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The nucleotide sequence of the coat protein genes and 3′ non-coding regions of two resistance-breaking tobamoviruses in pepper shows that they are different viruses

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Summary. The nucleotide sequence of the coat protein genes and 3′ non-coding regions of two different resistance-breaking tobamoviruses in pepper have been determined. The deduced coat protein of an Italian isolate of pepper mild mottle virus (PMMV-I) consists of 156 amino acids and its 3′ non-coding region is 198 nucleotides long. They have been found to be very similar in sequence and structure to those previously reported for a Spanish isolate (PMMV-S). In contrast, a Dutch isolate termed P 11 codes for a coat protein of 160 amino acids and its 3′ non-coding region is 291 nucleotides long, which may have arisen by duplication. The nucleotide and the predicted coat protein amino acid sequence analysis show that this isolate should be considered as a new virus within the tobamovirus group. The term paprika mild mottle virus (PaMMV) is proposed.

Introduction

Different tobamoviruses such as tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), or tobacco mild green mosaic virus (TMGMV) have been described infecting pepper crops [24, 37, 39, 61]. In addition, the introduction of commercial pepper cultivars and hybrids with incorporated resistance genes to common strains of TMV and ToMV has led to descriptions of several tobamoviruses that were able to infect these resistant pepper cultivars and were given different names [7, 8, 14, 17, 23, 36, 46, 49, 55, 57, 61–64].

These resistance-breaking tobamoviruses of pepper have been characterized to different extents by their biological and serological properties and most of them share similar properties such as the ability to induce mottling diseases in pepper, the inability to infect tomato plants, and the induction of necrotic local

lesions in some hosts which are much smaller than the ones produced by TMV or ToMV in the same plants [3, 20, 36, 59]. These viruses have been differentiated by breeders as pathotypes P_1, $P_{1,2}$, and $P_{1,2,3}$ based upon their ability to overcome the resistance conferred by an allelic series of *Capsicum* spp. genes known as L^1, L^2, and L^3, respectively [8, 55]. In every case, the hypersensitive resistance is manifested through the induction of necrotic local lesions.

In a previous work, a tobamovirus infecting TMV-resistant pepper crops grown under plastic in the southeast region of Spain [3] was identified as a $P_{1,2}$ pathotype [20] and based upon its biological, biochemical and serological properties, it was termed pepper mild mottle virus strain S (PMMV-S) to differentiate it from an Italian isolate [63], here referred as PMMV-I which was identified as a $P_{1,2,3}$ pathotype [20].

In order to define the regions of the viral genome involved in the induction of the hypersensitive resistance genes against the tobamoviruses in *Capsicum* spp., we have previously determined the complete genome sequence of PMMV-S. In this work, we present the sequences of the coat protein cistrons and 3′ non-coding regions of PMMV-I and of a Dutch virus isolate termed P 11, identified as a P_1 pathotype [49]. The data obtained made it possible to clarify the status of the tobamoviruses involved in resistance breakage of the different *Capsicum* spp. genes, which is important for resistance breeding. In addition, the nucleotide and amino acid sequence homology values found, indicate that the Dutch isolate P 11 should be considered as a new virus within the tobamovirus group, different from PMMV-S and PMMV-I which seem to be strains of the same virus.

Materials and methods

Viral sources, propagation, purification, and RNA extraction

The origin of PMMV-S has been reported [20]. PMMV-I is an Italian isolate of PMMV [63] kindly supplied by Dr. M. Conti (Italy). The Dutch P 11 isolate from pepper [49] was a kind gift from Dr. A. Th. B. Rast (Netherlands).

Viruses were purified from systemically infected *Nicotiana clevelandii* Gray plants and virion RNAs were prepared from purified virus particles by SDS-phenol extraction as previously described [4, 20]. The resistance-breaking characteristics of the purified viruses were confirmed by inoculation to the appropriate *Capsicum* spp. plants [20].

cDNA synthesis and cloning

The synthesis and cloning of cDNA was done essentially as described [25]. An oligonucleotide (5′ GGGGGATTCGAACCC), complementary to nucleotides (nt) 24 to 37 and 24 to 48 from the 3′ end of PMMV-I and P 11 isolate RNAs, respectively, was used for priming first strand synthesis. Double-stranded (ds) cDNA was size-fractionated in 4% polyacrylamide slab gels, ligated to Hin dII-digested pUC 18 plasmid and used to transform *E. coli* strain DH5α.

