMV) (Ohno et al., 1984) and tobacco mild green mottle virus (TMGMV) (Solis & García-Arenal, 1990) have already been reported. The tobamoviral RNA encodes four different proteins: 126K, 183K, 30K and 17.5K, in that order. The 126K and 183K proteins are involved in the replication processes (Young et al., 1987; Quadt & Jaspars, 1989), the 30K protein participates in the cell-to-cell spread of the virus (Deom et al., 1987; Mashi et al., 1987) and the 17.5K protein is the coat protein. The 126K and 183K proteins are directly translated from the viral RNA, whereas the 30K protein and coat protein are translated from subgenomic RNAs (Palukaitis & Zaitlin, 1986).

PMMV is one of the most destructive pathogens of protected pepper crops. It is found infecting pepper cultivars with genetically incorporated resistance to TMV and ToMV. The infection by this virus produces important economic losses all over the world in crops grown under plastic or glass (Wetter & Conti, 1988). Additionally, PMMV is unable to infect tomato plants and possesses a reduced capability to replicate and/or accumulate in tobacco plants when compared to TMV and ToMV (Wetter et al., 1984; Garcia-Luque et al., 1990).

To develop an understanding of the mechanism(s) involved in these biological properties, we have determined the nucleotide sequence of PMMV-S RNA, a Spanish isolate of PMMV (Alonso et al., 1989). The nucleotide sequences of its 5' and 3' non-coding regions have previously been reported (Avila-Rincón et al., 1989). In this paper, we present the cloning and complete nucleotide sequence of PMMV-S and the analysis of its deduced amino acid sequences.

Methods

Virus propagation, purification and RNA extraction. The origin of PMMV-S has been reported previously (Alonso et al., 1989). The virus was purified from Nicotiana clevelandii Gray plants as described (García-Luque et al., 1990). Virion RNA was prepared by conventional SDS-phenol extraction after heating of the particles in 20 mM-sodium phosphate buffer pH 7.0, 0.5% SDS for 20 s at 100 °C.

cDNA synthesis and cloning. cDNA was prepared as described by Gubler & Hoffman (1983), using a commercial cDNA synthesis kit (Boehringer-Mannheim). PMMV-S RNA was 3' polyadenylated in vitro with Escherichia coli poly(A) polymerase (0.25 unit/µg of RNA) for 7 min at 37 °C, under the conditions recommended by the manufacturer (Pharmacia LKB Biotechnology), and first-strand cDNA synthesis was primed with oligo(dT). Double-stranded cDNA was size-fractionated in 0.8% agarose gel, and the cDNA was eluted and ligated into plasmid pUC18 digested with HincII. In another experiment, EcoRI linkers were added to ds cDNA that had previously been treated with EcoRI methylase and EcoRI-digested prior to being size-fractionated in agarose gels. After elution, the cDNA was cloned into EcoRI-digested pUC18. Plasmids were tested for the presence of viral cDNA inserts by colony hybridization, using randomly primed...
Nucleotide sequence determination and analysis. The nucleotide sequence of cDNA clones was determined by the chemical degradation procedure (Maxam & Gilbert, 1980). Subclones used for sequencing were generated by deletions with nuclease Bal31 or restriction enzyme sites of pUC18. All recombinant DNA techniques were as described by Maniatis et al. (1982), using E. coli strains JM83 and DH5α.

Results and Discussion

Sequence determination and terminal non-coding regions

Fig. 1 shows the strategy used to determine the sequence of the genomic RNA of PMMV-S from a set of overlapping cDNA clones. They contain sequences representing all but the first 34 nucleotides located at the 5' end of PMMV-S RNA. Most of the sequence was obtained from at least two independent cDNA clones.

The nucleotides of the 5' and 3' non-coding regions of PMMV-S RNA have previously been sequenced directly on the viral RNA (Avila-Rincón et al., 1989). As with other tobamoviruses, PMMV-S possesses a 69 nt leader sequence, devoid of G residues, termed the Ω fragment (Richards et al., 1977; Avila-Rincón et al., 1989). Its 3' non-coding region is 199 nt long. It was previously proposed that some structural features in the tRNA-like conformation of PMMV-S RNA such as two unpaired nucleotides connecting the aminoacyl and anticodon arms could be related to its lower replicability observed in tobacco plants (Avila-Rincón et al., 1989) as described for certain chimeric tobamoviruses (Ishikawa et al., 1988).

