A New Pathotype of *Pepper mild mottle virus* (PMMoV) Overcomes the L^4 Resistance Genotype of Pepper Cultivars

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ABSTRACT

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The biological, serological, and molecular characteristics of a newly isolated L^4 resistance-breaking isolate of *Pepper mild mottle virus* (PMMoV) were studied. The new pathotype of PMMoV is closely related to the Israeli pathotypes $P_{1,2}$ and $P_{1,2,3}$ of the virus; however, the mosaic symptoms caused by this new pathotype on pepper plants with an L^4 genotype were more severe than the mild mosaic symptoms caused by other common pathotypes of the virus in susceptible plants. The predicted amino acid sequence of the putative coat protein (CP) of the newly described pathotype has two amino acid mismatches when compared with the CP of pathotype $P_{1,2}$, leucine to glutamine at position 47, and alanine to glycine at position 87. The CP of the new pathotype has one amino acid mismatch when compared with $P_{1,2,3}$, having alanine instead of glycine at position 87. Based on its biological characteristics, the new pathotype was designated $P_{1,2,3,4}$ of PMMoV-Is. A method is described for the differentiation among the three PMMoV pathotypes using restriction cleavage analysis of reverse-transcription polymerase chain reaction products made from virus-infected plants. An additional unique *MnII* site in the CP gene of the newly isolated $P_{1,2,3,4}$ allows its distinction from the other two isolates, while *BglII* cleaved only products of the $P_{1,2}$ pathotype.

Pepper mild mottle virus (PMMoV) was first described in Italy in 1984 (21). Since then, it has spread and become a significant pathogen of pepper crops worldwide. The devastating damage caused to *Capsicum* spp. by this virus spurred the introduction of resistance genes into commercial cultivars of pepper; however, new strains of the virus may be selected by the new *Capsicum* resistance genes (4). Resistance is governed by four different genes, known as L^1 to L^4 , which are considered to be alleles at the locus L (5,6). The “gene-for-gene” concept was proposed by Flor (10) to explain the resistance reaction in some pathogen–plant interactions. According to this model, complementary pairs of dominant genes, one in the host plant and one in the pathogen, are required to produce a host resistance reaction. Later studies have shown that recognition between a viral gene and a plant gene determines whether or not the virus can successfully infect the plant. The coat protein (CP) in several groups of plant viruses is by far the most abundant viral protein present in infected plant cells, and is likely to be a

recognition signal for the host defense response (8,9). The most common mechanism of resistance is mediated by a hypersensitive reaction (HR), manifested through the induction of necrotic local lesions. The *Tobamovirus* strains that were isolated from pepper have been classified into four groups (P_0 , P_1 , $P_{1,2}$, and $P_{1,2,3}$) on the basis of their interactions with the corresponding plant resistance genes. Virus pathotype $P_{1,2,3}$ infects the L^1 , L^2 , and L^3 plants systemically, and elicits HR only in the L^4 plants (5).

We describe a new resistance-breaking pathotype of PMMoV, designated $P_{1,2,3,4}$, that overcomes the L^4 -mediated resistance in pepper and a molecular procedure to distinguish it from the other common pathotypes of the virus.

MATERIALS AND METHODS

Virus source and maintenance. The new PMMoV pathotype ($P_{1,2,3,4}$) described in this article was isolated from infected pepper plants grown in commercial greenhouses in Israel. A culture of each of the studied pathotypes originated from a single local lesion taken from an inoculated *Nicotiana glutinosa*. *Capsicum annuum* differential hosts (5,6) served for pathotype identification. Cultures of the viruses under study were maintained by rubbing viral crude inoculum onto Carborundum-dusted leaves. The new pathotype and the formerly described pathotypes $P_{1,2}$ and $P_{1,2,3}$ (Y. Antignus, unpublished) were main-

tained on pepper cvs. Cuby (L^4) and Maor (L^0), respectively. All plants were kept in an insect-proof air-conditioned greenhouse (26 and 21°C during the day and night, respectively) that was treated regularly with insecticides.

Virus identification. The new pathotype under study was purified from infected plants as previously described (21). Serological identification was done by double-antibody sandwich enzyme-linked immunosorbent assay as described by Clark and Adams (7). Antisera against PMMoV $P_{1,2}$ and PMMoV $P_{1,2,3}$ were produced as described by Antignus et al. (2). Virus preparations were examined through transmission electron microscopy after staining with uranyl acetate.

