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SHORT COMMUNICATION / *COMUNICAÇÃO*

Complete genome nucleotide sequence of *Pepper mild mottle virus* isolated in the Federal District, Brazil

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ABSTRACT

Occurrence of *Pepper mild mottle virus* (PMMoV) on *Capsicum* plants has become common in Brazil. Despite the importance of this virus, genome information is still lacking for South American isolates. In this report, the first complete genome sequence of a Brazilian isolate of PMMoV (BR-DF01) was elucidated and compared with other PMMoV sequences. The nucleotide sequence of the complete genome of BR-DF01 isolate shared the highest nucleotide identity (99.54%) with the Japanese isolate JP-J, which does not overcome *L3* resistance gene of *Capsicum* plants, as also observed for BR-DF01. The coat protein (CP) amino acid sequence of these two isolates was identical, which suggested that CP is the key factor for *L*-based resistance breaking. Phylogenetic analysis implied that BR-DF01 and PMMoV isolates belonging to Cluster I, including JP-J isolate, may share a common ancestral origin.
Key words: *Tobamovirus*, *Capsicum.*

RESUMO

Seqüência nucleotídica completa do genoma de *Pepper mild mottle virus* isolado no Distrito Federal, Brasil

A ocorrência do *Pepper mild mottle virus* (PMMoV) em plantas de *Capsicum* tornou-se frequente no Brasil. Apesar da importância desse vírus, informações genômicas de isolados sul-americanos são ainda escassos. Neste trabalho, foi elucidada a sequência do genoma completo de um isolado brasileiro de PMMoV (BR-DF01) e esta foi comparada a outras sequências de PMMoV. A sequência de nucleotídeos do genoma completo do isolado BR-DF01 apresentou a maior identidade (99,54%) com o isolado japonês JP-J, que é capaz de contornar a resistência do gene *L3* de plantas de *Capsicum*, como foi também observado para BR-DF01. A sequência de amino ácidos da capa protéica (CP) desses dois isolados foi idêntica, sugerindo que a CP é o fator-chave para a quebra de resistência baseada no gene *L*. Análises filogenéticas indicaram que BR-DF01 e isolados de PMMoV pertencentes ao agrupamento I, incluindo o isolado JP-J, podem compartilhar de um ancestral em comum.
Palavras-chave: *Tobamovirus*, *Capsicum.*

Plants of the genus *Capsicum*, including hot (*Capsicum* spp.) and sweet pepper (*Capsicum annuum* L.), are important crops worldwide. Viruses such as *Potato virus Y*, *Pepper yellow mosaic virus* (*Potyvirus*); *Tomato spotted wilt virus*, *Groundnut ringspot virus*, *Tomato chlorotic spot virus* (*Tospovirus*); *Cucumber mosaic virus* (*Cucumovirus*); and *Pepper ringspot virus* (*Tobravirus*) are reported as important viral pathogens of capsicum plants in Brazil. Recently, an increasing occurrence of *Pepper mild mottle virus* (PMMoV, *Tobamovirus*) has been observed, possibly associated with the expansion of cultivation of peppers under plastic in this country in the last decade, indicating the importance of implementing a serious PMMoV control management in Brazil (Cezar et al., 2003a, b; Eiras et al., 2004; Kobori et al., 2001). The virus's transmissibility by seeds may also have contributed to its threatening spread in Brazil.

Isolates of PMMoV, as well as *Tobacco mosaic virus* (TMV), which were able to overcome the resistance genes controlled by *L1*, *L2*, *L3* and *L4* in capsicum plants, have been reported. In Brazil, the natural existence of resistance-breaking PMMoV isolates against *Capsicum* plants carrying *L1* and/or *L2* genes was also demonstrated (Eiras et al., 2004; Kobori et al., 2001). Despite the importance of the virus, the genome of PMMoV is poorly studied, not only in Brazil, but also in other South American countries. Thus, this work was planned to determine the complete genome sequence of one Brazilian isolate of PMMoV. Its genome sequence was compared with 10 complete genome sequences of PMMoV available in public databases, six of which were from Japan, two from Spain and one each from China and Korea.

