Research Note

Characterization of a Pepper Mild Mottle Tobamovirus Strain Capable of Overcoming the L^3 Gene-Mediated Resistance, Distinct from the Resistance-Breaking Italian Isolate

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Green pepper plants with the L^3 resistance gene usually develop necrotic lesions on leaves infected with a Japanese strain of pepper mild mottle tobamovirus (PMMoV-J). A recently discovered strain, PMMoV-Ij, has the ability to overcome L^3 resistance. Phytopathological responses of a variety of plant species to PMMoV-J and PMMoV-Ij were determined and the coat protein (CP) sequence comparisons revealed both amino acids 43 and 50 of PMMoV-Ij were unique. This led us to believe that substitutions at these residues would enable PMMoV-J to overcome L^3 resistance. This was confirmed by Western blot (immunoblot) detection of PMMoV-J containing both point mutations in upper uninoculated leaves of resistant plants. Computer models suggest the critical residues in overcoming resistance lie in CP regions that putatively interact with other subunits. These results contribute to our understanding of the virus's ability to circumvent plant resistance.

Additional keyword: elicitor.

A strain of pepper mild mottle tobamovirus (PMMoV) is one of the most significant causes of economic loss in Japanese green pepper fields (Kirita et al. 1997). Tobamoviral diseases are usually spread through seed transmission after insufficient virus sterilization treatments. Plants introgressed with the L^2 resistance gene from *Capsicum frutescens* by conventional breeding methods can successfully prevent tobamoviral diseases in most cases. However, a Japanese strain of PMMoV (PMMoV-J) has developed the ability to overcome the L^2 resistance gene and cause mosaic symptoms (Kirita et al. 1997). Similarly, a PMMoV virus strain, tentatively named PMMoV-Ij, has recently been discovered that is able to over-

come L^3 resistance and cause systemic infection. This virus can infect both wild pepper, *C. chinense* (PI159236), and a popular commercial cultivar carrying the L^3 gene, which is usually resistant to PMMoV-J. We have analyzed this tobamovirus to determine the changes allowing it to overcome the L^3 gene-mediated resistance.

Four allelic tobamoviral resistant genes, L^1 , L^2 , L^3 , and L^4 , are known to induce the hypersensitive response (HR) in *Cap-sicum* spp. (Boukema 1982). These genes were categorized by their response to four respective tobamovirus pathotypes, P₀, P₁, P_{1,2}, and P_{1,2,3}. Tobamovirus RNA genomes have four open reading frames encoding four proteins: the 126- and 183-kDa replicase proteins, which are involved in RNA replication (Ishikawa et al. 1986) and vascular-dependent accumulation (Derrick et al. 1997); the 30-kDa movement protein, which is required for cell-to-cell movement (Deom et al. 1987; Meshi et al. 1987); and the 17.5-kDa coat protein (CP), which is needed for long-distance movement (Osbourne et al. 1990). Similar to the L^2 gene (de la Cruz et al. 1997), the CP of PMMoV has been identified as the elicitor of the L^3 gene-mediated resistance by analyzing chimeric PMMoV Spanish (PMMoV-S; the P_{1,2} pathotype) and Italian strains (PMMoV-I; the P_{1,2,3} pathotype; Berzal-Herranz et al. 1995). PMMoV-S induces HR in pepper plants carrying the L^3 gene, while PMMoV-I infects systemically. Phytopathological studies have suggested that PMMoV-J and PMMoV-S are the same since both viruses induce HR in pepper plants carrying the L^3 gene and belong to the same pathotype, described by Boukema (1982). The complete cDNA sequences of PMMoV-S and PMMoV-J (GenBank accession no. AB000709) were determined by Alonso et al. (1991) and Kirita et al. (1997), respectively. Sequence comparisons confirmed that both viruses belong to the same virus species but differ in seven nucleotides. Of those, only three led to amino acid substitutions: two occurring in the 126- and 183-kDa replicase proteins, and one in the 30-kDa movement protein (Kirita et al. 1997).

To investigate the similarities and differences between PMMoV-Ij and PMMoV-J symptoms, we compared the responses of different plant species to inoculation. PMMoV-Ij

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isolated from systemic mosaic leaves of the cultivar carrying the L^3 gene was propagated in *Nicotiana benthamiana* after successive single-lesion isolations on *N. glutinosa*. Sap was then prepared by grinding inoculated *N. benthamiana* leaves in 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM Na_2SO_3 . The PMMoV-J employed as a control was supplied by the Genetic Resources Center, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries (MAFF) in Japan, no. 104032 (Kirita et al. 1997). Sap from PMMoV-Ij and PMMoV-J was used to mechanically inoculate plants belonging to two families and 16 species (Table 1 and data not shown). Test plants were maintained in a greenhouse at 25°C and symptoms were recorded through 30 days post inoculation.

The pathotype of PMMoV-Ij belongs to $P_{1,2,3}$ according to the description of Boukema (1982). *C. chacoense* carrying the L^4 gene developed necrotic lesions upon either PMMoV-J or PMMoV-Ij inoculation. PMMoV-Ij was able to cause systemic mosaic symptoms in *C. chinense* with L^3 gene-mediated resistance without eliciting an HR, whereas PMMoV-J induced an HR (Table 1). In contrast, both PMMoV-J and PMMoV-Ij induced an HR on *N. sylvestris*, in which the viral CP is necessary to elicit the N' gene-mediated resistance (Saito et al. 1987; Knorr and Dawson 1988; Berzal-Herranz et al. 1995). This suggests that although both gene products recognize the viral CP, the characteristics of the CP recognized are host specific. This phenomenon has also been observed in *Solanum melongena*, in which the CP of tobacco mosaic virus (TMV) U1 elicits an HR on primary inoculated leaves of eggplant, but the CP of cucumber green mottle mosaic tobamovirus does not (Dardick and Culver 1997). Although both PMMoV-J and PMMoV-Ij evoked mosaic symptoms in the upper leaves of *Petunia hybrida*, PMMoV-J induced chlorotic local lesions on inoculated leaves (Gebre-Selassie and Marchoux 1991; Kirita et al. 1997), whereas PMMoV-Ij was asymptomatic. This suggests that *P. hybrida* may contain protein(s) that only respond to PMMoV-J.