Due to specific reorganization in most of the cDNA clones which contained the coat protein coding region of the P 11 virus, another set of clones was prepared by using an oligonucleotide (5′ TGGGCCCCATACCCGGGG), complementary to the last 3′ end nt

of this RNA as primer for first strand cDNA synthesis. The ds cDNA was digested with Hin dIII, fractionated by electrophoresis in a 1% agarose gel, and the 888 nt long fragment was eluted and cloned into the Hin dIII-Hin dII sites of pUC 18.

Clones were analyzed for the presence of coat protein-related sequences by colony hybridization, using as radioactive probe a cDNA fragment which contained the last 780 nt of PMMV-S RNA labelled by nick-translation [34].

Nucleotide sequence determination and analysis

The 3′ end nucleotide sequences of both PMMV-I and P 11 RNAs were determined by the direct cleavage method [48] on fragments obtained by partial RNase T 1 digestion of the 3′ end labelled RNAs as described [6]. DNA sequences were determined by the chemical degradation procedure [38] and the dideoxy chain termination method [54]. For sequencing, subclones were obtained by restriction enzyme or Bal 31 digestions [34]. No sequence variation was found among the different clones analyzed.

Sequences were analyzed with the DNASTAR computer programs (DNASTAR, Inc., U.K.). Folding of the non-coding region was performed with the RNA/STAR computer program developed at the University of Leiden [1]. Multiple sequence alignments were obtained using the CLUSTAL V program of Higgins and Sharp [27] and Higgins et al. [28]. Trees were generated by the neighbor-joining method [53].

The nucleotide sequence data will appear in the EMBL, GenBank, and DDBJ nucleotide sequences databases and accession numbers X 72586 and X 72587.

Results

Coat protein gene and 3′ non-coding region of PMMV-I

PMMV-I RNA and cDNA clones (Fig. 1 a) were used to determine the nucleotide sequence of the 700 3′ terminal bases. From the nucleotide sequence analysis (Fig. 2 a), it is predicted that the coding region for the coat protein (CP) is 474 nt long, followed by a non-coding region of 198 bases. The CP is composed of 156 amino acids with a calculated M_r 17 145. The length of PMMV-I CP is, therefore, two amino acids shorter than the one previously reported for it (158 amino acids) [63].

The alignment of the CP open reading frame (ORF) and the 3′ non-coding region of PMMV-I and PMMV-S (Fig. 3) shows that both viruses have much sequence identity at the nucleotide level. There are a total of 27 nt changes in the CP coding region and 6 nt changes plus one deletion in the non-coding region. Most of the changes (25) take place at the third base of the codons. Only three amino acid exchanges are scattered along the protein (Fig. 4). Of these base changes, 19 are pyrimidine transitions, the most abundant substitution. These exchanges lead to a greater amount of T/A vs. C/G content in this particular region of PMMV-I respect to PMMV-S. It is also noticeable that the variability of the non-coding region (3.5%) is less than that in the CP ORF (5.7%), indicating that its nucleotide sequence may be under greater functional constraints.

Of the three amino acid exchanges detected in the CP of PMMV-I, two of them are of the conservative type (Ser to Thr and Ala to Val at positions 5