The determined nucleotide sequence of the cDNA clones coincides with that of the RNA except for a single base transition at position 6181 which would change the C/G pair (6197/6181) situated at the beginning of the V stem in the proposed secondary structure (Avila-Rincón et al., 1989) to a C/A pair. This nucleotide substitution was present in three of four sequenced cDNA clones. In other parts of the genome no sequence heterogeneity was detected in the clones analysed. The only nucleotide difference was found in clone 4, in which the insertion of a T between nt 5385 and 5386 could lead to a truncated protein.

Fig. 2 shows the sequence of PMMV-S RNA and that of its deduced amino acid. The genome of PMMV-S is 6357 nt long. It shares an overall sequence identity of 69.4% with the RNA of ToMV (Ohno et al., 1984), 68.5% with that of TMV (Goel et al., 1982) and 64% with TMGMV (Solis & García-Arenal, 1990), the other members of the tobamovirus group whose entire RNA sequences are already known. As shown in Table 1, also, PMMV-S shares a higher degree of amino acid sequence identity with ToMV than with TMV and TMGMV.

Organization of the 126K/183K gene sequence

The first open reading frame (ORF) of PMMV-S RNA begins at nt 70, in the first AUG encountered from the 5' end, and extends to nt 3423, encoding a protein of 1117 amino acids (126K), with a calculated Mr of 126304. The readthrough of the amber codon (UAG), possibly by insertion of tyrosine (Beier et al., 1984), results in a 183K protein which terminates at position 4908. It is composed of 1612 amino acids with a predicted Mr, of 183340. The nucleotide and amino acid sequences in the readthrough part of the 183K protein (nt 3421 to 4908, amino acids 1118 to 1612) (Fig. 2 and 3) are the most highly-conserved in all the genome, with only 14 and 15 non-conservative amino acid substitutions with respect to the corresponding proteins of ToMV and TMV, respectively.

The 126K and 183K proteins are thought to be involved in viral replication because they have been detected in partially purified preparations of the viral polymerase complex and because they contain several sequence motifs which are conserved in proteins known to act in replicative processes of plant and animal viruses (Young et al., 1987; Goldbach & Wellink, 1988; Strauss & Strauss, 1988; Quadt & Jaspers, 1989). The alignment of the 126K/183K proteins of PMMV-S with those from the more closely related tobamoviruses (ToMV and TMV) shows that the sequence is well conserved along all the protein (Fig. 3), except for three stretches (amino acids 155 to 191, 623 to 669 and 768 to 791) in which non-conservative substitutions as well as deletions and insertions occur. Other regions of weaker amino acid sequence identity correspond to positions 382 to 388, 537 to 555 and 991 to 1001 (Fig. 3).

Based upon the existence of conserved motifs between the tobamoviral 126K protein and those from other RNA viruses, two functional domains have been defined. The first one, in the amino part of the protein, has homology with the nsP1 protein of alphaviruses and with the amino part of other proteins implicated in the
replication of RNA viruses with a monopartite or divided genome (Ahlquist et al., 1985). In this region, Rozanov et al. (1990) have identified two conserved sequence motifs defined by the presence of an invariant His in the first motif and the sequence Asp-X-X-Arg in the second one, that are located at amino acid positions 76 to 81 and 134 to 138 in the 126K/183K protein of TMV (Fig. 3), respectively. By analogy with the nsP1 protein of Sindbis virus (Mi et al., 1989), this domain may be responsible for the methyltransferase activity necessary for the cap formation of the genomic and subgenomic RNAs. Of the two amino acid sequence motifs described by Rozanov et al. (1990), the predicted 126K/183K protein from PMMV-S possesses both, except for a conservative substitution Ile to Val at position 135 (Fig. 3). This domain is the best conserved with respect to TMGMV.

The second functional domain in the 126K/183K
protein has been mapped to amino acids 833 to 1086, and is known as the helicase domain (Hodgman, 1988; Gorbalenya & Koonin, 1989; Habili & Symons, 1989). It is implicated in nucleic acid unwinding, and possibly in other processes such as recombination and transcription.

