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Short Communication

Cukurova University, Adana, Turkey

Detection and Molecular Characterization of *Pepper Mild Mottle Virus* from Turkey

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Abstract

Using double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA), *pepper mild mottle virus* (PMMoV) was detected in 27 pepper (*Capsicum* spp.) plants of 3000 tested and found to be present in Adana, Antalya, Kahramanmaraş, Mersin and Şanlıurfa, all provinces devoted to pepper production in southern Turkey. Results of reverse transcription-polymerase chain reaction (RT-PCR) using primers specific to RNA-dependent RNA polymerase (RdRp) and capsid protein (CP) genes confirmed those of ELISA by amplifying all PMMoV-infected plants. Restriction fragment length polymorphism (RFLP) and PCR assays using sequence characterized amplified region (SCAR) marker primers showed that PMMoV from Turkey overcomes L^3-gene-mediated resistance, so pepper plantations are susceptible to PMMoV infection. Sequences of CPs showed high amino acid identities (92–99%) with their homologues in the database and, furthermore, to share a distinguished molecular print found common uniquely in pathotypes $P_{1,2,3}$. The phylogenetic tree allocated the Turkish isolates in one cluster together with PMMoV pathotypes $P_{1,2,3}$ of the Italian, Spanish and Israeli isolates, all reported to overcome the L^3-resistance-breaking gene in pepper. This is the first molecular information on PMMoV isolates present in Turkey, for which this information could have guiding significance in future pepper resistance breeding in the country.

Introduction

Sweet and hot peppers (*Capsicum* spp.) are among the most important vegetables used in Turkish cuisine and are consumed fresh processed or dried. Between 2004 and 2010, the production of peppers in the country increased from 710 000 tons to 1.84 million tons, placing Turkey 3rd in the world in terms of production (Anonymous 2010). This crop is commonly grown in the Black Sea region (southern Turkey) where Adana, Antalya, Kahramanmaraş, Mersin and Şanlıurfa provinces are the main producers and constitute 18% of the total Turkish production (Anonymous 2010). More than fifty cultivars are cultivated in the country and the most important are Karaisalı, Maraş and Urfa. Traditional and native pepper cultivars are grown in small plots whereas the new plantations, established mainly with new imported cultivars, are cultivated in greenhouses.

Several viruses are known to infect peppers, that is, *tobacco mosaic virus* (TMV), *cucumber mosaic* virus (CMV) (Heper 1979), *potato virus Y* (PVY) (Erkan 1991), *tobacco etch virus* (TEV) (Palloix et al. 1994a), *tomato spotted wilt virus* (TSWV) (Yurtmen et al. 1999) and *pepper mild mottle virus* (PMMoV) (Palloix et al. 1994b). PMMoV belongs to the genus *Tobamovirus* and its genome consists of a single-strand positive sense RNA of 6357 nucleotides (Garcia-Luque et al. 1990; Kirita et al. 1997). Like other Tobamoviruses, PMMoV is transmitted through seeds and soil (Komuro and Iwaki 1969; Lanter et al. 1982). Four pathotypes (P_0, P_1, $P_{1,2}$ and $P_{1,2,3}$) are found in pepper (Boukema 1982) and resistance against Tobamoviruses is conferred by the L^1, L^2, L^3 and L^4 genes that seem to be allelic forms of a single gene known to induce the hypersensitive response (HR) in *Capsicum* spp. (Boukema 1982; Rast 1982). In particular, resistance to PMMoV is provided by the L^3-resistance gene, and PMMoV is characterized as a pathotype (P) $P_{1,2}$ or $P_{1,2,3}$ that overcomes the L^2 or L^3 gene-mediated resistance in *Capsicum* spp. plants. To date, two isolates have been reported to overcome L^3-resistance: one from Italy (PMMoV-I; Garcia-Luque et al. 1993) and one from Japan (PMMoV-J; Tsuda et al. 1998). However, the cultivation of plants containing the *L* gene has led to the detection of new strains able to overcome virus resistance.

In 2009–2011, PMMoV-like symptoms were observed in local pepper cultivars grown in plastic tunnels and in open fields in Turkey, all exhibiting stunted growth, mottling, malformed leaves, deformed fruits with off-coloured sunken areas. With the exception of Şanlıurfa, there has been no further information on the presence of PMMoV in other Turkish provinces (Güldür and Çaglar 2006) or on the eventual PMMoV pathotypes existing in the country. For correct control and management of this virus disease in Turkey, the determination of the level of susceptibility or resistance of pepper plantations to PMMoV infections is fundamental, whose results are hereafter reported.