Pepper seed sources. The *C. annuum* cultivars used in this study were Maccabi and Maor (Hazera, Israel); Ray, Mazurka, and Bosanova (Raekzwaan, The Netherlands); and Luisiana and Cuby (S&G, The Netherlands). Seed of Plant Introduction (PI) 1080 and *C. chinense* PI 152225 were kindly provided by Dr. I. Paran, Department of Genetics, ARO, The Volcani Center, Israel.

RNA extraction. Virus preparations were incubated with RNase free DNase I (Fermentas UAB, Vilnius, Lithuania) for 30 min at 37°C prior to the extraction of RNA. Proteinase K (Boehringer, Mannheim, Germany) at a final concentration of 200 µg/ml was then added to a solution containing 0.1 M Tris-HCl buffer; pH 7.5; 1% sodium dodecyl sulfate; 0.15 M NaCl; and 12.5 mM EDTA. The reaction mixture was incubated for 30 min at 37°C. The viral nucleic acids were purified by extraction with an equal volume of Tris-EDTA-saturated phenol. The aqueous phase of the preparation was treated with ether to remove phenol residues, and nucleic acids were precipitated with 0.1 M Na acetate and ethanol at –20°C. Total RNA isolation from plant tissue was carried out by means of the lithium method. A 100-mg sample of fresh tissue was homogenized in 5 volumes of LiCl buffer. Proteins were removed using 6 M potassium acetate (pH 6.5) followed by nucleic acids precipitation by isopropanol (15). The dried pellet was washed with ethanol and suspended in 20 µl sterile water, and 1 µl of RNA was used as a template for reverse-transcription polymerase chain reaction (RT-PCR).

Cloning the CP genes of PMMoV isolates. The virus CP gene was amplified by

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PCR using the primers 5'-CGGACG CCGAGTCTTCTTCG-3' (forward, position 5,657 to 5,677) and 5'-TTAAGG AGTTGTAGCCCAGG-3' (reverse, position 6,139 to 6,159).

The nucleotide positions were numbered according to the full-length sequence of PMMoV (1), GenBank accession no. X72587. RT-PCR conditions were as described by Rosner and Maslenin (14).

PCR products of the virus isolates were cloned into a pGEM-TEasy plasmid vector (Promega Corp., Madison, WI) according to the manufacturer's instructions.

Sequence analysis. Both strands for each of five independent clones of each of the studied pathotypes were sequenced. Nucleotide sequencing was done by the

Sequencing Service of the Molecular Biology Center, Rehovot, Israel. The nucleotide sequence analysis was carried out using the software from the National Center for Biotechnology Information Basic Blast search, San Diego Supercomputer Center (SDSC), and DNAMAN (Lynnon BioSoft, Montreal, Canada). Multiple sequence alignments were done using ClustalX version 1.83 with its default parameters (17). The following nucleotide sequences were used for alignment: Israeli pathotypes PMMoV-Is, P_{1,2}, P_{1,2,3}, and P_{1,2,3,4} (accession nos. EF434393, EF432637, and EF422083, respectively). Spanish isolate P_{1,2} of PMMV-S, accession no. M81413, and Italian pathotype P_{1,2,3} of PMMoV-It, accession no.

X72587, also were used for sequence comparison.

Gel electrophoresis of PCR products. Loading buffer (50% glycerol, 1 mM EDTA, and 0.4% bromophenol blue, one-third by volume) was added to samples of PCR products (5 µl) and fractionation was carried out by electrophoresis in a 1% agarose gel in 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA at room temperature. Fractionation of the PCR products was also performed by running in 6% polyacrylamide gel electrophoresis as previously described (14).

Restriction endonuclease analysis. Aliquots (5 µl) of the amplified PCR products were used for the reaction. Restriction enzymes were purchased from Fermentas UAB, Vilnius, Lithuania and cleavage digestions were carried out according to the manufacturer's protocols. Reaction products were fractionated by electrophoresis on a 1% agarose gel.

RESULTS

Description of symptoms. The disease was first observed in a commercial pepper greenhouse located on the Israeli southern Mediterranean shore. The affected pepper cultivars were Maccabi (*L*⁰) and Cuby (*L*⁴). Disease incidence in both cultivars was about 40%. Leaves of infected plants of both cultivars showed strong yellow mosaic symptoms (Fig. 1), which differed markedly from the mild mosaic caused by the previously described strains of PMMoV. Fruits of cv. Maccabi were distorted and showed unusual, strong yellow mosaic symptoms, whereas fruit of cv. Cuby had rough skin with a discolored appearance or had necrotic, irregular lesions (Fig. 1).