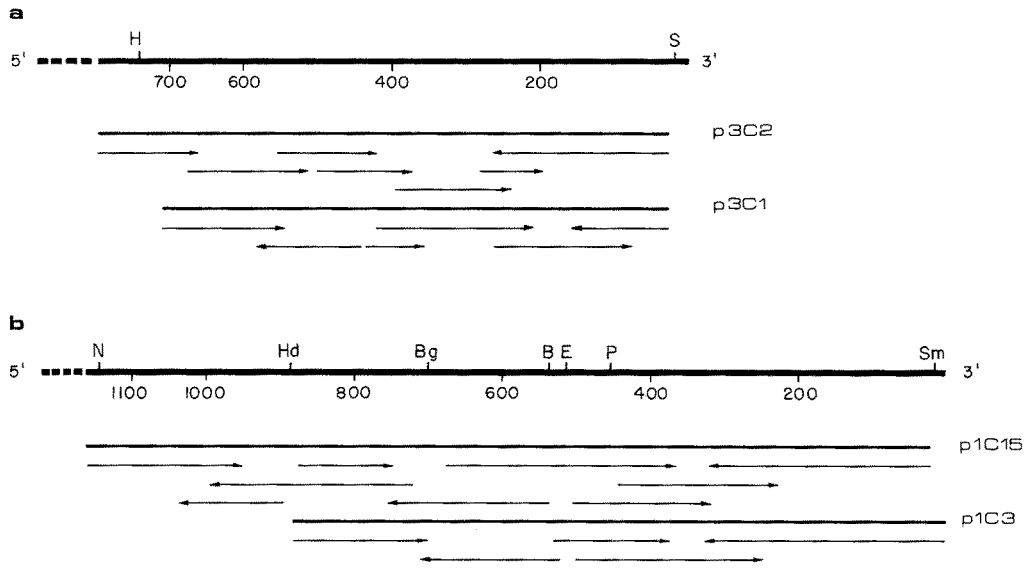

Fig. 1. Restriction maps and sequencing strategies for cDNA clones of PMMV-I (**a**) and P 11 (**b**) genomic RNAs. Sequences were read in the directions indicated by arrows. Restriction enzymes: *B* Bam HI; *Bg* Bgl II; *E* Eco RI; *H* Hin dII; *Hd* Hin dIII; *N* Nsi I; *P* Pvu II; *S* Sac II; *Sm* Sma I

and 147, respectively). The Met to Asn substitution at position 138 is the only non-conservative one (Fig. 4).

Table 1 shows a comparison of the amino acid composition of the CPs of PMMV-S and PMMV-I deduced from the nucleotide sequence and those determined for PMMV-I [63] and capsicum mosaic virus (CaMV), a tobamovirus isolated from pepper in Australia [13]. The differences observed in the amino acid contents may be ascribed to the methods employed for their determination, rather than to differences in the CPs, as different isolates of TMGMV [44, 56] have been found to have strictly conserved CPs. As the molar content of methionines in both the PMMV-S and CaMV CPs also coincide and are greater than that of PMMV-I, it can be inferred that CaMV is closer to PMMV-S than to PMMV-I.

The last 180 nt of PMMV-I RNA may be folded in the same possible structure as that previously proposed for other tobamoviruses [6, 19, 50, 60] which consist of three pseudoknots preceeding the tRNA-like structure (Fig. 5 a). The 3′ non-coding regions of both PMMV-I and PMMV-S can adopt the same overall structure because the nucleotide changes (the A to G transition at position 574 and the deletion at position 630) (Fig. 3) take place in loops of the structure (Fig. 5 a). The only structural difference will be caused by the A to T transversion at position 612 as this will modify the stem of the anticodon arm in the small bulging loop (Fig. 5 a) [6]. As this is a variable region within the tRNA-like structure [50], it is unlikely that biological differences between the viruses would be caused by this change.