PMMoV was isolated from a *Capsicum chinense* Jacquin. plant with mosaic symptoms in the experimental field at Embrapa Vegetables, Brasília, Federal District of Brazil. The isolate was denominated as PMMoV BR-DF01. The virus was identified by a combination of electron microscopy, ELISA and partial genomic information by direct sequencing of amplified cDNA by RT-PCR using

tobamovirus degenerated primers. The host responses in indicator plants after mechanical inoculation demonstrated that this isolate was an *L1/L2* breaker, but it could not infect *Capsicum chinense* PI159236 which carries the *L3* resistance gene.

Total RNA was extracted from mechanically inoculated and infected *Nicotiana benthamiana* Domin. plants using Plant RNA reagent (Invitrogen) according to the manufacturer's instructions. The whole genome divided into two regions of 5' (2497bp) or 3' (3989bp) was amplified by Reverse transcription (RT)-PCR. RT was done using specific primers (5' half end: PMMoV5'Rev 5'- TCC CCA TAT CTG AAT ACA CCA AAG A -3', and 3' half end: PMMoV3'Rev 5'- TGG GCC GCT ACC CGC GGT T-3') and Superscript III Reverse Transcriptase (Invitrogen) at 50◦C. PCR was done using LongAmp *Taq* DNA Polymerase (New England Biolabs, NEB) with the combination of forward primers (PMMoV5'For 5'-GTA AAT TTT TCA CAA TTT AAC A-3' and PMMoV3'For 5'-GCG CTT CTC ACA TAC GAT GGC GAG AAC A-3') and reverse primers used for the RT reaction. Adenosine overhang addition at 3' end of cDNA was enforced by adding *Taq* DNA polymerase (NEB) after finishing PCR cycles with incubation at 70◦C for 30 min. Amplified cDNA fragments were gel-purified, cloned into pCR4 plasmid vector using TOPO TA cloning kit for sequencing (Invitrogen) and sequenced by primer walking. A total of three clones for each region (5' and 3') were sequenced. The results were analyzed by Staden Package (Staden et al., 2003) for contig assembly, and multiple alignments and phylogenetic analysis were done by MEGA 4 (Tamura et al., 2007).

The sequences of three 5' half-end clones were identical, but there were 11 nucleotides of heterogenicity in three 3' half-end clones. Thus, for the 3' end region, clone 2 was selected for assembly of complete genome and further sequence analysis due to its highest identity with the consensus sequence. The overlapping region between 5' and 3' end sequences was identical for all clones. The total genome length was 6356 nucleotides, and no nucleotide insertion and deletion was observed in the coding regions compared to other sequences of PMMoV available in databases. The first ORF (126K protein) was encoded at nt 70-3423, terminated with an amber readthrough codon, so that ORF 2 (183K protein) finished at nt 4908. The movement protein gene (ORF3) started at nt 4909 without any nucleotide insertion after the stop codon of ORF2 and finished at nt 5682. The coat protein (CP) gene (ORF4) started at nt 5685, two nucleotides after the stop codon of ORF3 and finished at nt 6158. This ORF organization was identical to other PMMoV isolates (Alonso et al., 1991; Genda et al., 2007; Hamada et al., 2007; Wang et al., 2006; Yoon et al., 2006). The nucleotide sequence of PMMoV BR-DF01 is available in the DDBJ/EMBL/GenBank databases as accession number AB550911.

Pairwise comparison of PMMoV BR-DF01 with all 10 complete PMMoV genomes available in public databases revealed that the Brazilian isolate was the best-hit partner of a Japanese isolate, PMMoV JP–J, with nucleotide identity of 99.54 %. There were 30 nt differences between these two isolates, most of them being silent mutations. Most mutations (14 out of 30) were between pyrimidines (C-U), followed by those between purines (A-G) (11 out of 30). There was a total of only six different amino acids (a.a.): two within the 126K protein, one in the readthrough region of 183K protein, three in the movement protein and no a.a. change in the CP.

A phylogenetic tree based on the alignment of all PMMoV complete genome nucleotide sequences was constructed by MEGA4 program (Tamura et al., 2007) using the Neighbor-Joining method (Figure 1). There were three clusters formed and BR-DF01 was grouped within Cluster I, which consisted of one Spanish, four Japanese and one Chinese isolate. This clustering was the same as the one constructed using the CP nucleotide sequences (data not shown).