Host range. Partial host range of the virus was determined using mechanical inoculation of test plants. The following plants were susceptible to the virus under laboratory conditions: *C. annuum*, *Datura stramonium*, *Gomphrena globosa*, *N. benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. occidentalis*, *N. rustica*, *N. sylvestris*, *N. tabacum* var. *samsun*, *N. tabacum* var. *white burley*, and *Physalis floridiana*. The following plants were found unsusceptible to the virus: *Chenopodium morale*, *C. quinoa*, *C. tricolor*, *Cucumis sativus*, *Cucurbita pepo*, *Lycopersicon esculentum*, and *Petunia hybrida*. The new pathotype of PMMoV and pathotype P_{1,2,3} shared an identical host range. However, there were differences in the type of symptoms on some of the host plants. The new pathotype induced large, brownish local lesions in *N. glutinosa*, whereas those produced by P_{1,2,3} were smaller and lighter in color (Fig. 2).

PMMoV infection of *Capsicum annuum* cultivars carrying different alleles of the resistance *L* gene. Different *Capsicum* genotypes were mechanically inoculated with the different PMMoV pathotypes at the seven-leaf stage. All the tested

Fig. 1. Symptoms induced on the fruit and leaves of greenhouse-grown pepper plants by the new Israeli pathotype of *Pepper mild mottle virus*. **A** and **D**, Fruit and leaves from healthy *Capsicum annuum*; **B** and **E**, fruit and leaves from infected *C. annuum* cv. Maccabi (*L*⁰); **C** and **F**, fruit and leaves from infected *C. annuum* cv. Cuby (*L*⁴).

pepper genotypes, including the L^4 (homozygous and heterozygous), were systemically infected when inoculated with PMMoV- $P_{1,2,3,4}$, but produced local lesions when infected with the previously described PMMoV- $P_{1,2,3}$ pathotype, as expected (Table 1).

The systemic reaction caused by the new pathotype was more severe than those produced by the other virus pathotypes. Pathotype $P_{1,2,3}$ systemically infected L^0 , L^1 , and L^3 genotypes, and induced a local lesion reaction in the plants carrying the L^4 resistance gene. Infection with pathotype $P_{1,2}$ of pepper plants carrying the L^3 and L^4 genes in heterozygous configuration resulted in a combination of necrotic and systemic reactions, which led to the plants' collapse (Fig. 3). However, homozygous L^4 and L^3 genotypes were found resistant to infection with $P_{1,2}$, reacting by local lesion production on the inoculated leaves (*not shown*).

Sequence analysis of the putative viral CP. The CP genes of the Israeli pathotypes $P_{1,2}$ and $P_{1,2,3}$ and the new pathotype of the virus were cloned and their nucleotide and deduced amino acid sequences were determined. Few differences were seen among the various pathotypes compared. In total, 100% identity was found between the CP amino acid sequences of the Israeli PMMoV pathotypes $P_{1,2}$ and $P_{1,2,3}$ and the published amino acid sequence of the CP of the Spanish $P_{1,2}$ and the Italian $P_{1,2,3}$ pathotypes of PMMoV, respectively (*data not shown*). The new pathotype contained a single variant amino acid compared with the amino acid sequence of the CP of the common Israeli pathotype $P_{1,2,3}$. This change was located at position 87, where alanine was replaced by glycine (Fig. 4). Pairwise comparisons of the putative CPs $P_{1,2,3,4}$ and $P_{1,2}$ showed two amino acid modifications, one at position 47, where leucine was substituted to glutamine, and one at position 87, where glycine replaced alanine. One amino acid difference was found between the CPs of pathotypes $P_{1,2,3}$ and $P_{1,2}$ (Fig. 4).