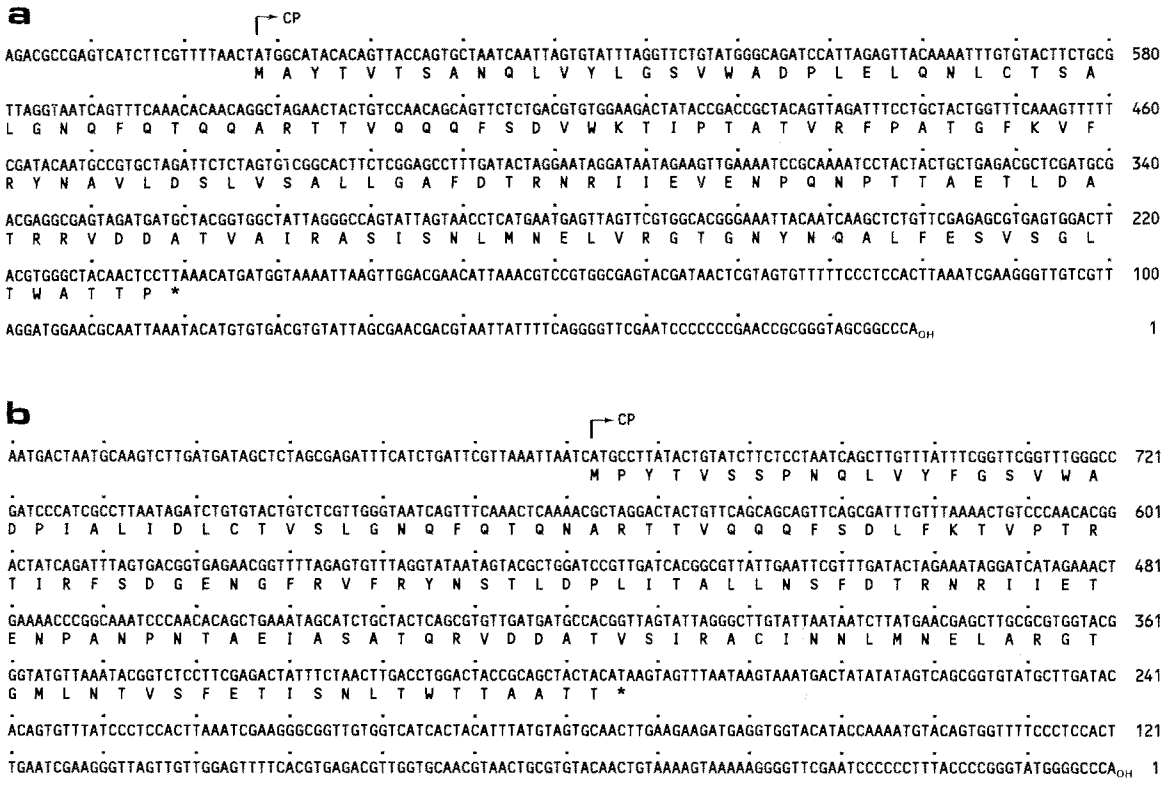

Fig. 2. Nucleotide and deduced amino acid sequences of the coat protein cistrons and 3′ non-coding regions of PMMV-I (**a**) and P 11 (**b**). The initiation codons are marked by arrows, the termination codons by an asterisk

Fig. 3. Alignment of the nucleotide sequences of the coat protein genes and the 3′ non-coding regions of PMMV-I and PMMV-S. Initiation and termination codons are underlined. Only nucleotide exchanges are shown. Gaps are designated by dashes

Coat protein gene and 3′ non-coding region of the Dutch isolate P 11

The nucleotide sequence of the 3′ terminal region of the P 11 viral RNA determined from its RNA and from cDNA clones (Figs. 1 b and 2 b) showed that

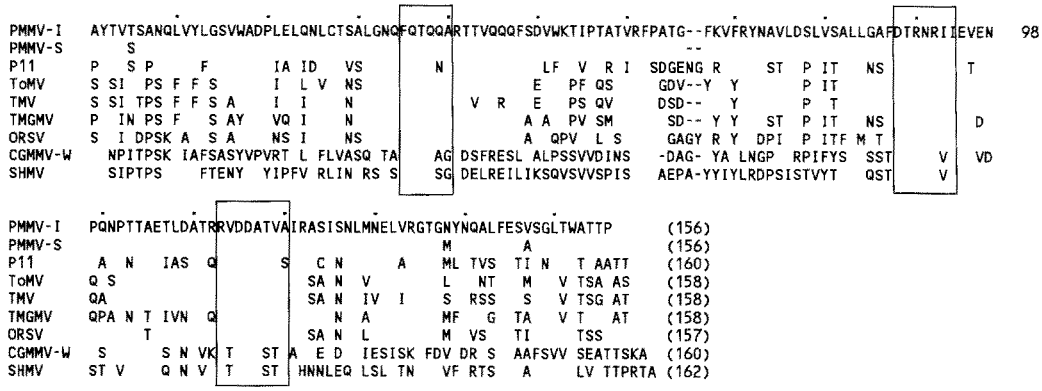

Fig. 4. Comparison of the coat proteins of PMMV-I and P11 with those from other tobamoviruses. Only different amino acids are shown. Gaps are designated by dashes. The RNA-binding domains are boxed. Sources of data as in Table 2