Seven out of 11 of the genomes compared here, including BR-DF01, did not contain the T residue at nt position 6317, which is located in the 3' untranslated region (UTR). This T residue was present after four repeated T residues, so for the four other isolates (SP-S, SP-Ia, JP-J and JP-C1421), this additional T residue could be a result of a misinterpretation by the program used. Recombination search by RDP3 (Martin, 2009) did not show any significant recombination events among these 11 sequences (data not shown).

It is possible that this Brazilian isolate shares a common ancestor with isolates of Cluster I, but the low number of complete genome sequences available did not permit a thorough geographical analysis from this tree. It is interesting to note that the deduced CP a.a. protein sequence of BR-DF01 and JP-J (both viruses break the *L1/L2* resistance genes, but not *L3*) was exactly the same. It is believed that the CP sequence mutation is related to the breakage of *L* resistance genes (Genda et al., 2007; Gilardi et al., 1998, 2004; Hamada et al., 2002, 2007; Tsuda et al., 1998). If this holds true, it is another confirmation that the CP a.a. sequence is a good indicator of the interaction between the virus and *L* resistance genes.

Japanese isolates were divided into two different clusters (Figure 1), suggesting a diverging line of evolution between them. The two Spanish isolates (SP) were variable: the first (SP-S) was clustered within Cluster I; however, another (SP-Ia) was located in a far-separated branch (Cluster III). The Chinese isolate clustered with the Spanish and Brazilian group (Cluster I), while the Korean one was within the second cluster with two Japanese isolates (Cluster II). Taken together (genetic variability of Spanish isolates and the position

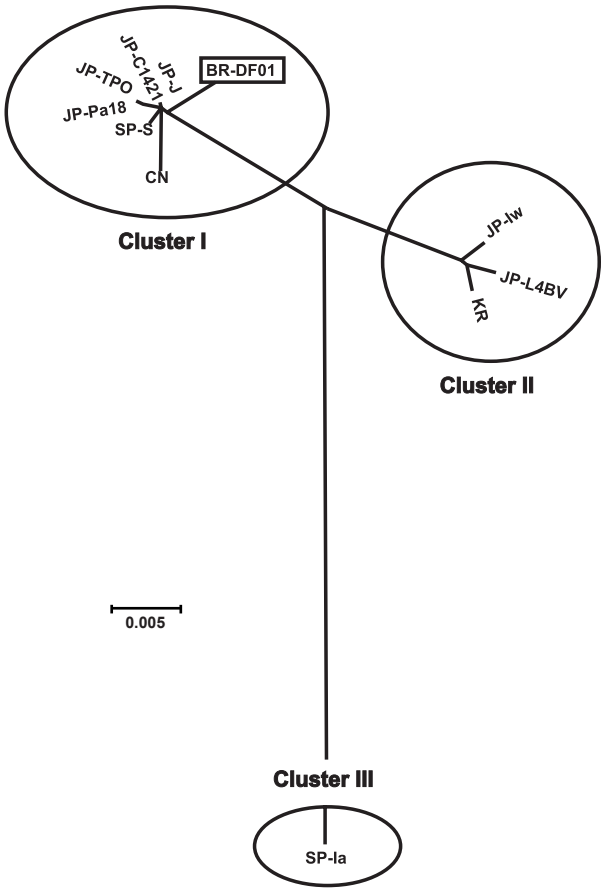

FIGURE 1 - Unrooted phylogenetic tree of PMMoV sequences constructed by MEGA4 using Neighbor-Joining method. The position of BR-DF01 isolate is indicated with an open box. Accession numbers of isolates are: JP-J (AB000709), JP-C1421 (AB069853), JP-TPO (AB113117), JP-Pa18 (AB113116), SP-S (M81413), CN (AY859497), BR-DF01 (AB550911), JP-Iw (AB254821), JP-L4BV (AB276030), KR (AB126003), SP-Ia (AJ308228). JP=Japan, SP=Spain, CN=China, KR=Korea

of the Chinese isolate in the phylogenetic tree), it is hypothesized that the spread of PMMoV occurred due to global trading of pepper plants and seeds. The complete genome sequences of PMMoV from other countries are a prerequisite to conclude this hypothesis. The translocation of humans themselves may act as another source of distribution mediated by fecal transmission, since PMMoV particles could be detected frequently from human stool samples (Rosario et al., 2009) and, interestingly, PMMoV extracted from human stool was still infective (Zhang et al., 2006).