Differentiation of PMMoV isolates using restriction analysis. In order to be able to distinguish among the three PMMoV pathotypes, the CP encoding sequences were screened by computer analysis for strain-specific restriction endonucleases (Fig. 5A). PCR products made from cloned CP genes of the three PMMoV pathotypes $P_{1,2}$, $P_{1,2,3}$, and $P_{1,2,3,4}$ were cleaved with restriction endonucleases: *Bgl*I, *Mn*I and *Bsp*HI (Fig. 5B). A single unique *Bgl*I restriction endonuclease site was found in the products of isolate $P_{1,2}$. All three pathotypes were cleaved by *Mn*I; however, pathotype $P_{1,2,3,4}$ had an additional *Mn*I site and its cleavage pattern was different (Fig. 5A and B). The cleavage patterns by *Bsp*HI were similar (Fig. 5A). We have further studied the corresponding digests of RT-PCR products

made from total RNA extracted from virus-infected plants. The cleavage patterns of *Bgl*I and *Mn*I were unique and differentiate isolates $P_{1,2}$ from $P_{1,2,3,4}$, respectively, whereas isolate $P_{1,2,3}$ was not cleaved by either of these enzymes. *Bsp*HI similarly cleaved all the three PMMoV isolates (Fig. 5A and B).

DISCUSSION

In this report, we describe the emergence of a new pathotype of PMMoV that can overcome the resistance conferred by the L^4 allele in *C. annuum*. The host range of the new strain does not differ from that of the common Israeli pathotype $P_{1,2,3}$. However, the pattern of symptoms produced by the new pathotype differed slightly in some of the tested host plants (e.g., *N. glutinosa*; Fig. 2). The new pathotype induced symptoms on pepper plants which were more severe than those induced by the virus strains $P_{1,2}$ and $P_{1,2,3}$ on the same pepper cultivars (Fig. 3).

Several viral CPs have been shown to be elicitors recognized by the host plants and capable of activating plant defense response (3,8,12,13). Our results support previous findings that indicated that altering a single, or a few, amino acids in the CP may lead to the loss of a recognition site and, consequently, to a shift from a resistant response in the host plant to a systemic infection (1,11). The L^4 resistance breaking pathotype described in the present study has a single amino acid substitution; alanine to glycine, at position 87 of its CP (Fig. 4), leading to speculation that the resistance breaking of the L^4 genotype may be associated with this amino acid change. The distinct biological reaction of $P_{1,2,3,4}$ indicates that it is an L^4 resistance-breaking member of *Tobamovirus*, and constitutes a distinct pathotype of PMMoV. A recent report from Japan (10) brings evidence for another newly emerged pathotype of PMMoV that overcomes the L^4 gene-mediated resistance in *Capsicum* spp.

Fig. 2. Differentiation between the common Israeli pathotypes of *Pepper mild mottle virus* (PMMoV) and the new Israeli pathotype of the virus using a bioassay on *Nicotiana glutinosa*. Left: Big, dark-brown local lesions induced by the new pathotype (PMMoV-Is- $P_{1,2,3,4}$). Right: Small, light-brown local lesions induced by the common Israeli pathotype PMMoV-Is- $P_{1,2,3}$.

Table 1. Reactions of different *Capsicum* genotypes to infection with *Pepper mild mottle virus* $P_{1,2,3}$ and the L^4 -breaking pathotype $P_{1,2,3,4}$

<i>Capsicum</i> spp.	Resistance genotype	Type of symptoms	
		$P_{1,2,3}$	$P_{1,2,3,4}$
<i>Capsicum annuum</i> 'Maor'	L^0/L^0	Systemic	Systemic
<i>C. annuum</i> 'Mazurka'	L^1/l^1	Systemic	Systemic
<i>C. annuum</i> 'Bosanova'	L^3/l^3	Systemic	Systemic
<i>C. chinense</i> 159236	L^3/L^3	Systemic	Systemic
<i>C. annuum</i> 'Luisiana'	L^4/l^4	Local lesions	Systemic
<i>C. annuum</i> 'Cuby'	L^4/l^4	Local lesions	Systemic
<i>C. annuum</i> PI 1080	L^4/L^4	Local lesions	Systemic

A previous report by Verhoeven et al. (18) described the ability of the $P_{1,2}$ pathotype to overcome L^4 resistance in *C. annuum* cv. Tasty. However, no explanation for this phenomenon was provided. Inter-

estingly, similar results were observed by us when pepper cultivars with heterozygous L^3 and L^4 resistance genes were inoculated with the Israeli pathotype $P_{1,2}$. The inoculated plants reacted with a com-

bination of systemic necrosis and mosaic symptoms that led to plant collapse (Fig. 3). This phenomenon can be explained by differences in the genetic background that may interfere with the expression of resis-

Fig. 3. Characterization of plant reactions to *Pepper mild mottle virus* (PMMoV) infection in *Capsicum annuum* cultivars carrying various alleles of the resistance L gene. The following pepper cultivars were used in the experiment: row A, *C. annuum* cv. Ray (L^0); row B, *C. annuum* cv. Mazurka (L^1); row C, *C. annuum* cv. Bosanova (L^3); row D, *C. annuum* cv. Luisiana (L^4). Plants of each cultivar were inoculated with the following PMMoV pathotypes: column 1, PMMoV-Is- $P_{1,2}$; column 2, PMMoV-Is- $P_{1,2,3}$; column 3, PMMoV-Is- $P_{1,2,3,4}$.