Table 1. Comparison of the amino acid composition (mole%) of CaMV and PMMV-I coat proteins determined by amino acid analysis of virions and those of PMMV-I and PMMV-S deduced from their nucleotide sequences

	Amino acid sequence		Nucleotide sequence	
	CaMV[a]	PMMV-I[b]	PMMV-S	PMMV-I
Ala	12.0	10.8	11.5	10.9
Arg	5.1	5.7	5.8	5.8
Asp (Asn)	12.0	11.4	10.9	11.5
Cys	0.6	0.6	0.6	0.6
Glu (Gln)	11.4	11.4	10.9	10.9
Gly	4.4	5.1	4.5	4.5
His	0.0	0.0	0.0	0.0
Ile	2.5	3.2	3.2	3.2
Leu	10.1	10.1	9.6	9.6
Lys	1.3	1.3	1.3	1.3
Met	1.3	0.6	1.3	0.6
Phe	4.4	4.4	4.5	4.5
Pro	3.2	3.8	3.8	3.8
Ser	7.6	6.3	7.1	6.4
Thr	12.0	12.7	12.2	12.8
Trp	1.9	1.3	1.9	1.9
Tyr	1.9	2.5	2.5	2.5
Val	8.5	8.9	8.3	8.9

[a] From Creaser et al. [13]
[b] From Wetter et al. [63]