Materials and Methods

Plant material and virus isolates

During 2009–2011, three thousand samples from pepper plants, symptomatic and asymptomatic, were collected from fields and greenhouses of Adana (1250), Antalya (150), Kahramanmaraş (1250), Mersin (300) and Şanlıurfa (50) provinces. All samples were tested by double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA); molecular and mechanical transmission assays were limited only to ELISA-positive samples.

Mechanical transmission of PMMoV

Sap was extracted from 27 PMMoV-infected plants by grinding tissue of young leaves into 0.3 M potassium phosphate buffer (1 : 10; w/v, pH 7.2) and using a pre-chilled mortar. The homogenate was centrifuged at 10 000 ***g*** for 5 min, and the supernatant inoculated by gently rubbing the bottom leaves of Celite-dusted *Nicotiana glutinosa* and *Chenopodium amaranticolor* plants. Control plants were treated in the same manner using distilled water for inoculation. Healthy sweet pepper (cv. Qiemen) plants were grafted on 10 PMMoV-infected plants and propagated for symptoms expression.

Double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA)

All samples were tested by DAS-ELISA as described by Clark and Adams (1977) using a specific kit of antibodies to PMMoV (Tsuda et al. 1998). Absorbance at 405 nm (A_{405}) was determined using an ELISA plate reader (Model 550; Bio-Rad, Hercules, CA, USA).

Total nucleic acids extraction (TNA) and reverse transcription –polymerase chain reaction (RT-PCR)

Total nucleic acids were extracted from infected samples using a procedure described by Hadidi et al. (1993). Reverse transcription reactions were applied using 1 μg of TNA extract and 0.5 μg of random primer (Promega, Madison, WI, USA) in the presence of RT 5× Buffer and M-MLV enzyme, according to the manufacturers' instruction (Invitrogen, Carlsbad, CA, USA).

Two pairs of specific primers (CP/s: 5′-ATGGCATACACAGTTACCAGT-3′ and CP/a: 5′TTAAGGAG TTGTAGCCCACGTA3′, designed in this work, and P12/3 and P12/3A) (Velasco et al. 2002) were used in RT-PCR for the amplification of the capsid protein (CP) (474 bp) and partial RNA-dependent RNA polymerase (RdRp) (830 bp) genes, respectively.

Two codominant sequence characterized amplified region (SCAR) marker pairs (PMF1/PMR1 and PMF2/PMR1) (Sugita et al. 2004) were also used to investigate the L^3-resistance gene in pepper. Genomic DNA of pepper was extracted from young leaf tissues of collected samples, following a procedure described by Murray and Thompson (1980).

Capsid protein – and RdRp-generated amplicons were ligated into pGEM-T easy vector (Promega), cloned in *Escherichia coli* DH5α or SoloPACK cells, and custom sequenced (ABI 3130xl Genetic Analyzer, Applied Bio. REFGEN Gen Araştırmaları ve Biyoteknoloji Ltd. Şti., Ankara, Turkey). Nucleotide and deduced amino acid sequences were analysed with the assistance of the DNA STRIDER 1.1 program (Marck 1988). Multiple alignments of nucleotide and amino acid sequences were obtained using the default options of CLUSTALX 1.8 (Pearson and Lipman 1988). Search for homologies with proteins from the Protein Information Resources database (PIR, release 47.0) was performed with the FASTA, BLASTX and BLASTP (Altschul et al. 1990) programs. Tentative phylogenetic tree was constructed using the NJPLOT package (Perrière and Gouy 1996).

Restriction fragment length polymorphism (RFLP) analyses

PCR amplicons, primed with P12/3A and P12/3, generated from infected pepper were analysed with restriction enzymes digestion. Five μl of each amplicon in a RFLP mix consisting of 8.5 μl ddH_2O, 1.0 μl FastDigest buffer (10×), 0.5 μl FastDigest *Eco*RI (10 u/μl) restriction enzyme was digested at 37°C for 20 min (Promega). The restriction patterns were compared after electrophoresis on a 2.5% agarose gel followed by ethidium bromide staining and photographed under UV at 312 nm using a transilluminator.