The conservative type and the other three of the non-conservative type. It is therefore possible that the amino acid exchanges occurring in this domain in TMGMV could result in its lower replicability, in comparison with TMV or ToMV (Wetter, 1986). In the domain II to V motifs (amino acid positions 902 to 913, 930 to 940, 966 to 974, 1038 to 1055 and 1070 to 1085, respectively), all of the amino acid substitutions that occur in the PMMV-S protein with respect to the ToMV and TMV ones are of the conservative type and the other three of the non-conservative type. It is therefore possible that the amino acid exchanges occurring in this domain in TMGMV could result in its lower replicability, in comparison with TMV or ToMV (Wetter, 1986). In the domain II to V motifs (amino acid positions 902 to 913, 930 to 940, 966 to 974, 1038 to 1055 and 1070 to 1085, respectively), all of the amino acid substitutions that occur in the PMMV-S protein with respect to the ToMV and TMV ones are of the conservative type: Ile to Leu (position 906), Tyr to Phe (position 930), Val to Ile (position 974), Tyr to Phe (position 966), Ala to Glu (position 1049), Val to Leu (position 1048), Ala to Glu (position 1049), Val to Leu (position 1070), Lys to Arg (position 1079) and Leu to Ile (position 1081). The same occurs with the amino acid substitutions in TMGMV (Fig. 3).

The carboxyl end of the 183K protein results from the conservative type and the other three of the non-conservative type. It is therefore possible that the amino acid exchanges occurring in this domain in TMGMV could result in its lower replicability, in comparison with TMV or ToMV (Wetter, 1986). In the domain II to V motifs (amino acid positions 902 to 913, 930 to 940, 966 to 974, 1038 to 1055 and 1070 to 1085, respectively), all of the amino acid substitutions that occur in the PMMV-S protein with respect to the ToMV and TMV ones are of the conservative type: Ile to Leu (position 906), Tyr to Phe (position 930), Val to Ile (position 974), Tyr to Phe (position 966), Ala to Glu (position 1049), Val to Leu (position 1070), Lys to Arg (position 1079) and Leu to Ile (position 1081). The same occurs with the amino acid substitutions in TMGMV (Fig. 3).

The carboxyl end of the 183K protein results from
sequences as in Table 1. Only amino acid exchanges are indicated. Gaps are indicated by (-). Numbering corresponds to that from (1989), Poch et al. Numbers in parentheses indicate the total length of each protein. The sequence motifs defined by Gorbalenya & Koonin et al. (1990) are boxed.

Fig. 3. Alignment of the deduced amino acid sequences of the 126/183K proteins from different tobamoviruses. Source of amino acid sequences: as in Table 1. Only amino acid exchanges are indicated. Gaps are indicated by (-). Numbering corresponds to that from (1989), Poch et al. Numbers in parentheses indicate the total length of each protein. The sequence motifs defined by Gorbalenya & Koonin et al. (1990) are boxed.
readthrough of the UAG stop codon at nt 3421 to 3423. The suppression of a termination codon is a widespread phenomenon among animal and plant RNA viruses, and is related to the regulation of the expression of the different components of the viral RNA polymerase (Ishikawa et al., 1986; Strauss et al., 1988). The fact that the surrounding nucleotide sequences (ATAGCAAT- TACAG) at positions 3420 to 3432 (Fig. 2) are strictly conserved in all the tobamoviruses reveals the functional importance of this particular region in their genomes, as it also occurs in alphaviruses (Strauss et al., 1988). The so-called polymerase module is found in the carboxy portion of the protein, in which four domains (A to D) have been defined (Poch et al., 1989). This module is common to all of the DNA- and RNA-dependent RNA polymerases, and it is known to be involved in the elongation of pre-existing chains (Quadt & Jaspers, 1989). The Gly-Asp-Asp motif first identified by Kamer & Argos (1984) is found in the C domain surrounded by hydrophobic residues. The alignment of the PMMV-S 183K protein with those from other tobamoviruses (Fig. 3) also shows that all the amino acid substitutions in these domains are of the conservative type.

The only non-conservative differences between TMGMV and PMMV-S occur in the D domain, Cys to Gly (position 1496) and Asn to Leu (position 1500). The higher sequence variability in this readthrough part of the 183K protein is found in the region located at amino acids 1250 to 1291. There is also lower amino acid sequence identity in both the N- and C-terminal segments (Fig. 3), a feature common to other RNA viruses (Haseloff et al., 1984; Allison et al., 1989).