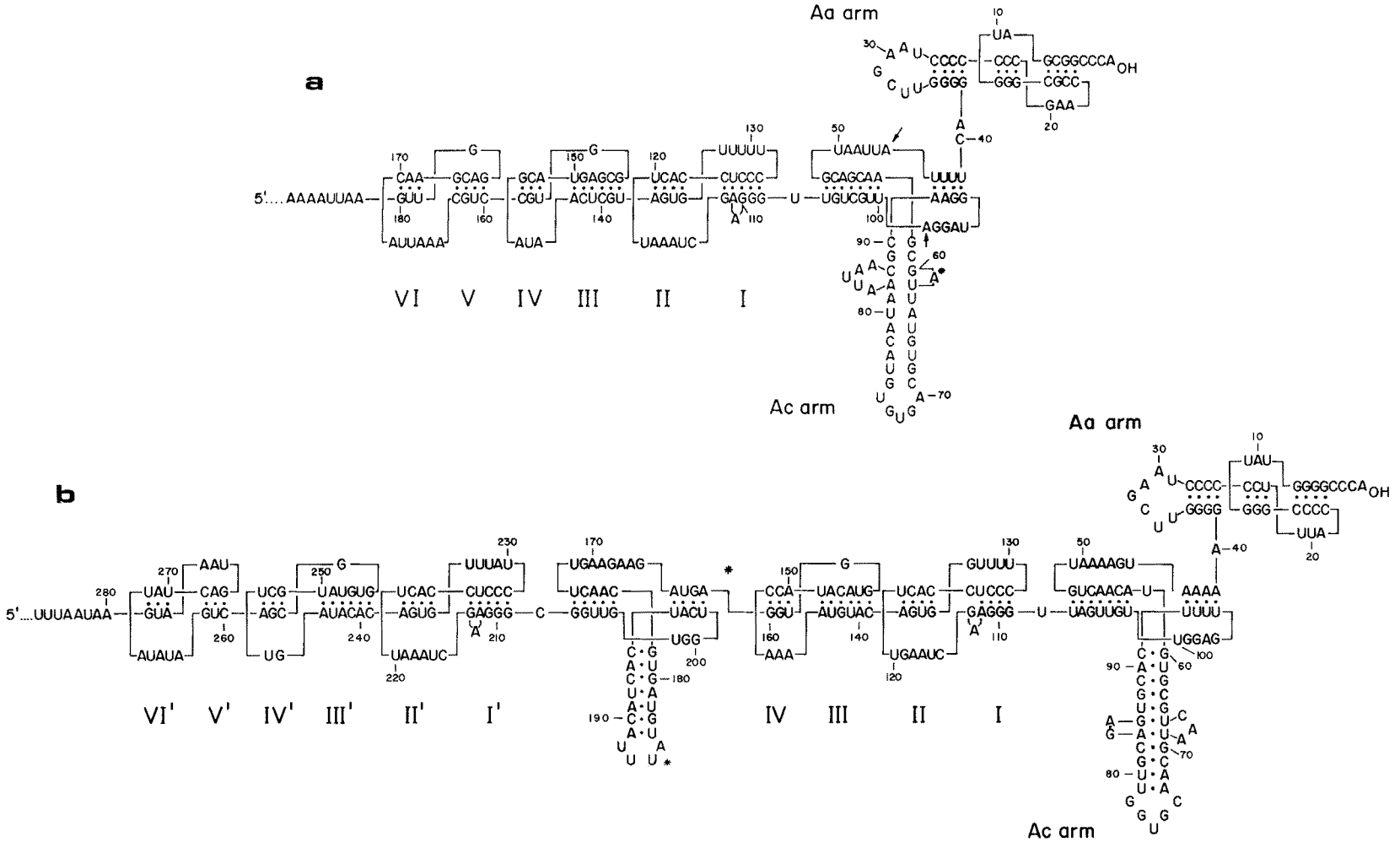

Fig. 5. Possible secondary structure of the 3′ non-coding regions of PMMV-I ((**a**) and P11 (**b**). Asterisk indicates the nucleotide substitution in PMMV-I which alters the predicted structural model of PMMV-S [6]. Arrows indicate nucleotide changes which do not affect the structure. Duplicated sequences in **b** with altered secondary structure are marked by an asterisk. The computer-predicted structure was slightly modified in order to maintain the folding previously determined for other tobamoviruses [19, 60]

its CP ORF is 486 bases long, followed by a non-coding region of 291 nt. The predicted CP is composed of 160 amino acids with a calculated M_r 17 706.

Comparisons of the amino acid and nucleotide sequence of its CP coding region with those from other tobamoviruses (Table 2) shows a greater degree of amino acid sequence identity with those of PMMV-S (69.2%), TMGMV (68.3%), and PMMV-I (67,3%) than with other tobamoviruses, and its nucleotide sequence identity being greatest with that of TMGMV (Table 2). In contrast, the greatest nucleotide sequence identity in the non-coding region was with those of ToMV and odontoglossum ringspot virus (ORSV) (Table 2).

The alignment of the P 11 CP amino acid sequence with those from the most closely related tobamoviruses (Fig. 4) shows that, in addition to the RNA-binding domains [5], there is a well-conserved domain located at residues 32 to 50, with only one amino acid substitution (Asn to Gln), which is unique for this virus among the closest tobamoviral CPs. The sequence is more variable in the central part of the CP coding region which includes a 6 nt insertion that gives a longer protein. This region has been previously shown to be of very variable length [5].

The P 11 CP have 5, 9 and 13 non-conservative amino acid substitutions compared with those from TMGMV, TMV and ToMV, respectively (Fig. 4). They are concentrated at position 29 (Val to Asn), in the region located between residues 55 to 70, and in the one-third carboxy part of the protein. The number of non-conservative amino acid substitutions with respect to PMMV-S and PMMV-I CPs are 7 and 6, respectively (Fig. 4). They correspond to amino acid residues 24 (Ile to Gln), 29 (Ser to Val), 58 (Ala to Arg), 110 (Leu to Ala), 139 (Tyr to Leu), 143 (Leu to Ser), and 138 (Met to Asn). This last exchange

Table 2. Nucleotide (N) and amino acid (A) sequence identities of the P 11 and PMMV-I coat protein (CP) genes and the 3′ non-coding regions (NC) and those of other tobamoviruses