Results and Discussion

DAS-ELISA results showed that 27 of 3000 tested pepper plants were infected with PMMoV, the presence of which has now extended to three provinces instead of two (Antalya and Şanlıurfa), based on previous reports (Güldür et al. 1994; Palloix et al. 1994a; Güldür and Çaglar 2006). The three provinces had different levels of infection ranging from 0.8% to 4% [Adana (10 of 1250), Antalya (2 of 150), Kahramanmaraş (10 of 1250), Mersin (3 of 300) and Şanlıurfa (2 of 50)]. DAS-ELSA and RT-PCR detected all PMMoV infections and results yielded from both assays agreed (Fig. 1a). The level of infection found in the tested samples is particularly low, considering that Tobamoviruses generally spread quickly in nature. This could be due to the kind of practice adopted by local growers who produce their own seeds from healthy plants and to the limited importation of new cultivars (seeds and plantlets) into the country.

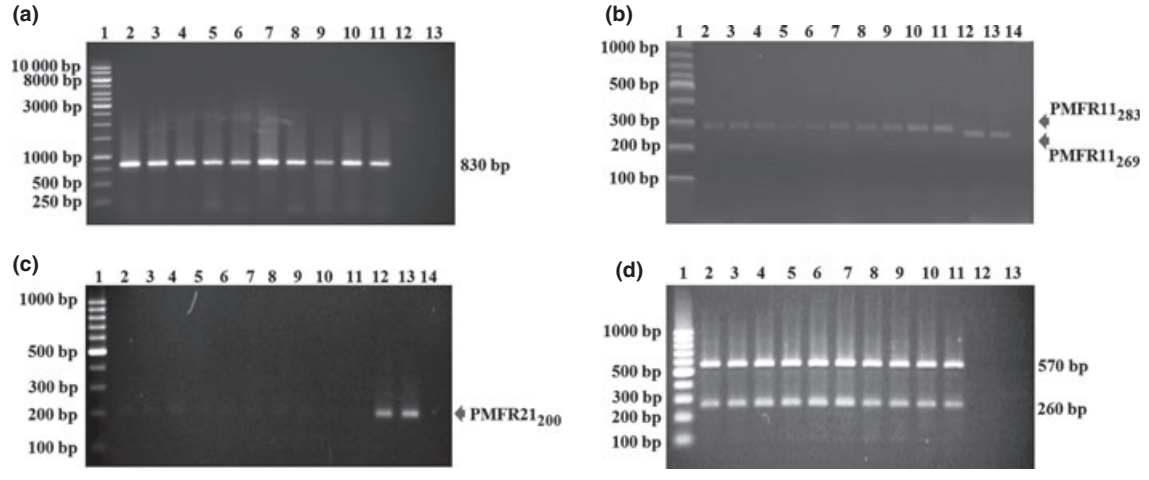


Fig. 1 (a) Electropherogram showing RNA-dependent RNA polymerase (RdRp)-PCR amplicons primed with P12/3A and P12/3. Lane 1: DNA Marker 1 kb; lanes 2–11: representatives of *pepper mild mottle virus* (PMMoV)-infected samples; lane 12: healthy pepper plant and lane 13: water control reaction. (b) Electropherogram showing sequence characterized amplified region (SCAR) PCR amplicons of 283 bp (designated as $PMFR11_{283}$), obtained from pepper samples collected from fields (lanes 2–11). Lane 1: DNA Marker XIV; lanes 12 and 13: PCR products of 269 bp (designated as $PMFR11_{269}$) obtained from *C. Chinense* resistant plants; lanes 14: water control reaction. (c) Electropherogram showing PCR positive reactions (200 bp), (designated as $PMFR21_{200}$), uniquely with resistant plants (lanes 12–13), while no reactions were obtained with tested pepper samples (lanes 1–11). (d) Electropherogram showing restriction fragment length polymorphism (RFLP) pattern generated from a digestion with *Eco*RI restriction enzyme of RdRp amplification from PMMoV-infected pepper plants. Lane 1: DNA Marker 1 kb; lanes 2–11: PMMoV-infected samples; lane 12: healthy pepper plant and lane 13: water control reaction