PMMV-P4-Is	MAYTVTSANQLVYLGSVWADPLELQNLCT	SALGNQFQTQQARTTVQ	QFSDVWKTIPTATVRFPATGF
PMMV-P3-Is	MAYTVTSANQLVYLGSVWADPLELQNLCT	SALGNQFQTQQARTTVQ	QFSDVWKTIPTATVRFPATGF
PMMV-P2-Is	MAYTVTSANQLVYLGSVWADPLELQNLCT	SALGNQFQTQQARTTVQ	LQFSDVWKTIPTATVRFPATGF
PMMV-P4-Is	KVFRYNAVLD	SLVSALLG	FDTRNRIIEVENPQNPTTAETLDATRRVDDATVAIRASISNLMNELVRG
PMMV-P3-Is	KVFRYNAVLD	SLVSALLG	FDTRNRIIEVENPQNPTTAETLDATRRVDDATVAIRASISNLMNELVRG
PMMV-P2-Is	KVFRYNAVLD	SLVSALLG	FDTRNRIIEVENPQNPTTAETLDATRRVDDATVAIRASISNLMNELVRG
PMMV-P4-Is	TGNYNQALFESVSGLTWATTP		
PMMV-P3-Is	TGNYNQALFESVSGLTWATTP		
PMMV-P2-Is	TGNYNQALFESVSGLTWATTP		

Fig. 4. Amino acid sequence alignment of the putative coat proteins of *Pepper mild mottle virus* (PMMoV)-Is- $P_{1,2}$, PMMoV-Is- $P_{1,2,3}$, and the new Israeli strain of the virus (PMMoV-Is- $P_{1,2,3,4}$). Modified amino acids are shaded.

Fig. 5. Differentiating the *Pepper mild mottle virus* (PMMoV) pathotypes by restriction endonuclease cleavage analysis. **A**, Restriction endonuclease maps of the cloned coat protein genes of the three virus pathotypes $P_{1,2}$ (P2), $P_{1,2,3}$ (P3), and $P_{1,2,3,4}$ (P4). Nucleotide sequences of the virus pathotypes were computer screened for unique restriction endonuclease sites. The position of Bgl I, Mnl I, and $BspH$ I cleavage sites were marked by numbers along the nucleotide sequence of the coat protein gene. **B**, Reverse-transcription polymerase chain reaction products made from total RNA extracted from plants infected with PMMoV pathotypes $P_{1,2}$, $P_{1,2,3}$, and $P_{1,2,3,4}$ were cleaved with the restriction endonucleases Bgl I, Mnl I, and $BspH$ I and fractionated by polyacrylamide gel electrophoresis. M = DNA size marker; 100-bp DNA ladder.

tance genes (19), as well as by a “dosage effect” of the resistance gene. In heterozygous plants, resistance sometimes is not inherited in a complete dominant fashion, resulting in a lower level of resistance (6). Our results support this latter possibility, showing that inoculation of plants with heterozygous configuration of L^3 and L^4 are susceptible to P_{1,2} whereas homozygous genotypes of this type were resistant.

The emergence of a new L^4 resistance-breaking pathotype highlighted the need for a simple and reliable diagnostic procedure for the differentiation among the virus pathotypes. Restriction cleavage analysis of RT-PCR products of the CP genes facilitated differentiation among pathotypes P_{1,2}, P_{1,2,3}, and P_{1,2,3,4} (Fig. 5) as was previously shown by others for the common PMMoV pathotypes (16).

The potential devastating impact of PMMoV in pepper crops is high. However, the introduction of resistant commercial cultivars has been effective in protecting the crop from the disease (20). The emergence of a new L^4 resistance-breaking pathotype of PMMoV calls for a search for novel sources of genetic resistance against this new virus pathotype.

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