It remains to be determined whether the attenuated biological behaviour of PMMV-S in tobacco, in comparison with TMV and ToMV, could be ascribed to regions of maximum sequence heterogeneity or to segments of the non-highly conserved sequence of the 126K/183K protein, as previously described for the attenuated L1,1A strain of ToMV (Nishiguchi et al., 1985) in which the amino acid substitutions responsible for this characteristic have been mapped in regions, not highly conserved, of the 126K protein (amino acid residues 348, 759 and 894). It is also unknown whether the ability of PMMV-S to break the resistance against tobamoviruses conferred by the L1 and L2 genes in pepper (Boukema et al., 1980; Garcia-Luque et al., 1990) is due to any of the amino acid changes which take place in this protein, as described for the L1a1 strain of ToMV, in which two amino acid substitutions (Glu to Gln and Tyr to His) at positions 979 and 984, respectively (980 and 985 in the PMMV-S protein) have been identified as those responsible for the ability of this strain to break the Tm-1 resistance gene in tomato (Meshi et al., 1988). However, since none of the resistance conferred by the L1 and L2 genes in pepper is expressed in protoplasts (unpublished results), it is plausible to consider that other regions of the PMMV-S genome may be implicated also.

**Organization of the 30K protein gene**

The third ORF of PMMV-S encodes the 30K protein (Fig. 2). Translation initiates at nt 4909 and terminates at nt 5682; thus the coding region for this protein overlaps with neither the coding region for the 183K protein nor with that for the coat protein. Its putative translation product is 257 amino acids long with a calculated Mr of 28347. Therefore, this is the first tobamovirus in which none of the reading frames overlap, since in ToMV, TMV, cucumber green mottle mosaic virus (CGMMV) and TMGMV the 5′ end of the genes encoding the 30K protein overlap with the 3′ end of those encoding the 183K proteins (Goelet et al., 1982; Ohno et al., 1984; Saito et al., 1988; Solis & Garcia-Arenal, 1990), and in sunn-hemp mosaic virus (SHMV) as well as in CGMMV (Meshi et al., 1982; Saito et al., 1988) their 30K-coding regions overlap at the 3′ end with their coat protein genes.

The 30K proteins of tobamoviruses are responsible for cell-to-cell spread of the viral infection (Deom et al., 1987; Meshi et al., 1987), by modifying the plasmodesmata (Wolf et al., 1989). Although the exact mechanism of action is unknown (Hull, 1989), a domain responsible for binding to nucleic acids which maps between amino acid positions 65 and 87 has been defined (Citovsky et al., 1990). As with the consensus sequences in the 126K/183K proteins, the amino acid substitutions that take place in this region of the PMMV-S 30K protein are of the conservative type with respect to TMV, ToMV and TMGMV, except for the semi-conservative exchange Ala to Val (position 70) in the ToMV 30K protein (Fig. 4).

As in other tobamoviruses (Ohno et al., 1984; Solis & Garcia-Arenal, 1990) the PMMV-S 30K protein is encoded in the least conserved part of the entire genome, both at the nucleotide and amino acid levels (Table 1). Its alignment with those from the most closely related tobamoviruses (Fig. 4) shows that the PMMV-S 30K protein shares a higher degree of amino acid sequence identity with that of TMV than with those of ToMV or TMGMV, in contrast to other proteins encoded by PMMV-S. It contains two well conserved regions located at amino acid positions 46 to 125 and 151 to 204. In the first one, only one non-conservative amino acid substitution takes place in the PMMV-S 30K protein with respect to ToMV and TMV (Arg to Tyr, both at position 109) and TMGMV (Ala to Cys at position 113). In the second well-conserved region, all of the amino acid changes among the TMV, ToMV and PMMV-S 30K proteins are of the conservative type, but it is less conserved compared to TMGMV, with three non-
conservative substitutions (Leu to Lys, Thr to Leu and Ala to Lys at positions 172, 179 and 192, respectively) (Fig. 4). Several amino acid changes in the central segment of the tobamoviral 30K proteins have been identified in temperature-sensitive mutants defective in cell-to-cell movement (Ohno et al., 1983; Zimmern & Hunter, 1983) as well as in the Ltbl strain of ToMV, known to overcome the resistance conferred by the Tm-2 gene in tomato plants (Meshi et al., 1989). This capability resides in two amino acid substitutions, at positions 68 and 133 (Cys to Phe and Glu to Lys, respectively). In this region of the PMMV-S 30K protein (Fig. 4), there are three non-conservative amino acid changes with respect to the proteins of TMV (Glu to Met, Ala to Lys and Lys to Ala at positions 133, 147 and 150, respectively) and of ToMV (Ala to Lys, Ala to Lys and Lys to Ala at positions 130, 147 and 150) but only one (Val to Pro at position 136) with respect to TMGMV. Although some of these substitutions may be of a compensatory type, they could be responsible for the ability of PMMV-S to overcome the pepper L^1^ and L^2^ resistance genes. The carboxy region of the tobamoviral 30K proteins are the most variable in terms of length or amino acid sequence. However, Saito et al. (1988) found that all of these proteins have a particular charge distribution with a basic domain flanked by two acidic domains. In this sense, the content of acidic amino acids (Glu, Asp) in the extreme C terminus of the 30K protein of PMMV-S is lower (four) than that of TMV and ToMV (six and seven, respectively). These changes could be involved in the adaptability of PMMV-S to its pepper host, although they may only represent, as stated above, the high degree of variability in this area of the tobamoviral 30K proteins.