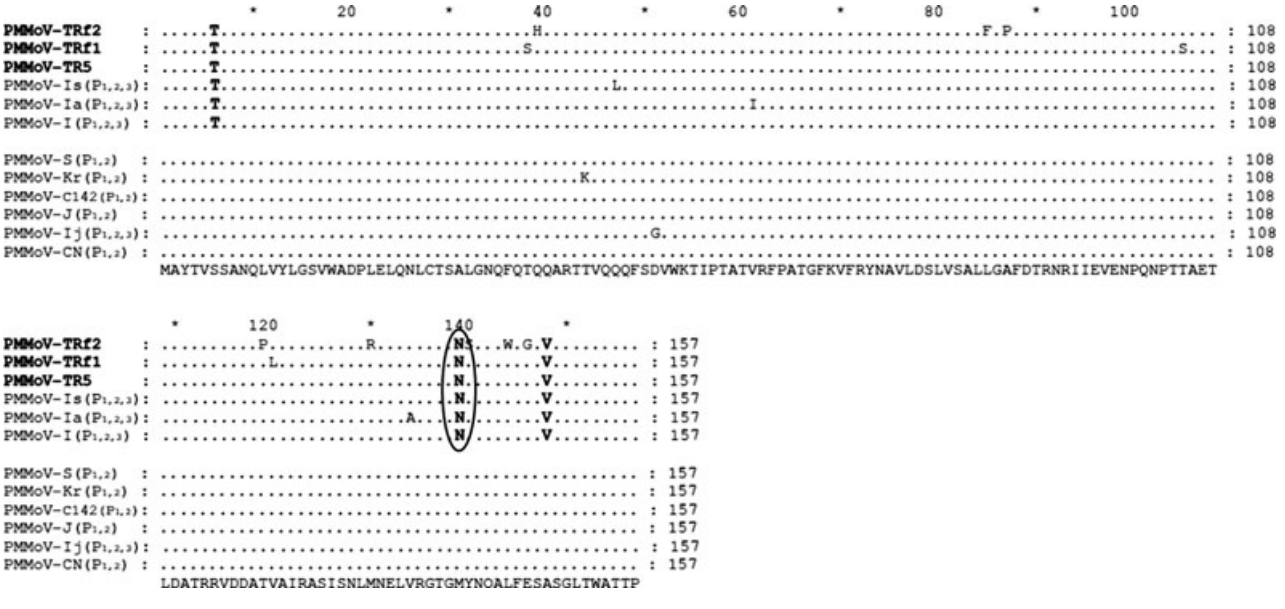


Fig. 2 Amino acids sequence alignment of capsid protein (CP) gene of different *pepper mild mottle virus* (PMMoV) pathotypes (P). Accession numbers of sequences retrieved from GenBank are reported between brackets, respectively. Accessions of Turkish isolates (TR5, TRf1, TRf2) are highlighted in bold. PMMoV-TR5 (HE963026), PMMoV-TRf1 (HE963027), PMMoV-TRf2 (HE963028), PMMoV-Ia ($P_{1,2,3}$) (AJ308228), PMMoV-I ($P_{1,2,3}$) (X72587), PMMoV-Is (ABO15562) and PMMoV-Ij ($P_{1,2,3}$) (BAB55796) are L^3-resistance-overcoming isolates, whereas PMMoV-S ($P_{1,2}$) (M81413), PMMoV-Kr ($P_{1,2}$) (AB126003), PMMoV-C-142 ($P_{1,2}$) (AB069853), PMMoV-J ($P_{1,2}$) (AB000709) and PMMoV-CN ($P_{1,2}$) (AY859497) are not. The key amino acid responsible for this phenotype in PMMoV-Ia, PMMoV-I and PMMoV-Is is indicated by an ovoid circle. Only amino acid substitutions are shown

All inoculated peppers and herbaceous hosts exhibited typical PMMoV leaf symptoms that varied from necrotic lesions to mosaic, and infections were checked by DAS-ELISA and RT-PCR using RdRp- and CP-specific primers.

PCR conducted with the combination of PMF1 and PMR1 primers generated amplicons of 283 bp, designated as $PMFR11_{283}$, in all pepper samples emphasizing the presence in pepper plantations of susceptible gene(s) to PMMoV infections, while the two resistant peppers used as controls generated a lower size DNA fragment (269 bp), designated as $PMFR11_{269}$ (Fig. 1b). These results were further confirmed by the second set of primers (PMF2 and PMR1), which generated positive reactions, a 200 bp DNA fragment, designated as $PMFR21_{200}$, uniquely in resistant peppers