	P 11			PMMV-I		
	CP		NC	CP		NC
	N	A		N	A	
PMMV-I	64.8	67.9	47.3	–	–	–
PMMV-S[a]	64.9	69.2	55.2	94.3	98.1	96.5
ToMV[b]	63.6	60.9	58.0	67.1	74.5	59.6
TMV[c]	65.1	60.9	56.4	65.6	72.6	60.1
TMGMV[d]	65.5	68.3	51.2	65.8	68.8	59.3
ORSV[e]	62.1	66.5	62.9	62.7	70.3	63.5
CGMMV-W[f]	46.6	35.4	54.4	49.9	35.9	55.3
SHMV[g]	48.5	36.9	41.7	48.7	40.1	43.8

Data from [a] Alonso et al. [4]; [b] Ohno et al. [45]; [c] Goelet et al. [22]; [d] Solís und Carcía-Arenal [56]; [e] Isomura et al. [29]; [f] Meshi et al. [41]; [g] Meshi et al. [40]

coincides with the only non-conservative substitution present in the PMMV-S and PMMV-I CPs (Fig. 4).

The 3′ non-coding region of the P 11 viral RNA (Fig. 2 b) is unusually long and contains 291 bases, with a duplicated sequence domain (positions 109 to 140 and 209 to 240) (Fig. 5 b), which has also been recently found to be repeated in the 3′ non-coding region of ORSV [29]. Similar duplication features have also been reported in the 3′ non-coding regions of viruses belonging to the tobravirus group [26] as well as in viroids [30].

The 160 3′ terminal nt can be folded in a similar structure to that of other tobamoviruses [19, 50, 60] (Fig. 5 b). In the tRNA-like structure, the anticodon arm (Ac arm) consists of a hairpin with a five nt bulging loop. As the GUG anticodon in the loop is an anticodon for histidine, this RNA may also be charged with histidine like most other tobamoviruses [19, 50]. However, in the stalk structure the double helical segments V and VI are not present (Fig. 5 b). The double helical segment IV is preceded at its 5′ end by a 120 bases long fragment which may also adopt a similar configuration with six double helical segments, I′–VI′ (Fig. 5 b), able to form three pseudoknots and a second truncated tRNA-like structure at its 3′ end, in which the aminoacylacceptor arm (Aa arm) is absent, and the Ac arm is much shorter than the one found downstream.

Phylogenetic tree of the coat protein gene

The tobamovirus phylogenetic tree obtained (Fig. 6) shows that both PMMV-I and PMMV-S are located in the same branch as TMV and ToMV, the two most closely related tobamoviruses so far described. This clustering corresponds with those previously proposed [18, 21], even though different types of data were used for constructing the dendrograms; namely amino acid compositions of the CPs, the peptide patterns of the 126 K proteins, and the amino acid sequences of the CPs deduced from their nucleotide sequences. The P 11 isolate is linked with TMGMV in a different branch indicating that it has evolved independently from both PMMV-S and PMMV-I. In this sense, and based upon previous serological findings [36, 59; our unpubl. data], the Hungarian

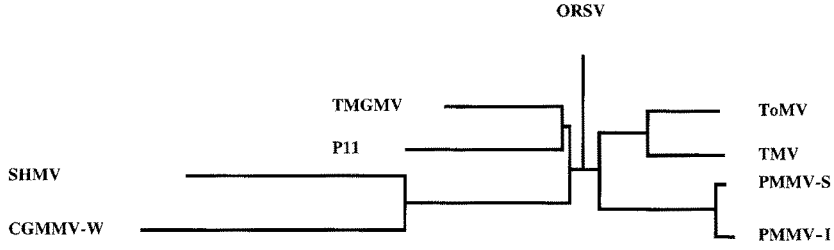

Fig. 6. Dendogram showing phylogenetic relationships between the tobamoviral coat proteins whose amino acid sequence have been deduced from the nucleotide sequence

ToMV-Ob [14] should map in the same branch as P 11 since the grouping of tobamoviruses correlates well with their serological relationships.