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REFERENCES

Alonso E, Garcia-Luque I, de la Cruz A, Wicke B, Avila-Rincon MJ, Serra MT, Castresana C, Diaz-Ruiz JR (1991) Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper. Journal of General Virology 72:2875-2884.

Cezar MA, Krause-Sakate R, Mezzena L, Kobori RF, Pavan MA (2003a) Caracterização biológica e identificação molecular de vírus pertencentes ao gênero *Tobamovirus* provenientes de *Capsicum* spp. Summa Phytopathologica 29:359-361.

Cezar MA, Pavan MA, Kobori RF, Krause-Sakate R (2003b) Detecção de tobamovírus em pimentão (*Capsicum annuum*) por meio de RT-PCR. Summa Phytopathologica 29:59.

Eiras M, Chaves ALR, Moreira SR, Araujo J, Colariccio A (2004) Caracterização de um isolado do Pepper mild mottle virus que não quebra a resistência do gene L3 em *Capsicum* sp. . Fitopatologia Brasileira 29:670-675.

Genda Y, Kanda A, Hamada H, Sato K, Ohnishi J, Tsuda S (2007) Two amino acid substitutions in the coat protein of Pepper mild mottle virus are responsible for overcoming the L-4 gene-mediated resistance in Capsicum spp. Phytopathology 97:787-793.

Gilardi P, Garcia-Luque I, Serra MT (1998) Pepper mild mottle virus coat protein alone can elicit the Capsicum spp. L-3 gene-mediated resistance. Molecular Plant-Microbe Interactions 11:1253-1257.

Gilardi P, Garcia-Luque I, Serra MT (2004) The coat protein of tobamovirus acts as elicitor of both L2 and L4 gene-mediated resistance in Capsicum. Journal of General Virology 85:2077-2085.

Hamada H, Takeuchi S, Kiba A, Tsuda S, Hikichi Y, Okuno T (2002) Amino Acid Changes in Pepper mild mottle virus Coat Protein That Affect L 3 Gene-mediated Resistance in Pepper. Journal of General Plant Pathology 68:155-162.

Hamada H, Tomita R, Iwadate Y, Kobayashi K, Munemura I, Takeuchi S, Hikichi Y, Suzuki K (2007) Cooperative effect of two amino acid mutations in the coat protein of Pepper mild mottle virus overcomes L3-mediated resistance in Capsicum plants. Virus Genes 34:205-214.

Kobori RF, Wierzbicki R, Della Vecchia PT, Pavan MA, Rezende JAM (2001) Ocorrência do Pepper mild mottle virus (PMMoV) em pimentão (*Capsicum annuum*) cultivado sob estufas no Estado de São Paulo. Fitopatologia Brasileira 26:516. Resumo

Martin DP (2009) Recombination detection and analysis using RDP3. Methods of Molecular Biology 537:185-205.

Rosario K, Symonds EM, Sinigalliano C, Stewart J, Breitbart M (2009) Pepper Mild Mottle Virus as an Indicator of Fecal Pollution. Applied and Environmental Microbiology 75:7261-7267.

Staden R, Judge DP, Bonfield JK (2003) Analysing sequences using thes Staden package and EMBOSS. In: Krawetz SA, Womble DD (Eds.) Introduction to Bioinformatics: A Theoretical and practical approach Totawa. Human Press Inc. pp. 393-410.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biolology and Evolution 24:1596-1599.

Tsuda S, Kirita M, Watanabe Y (1998) Characterization of a pepper

mild mottle tobamovirus strain capable of overcoming the L-3 gene-mediated resistance, distinct from the resistance-breaking Italian isolate. Molecular Plant-Microbe Interactions 11:327-331.

Wang X, Liu F, Zhou G, Li XH, Li Z (2006) Detection and molecular characterization of Pepper mild mottle virus in China. Journal of Phytopathology 154:755-757.

Yoon JY, Ahn HI, Kim M, Tsuda S, Ryu KH (2006) Pepper mild mottle virus pathogenicity determinants and cross protection effect of attenuated mutants in pepper. Virus Research 118:23-30.

Zhang T, Breitbart M, Lee WH, Run JQ, Wei CL, Soh SW, Hibberd ML, Liu ET, Rohwer F, Ruan Y (2006) RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biology 4:e3. doi:10.1371/journal.pbio.0040003

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