(Fig. 1c). In addition, RT-PCR using a common primer pair for PMMoV (P12/3A and P12/3) followed by restriction enzyme analysis with *Eco*R I, generated a RFLP pattern (doublet of DNA band) which is characteristic of PMMoV strains able to break L-resistance gene in pepper (Velasco et al. 2002) (Fig. 1d). All RdRp- and CP-PCR products obtained from PMMoV-infected samples from Turkey were sequenced. RdRp sequences shared high nucleotides similarity (98–100%) with its homologue of PMMoV isolates from different origins present in GenBank. The identity was lower in the CP gene, while 95–100 and 92–100% were found at nts and a.a levels, respectively. These ranges were based on three distinct CP clones, denoted as TRf1, TRf2 and TR5 (deposited in GenBank under the accession numbers HE963026, HE963027 and HE963028, respectively), found to be representatives of all the 27 analysed sequences. The highest variability was encountered in the 'TRf2′ isolate (92% of a.a identity) originating from Kahramanmaraş province. All CP sequences of PMMoV from *Turkey shared* identity (95–98%) with PMMoV-I pathotype $P_{1,2,3}$ from Italy (acc. no. X72587), PMMoV-Is pathotype $P_{1,2,3}$ from Israel (acc. n°. ABO15562) and PMMoV-Ia pathotype $P_{1,2,3}$ from Spain (acc. n°. CAC59958). The amino acid alignment constructed with CP sequences of TRf1, TRf2, TR5 and of different PMMoV pathotypes, most related to Turkish isolates, shared molecular prints defined by three amino acids at positions 6, 139 and 148 that were all uniquely common to PMMoV pathotype $P_{1,2,3}$ (Fig. 2). Notably, pathotype $P_{1,2,3}$ infects the L^1, L^2, and L^3 plants systemically and elicits HR (necrotic local lesions) only in the L^4 plants (Berzal-Herranz et al. 1995; Tsuda et al. 1998; Hamada et al. 2002; Antignus et al. 2008).

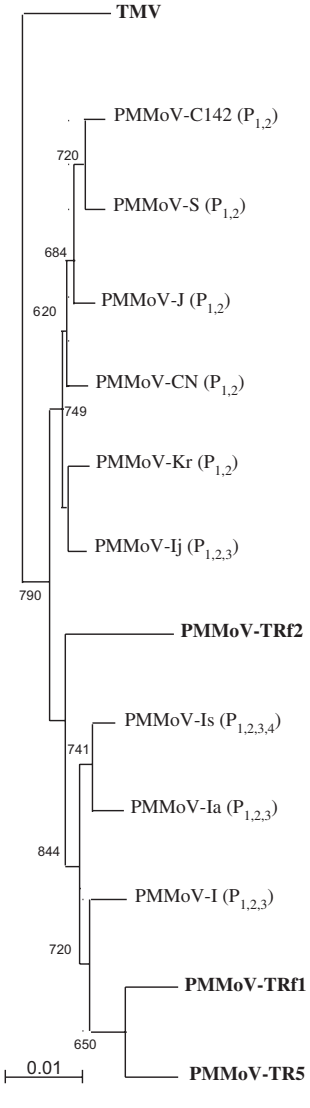


Fig. 3 Phylogenetic tree constructed with the nucleotide sequences of capsid protein (CP) gene of *pepper mild mottle virus* (PMMoV) from Turkey (TR5, TRf1, TRf2) together with homologue sequences of PMMoV from different origins and pathotypes (P). *Tobacco mosaic virus* (TMV) (AB669002) was used as an outgroup species

The CP-based phylogenetic tree, constructed with nucleotide sequences of PMMoV of different pathotypes, allocated the Turkish isolates close to $P_{1,2,3}$ strains, with the exception of the TRf2 isolate which showed a distant allocation (Fig. 3). However, PMMoV of Turkish isolates have been ordered on the basis of their molecular features together with pathotypes that are able to overcome the *L* gene for resistance in pepper genotype; Nevertheless, inoculation on standard pepper plants for L gene resistance is required for an accurate and definitive pathotype categorization. Based on the limited symptoms induced on pepper and herbaceous hosts, PMMoV from Turkey is presumably biologically identical to those of pathotype $P_{1,2,3}$. This claim is somehow supported by the phylogeny which has correlated the Turkish isolates to those originating from Italy and Spain, all of pathotype $P_{1,2,3}$, and in particular TRf2, to the PMMoV Israeli isolate (Is) of pathotype $P_{1,2,3,4}$ recently reported to overcome L^4 resistance in pepper. However, this suggestion remains hypothetical until a confirmation from artificial construct of TRf2 infectious cDNA clone sort-out symptoms of HR in pepper (experiment in progress).

In conclusion, this is the first molecular information on PMMoV isolates present in Turkey, which belong putatively to pathotype $P_{1,2,3}$. The information on the occurrence of such breaking resistance isolates investigated in pepper plantations and PMMoV should be helpful for a future control strategy against this virus and for the national breeding programmes in the country.

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