Discussion

The comparison of the nucleotide and amino acid sequence here presented for PMMV-I with that of PMMV-S [4] shows that they are strains of the same virus even though their differential ability to overcome the L^3 and L^2 resistance genes of *Capsicum* spp., respectively [8, 20]. Therefore, we think that other resistance-breaking tobamoviruses of pepper which have been previously shown to be similar in biological and serological properties to these viruses [8, 36, 47, 55, 59, 61] should also be referred to as strains of PMMV.

The degree of dissimilarity found in the nucleotide sequence and amino acid content of the coat protein of the Dutch P 11 isolate, another tobamovirus of pepper that can break the resistance conferred by the *Capsicum* L^1 gene, with respect to either PMMV-I or PMMV-S and the rest of the known tobamoviruses, indicates that this virus should be considered as a distinct virus within the tobamovirus group. We propose the term of paprika mild mottle virus (PaMMV) to designate it because the mottling symptoms induced by this virus in pepper cultivars are hardly distinguishable form the ones induced by PMMV-S and PMMV-I.

It is possible that the amino acid exchanges observed among the CPs of these tobamoviruses could be involved in their differential ability to overcome the resistance conferred by the different hypersensitive resistance genes in *Capsicum* spp. plants as it has been previously shown for similar structural changes in the CP of TMV and ToMV [15, 52] which are responsible for the induction of the hypersensitive reaction in *N. sylvestris* plants. However, it is also possible that other regions of the viral genome could be implicated in this characteristic as is found with some strains of ToMV which are able to break the *Tm 1* and *Tm 2* resistance genes in tomato plants [11, 42, 43]. It is possible that the unique non-conservative Met to Asn substitution at position 138 in the CP could be responsible for PMMV-I being able to overcome the resistance conferred by the *Capsicum* L^3 gene. Similarly, it has been previously reported that a single amino acid substitution at position 148 (Ser to Phe) in the CP of TMV confers the ability to induce the hypersensitive response in *N. sylvestris* plants which carry the N′ resistance gene [32].

It is noticeable that PMMV-S is located closer to the branch point than PMMV-I in the phylogenetic tree (Fig. 6). Thus, PMMV-I seems to have evolved further from its common ancestor PMMV-S. This might have occurred because PMMV-I has been positively selected since the introduction of the L^3 resistance gene in pepper crops.

As mentioned above, part of the 3′ non-coding region of the P 11 virus seems to have arisen by duplication. In the tRNA-like structure of brome mosaic virus (BMV), it has been found that certain regions such as the anticodon nucleotide sequence [16] or the sequences which correspond to the internal transcription

promoter of eukaryotic tRNAs, often referred as B-box, are necessary for the efficient replication of the virus [10, 35, and references herein]. The sequences equivalent to the B-box (GGTCGNNC) are located in the Aa arm of the tRNA-like structure of the tobamoviruses. Interestingly, none of the equivalent regions are repeated in the P 11 viral RNA.

Takamatsu et al. [58] have recently demonstrated that the double helical segments V and VI in the 3′ non-coding region of TMV are not necessary for its replication, while the maintenance of stem region I and the UAAAU sequence in segment II are essential for its efficient replication. In contrast with the tRNA-like structure, double-helical segments I and II correspond to the duplicated sequences of P 11 and ORSV. The A to G substitution in the segment II of the P 11 virus (UGAAU) is the only difference found in the duplicated structural elements. The functional significance, if any, of the conservation of these motifs is unknown at present. It is possible that this region may be recognized by host and/or viral factors involved in the viral replication mechanism and also help maintain the structure of the encoded motif.

Recombination phenomena have been described in many animal and plant RNA viruses [2, 9, 12, 31, 33, 51], and it is thought that it occurs as a copy-choice mechanism, when the replicase is synthesizing one of both strands involved in secondary structures [31, 65]. The phenomenon described here, indeed, seems similar. Therefore, the nucleotide differences between the upstream and downstream sequences in the 3′ non-coding regions of P 11 and ORSV may result from recombination with another tobamovirus coinfecting the same cell as has been described for polioviruses [31], although they also could be caused by a greater evolutionary rate in the duplicated sequences.

Acknowledgements

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