**Coat protein gene**

The fourth ORF of PMMV-S encodes the coat protein. It ranges from nt 5685 to 6158, with an intergenic region of two nucleotides between the 30K and coat protein ORFs (Fig. 2). The resulting protein consists of 156 amino acids, with a calculated Mr of 17110. This value differs from the previous report of 158 amino acids for the coat protein of an Italian isolate of PMMV (Wetter et al., 1984), determined by amino acid analysis. Although PMMV-S and PMMV are different isolates, which can be distinguished by their responses in *Capsicum* spp. with different resistance genes and therefore have been identified as different pathotypes (Garcia-Luque et al., 1990), sequencing of the Italian PMMV coat protein gene has shown that it also consists of 156 amino acids (M. L. Ferrero, I. Garcia-Luque, E. Alonso, A. de la Cruz, J. F. Rodriguez, M. T. Serra & J. R. Diaz-Ruiz, unpublished results).

The alignment of the deduced amino sequence for the coat protein gene of PMMV-S with those of other tobamoviruses (Fig. 5) shows that there is a strict conservation of those amino acid sequence motifs (36 to 41, 88 to 94, 113 to 120) which correspond to the RNA-binding site in the coat protein (Altschuh et al., 1987). These differences in the sequence motifs and therefore have been identified as different pathotypes (Garcia-Luque et al., 1990), sequencing of the Italian PMMV coat protein gene has shown that it also consists of 156 amino acids (M. L. Ferrero, I. Garcia-Luque, E. Alonso, A. de la Cruz, J. F. Rodriguez, M. T. Serra & J. R. Diaz-Ruiz, unpublished results).

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Dawson, 1988; Saito et al., 1988, 1989; Culver & Dawson, 1989).

Based upon nucleotide sequence homology with the TMV origin of assembly (Zimmern, 1977), the predicted position of this region in PMMV-S is located between nt 5458 and 5517, in accordance with the absence of encapsidation of its coat protein mRNA as shown by electron microscopy observation (Wetter et al., 1984) and electrophoretic analysis of the virion particles (Garcia-Luque et al., 1990).

Functional and evolutionary considerations

The determination of the nucleotide sequence of PMMV-S RNA has allowed us to confirm that the entire genome of this virus has diverged from other related tobamoviruses at a similar rate. The grouping of the tobamoviruses based on the amino acid composition of their coat proteins (Gibbs, 1986) and on the basis of the peptide pattern of the 126K proteins (Fraile & Garcia-Arenal, 1990) corresponds well to what is deduced from the entire genome, i.e. PMMV is located in the same cluster as TMV, ToMV and TMGMV, being more closely related to ToMV and TMV. These data also confirm the relationship between ToMV and PMMV-S previously found by serological analysis (Wetter et al., 1984;alonso et al., 1989).

The possession by PMMV-S RNA of all the conserved sequence motifs necessary for replication and virion stability, its biological properties such as the diminished capability to replicate and/or accumulate in tobacco plants, its ability to overcome the tobamoviral resistance genes in pepper and its inability to infect tomato plants, should be ascribed to the requirements for the establishment of a functional interaction(s) with the host factor(s) known to be necessary for the efficient multiplication of the viruses in their host plants. Whether these requirements are related to changes at the amino acid level (polymerase, 30K and/or coat proteins) or at the nucleotide level (3' non-coding region) is under current study.

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