The L^3 gene resistance is elicited by the tobamovirus CP (Berzal-Herranz et al. 1995). By changing a single amino acid from Asn in PMMoV-I to Met in PMMoV-S at position 138 on the CP, elicitor activity and the ability to contain the virus within necrotic lesions were conferred to peppers carrying the L^3 gene. We analyzed the nucleotide sequence of the CP region of PMMoV-Ij by reverse transcription-polymerase chain reaction (RT-PCR; Tsuda et al. 1994), using *Pfu* polymerase (Stratagene, La Jolla, CA). Primers designed for amplification of the entire CP region were both based on the known

PMMoV-J sequence (Kirita et al. 1997), and included a forward primer (5'-dGG ATCCCCGAGTCTTCTTCGTTTAA ACT) containing a *Bam*HI site (underlined), and a reverse primer (5'-dGGGCCC CAACTTATTTATGCCATCATGTTT) with an *Apa*I site (underlined). The resulting DNA fragments of approximately 500 bp were ligated between the *Bam*HI and *Apa*I sites in the plasmid pBluescriptSK(+) (Stratagene). The ligated mixtures were then transformed into *Escherichia coli* (JM109) with isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) selection. The cDNA sequence analysis was performed by fluorescence dideoxynucleotide chain termination on three clones with Taq Dye Primer Cycle Sequencing Core Kit (Perkin Elmer, Norwalk, CT). The nucleotide sequence of the CP gene was determined with a fluorescent DNA sequencer (373S, Perkin Elmer) and compared with GENETYX computer program (Software Development, Tokyo). The predicted amino acid sequences of PMMoV-Ij, PMMoV-J, and PMMoV-I CPs, based on the nucleotide sequence of each, are presented in Figure 1. The nucleotide sequence of PMMoV-J differs from that of PMMoV-Ij at five positions, leading to two amino acid substitutions. Whereas PMMoV-J has a Thr at position 43 and Asp at position 50 from the N terminus of the CP, PMMoV-Ij has an Lys and Gly, respectively. The Italian isolate differs from PMMoV-Ij at positions 5, 138, and 147, changing the corresponding amino acids from Ser to Thr, Met to Asn, and Ala to Val, respectively (Garcia-Luque et al. 1993). Although both PMMoV-Ij and PMMoV-I possess the ability to overcome the L^3 gene, their amino acid sequences do not differ from PMMoV-J in the same positions.

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PMMoV-Ij  AYT VSSANQLVYLGSVWADPLELQNLCTSLGNQFQTQQA
PMMoV-J   -----
PMMoV-I    ---T-----

RTKVQQQFSGVWKTIPATVRFPATGFKVFRYNAVLDSLVL
--T-----D-----
--T-----D-----

SALLGAFDTRNRIIEVENPQNPTTAETLDRRVDATVA
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IRASISNLMNELVRGTGMYNQALFESASGLTWATTP
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-----N-----V-----

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Table 1. Comparison of symptoms induced by PMMoV-Ij and PMMoV-J in indicator plants^a

Host	PMMoV-Ij	PMMoV-J
<i>Capsicum annuum</i> cv. New-Tosahikari (L^2 / -)	+ / M	+ / M
<i>C. frutescens</i> (L^2 / L^2)	+ / M	+ / M
<i>C. annuum</i> cv. Berumasari (L^3 / -)	+ / M	nll / -
<i>C. chinense</i> PI159236 (L^3 / L^3)	+ / M	nll / -
<i>C. chacoense</i> PI260429 (L^4 / L^4)	nll / -	nll / -
<i>Nicotiana glutinosa</i>	nll / -	nll / -
<i>N. sylvestris</i>	nll / -	nll / -
<i>Petunia hybrida</i>	+ / M	cil / M

^a Local / systemic reaction: + = symptomless infection; M = mosaic; nll = necrotic local lesion; cil = chlorotic local lesion; - = no reaction.

Fig. 1. Comparison of predicted amino acid sequences of the coat protein gene of tobamoviruses infecting green pepper. PMMoV-Ij and PMMoV-I infect systemically in *Capsicum chinense* carrying the L^3 resistance gene as a homozygote, but PMMoV-J is localized to necrotic lesions by the hypersensitive response.

In order to determine whether substitutions of amino acids 43, 50, or both, enable PMMoV-Ij to overcome the L^3 gene-mediated resistance, we constructed three mutants based on these differences. Point mutations were introduced into the PMMoV-J infectious cDNA clone, pTPW1 (Kirita et al. 1997), by PCR mutagenesis to determine which mutations allow the virus to overcome the L^3 resistance gene (Higuchi et al. 1988). Five cDNA infectious clones were constructed as shown in Table 2. pTPW1 contains the full-length, 6,357 nucleotide cDNA of PMMoV-J RNA (GenBank accession no. AB000709), immediately downstream of the T7 RNA promoter sequence in the pUC19 plasmid vector. To introduce

Table 2. Symptoms on *Capsicum frutescens* and *C. chinense* plants caused by the viral progeny corresponding to cDNA clones with amino acid substitutions in the coat protein (CP) gene^a

Clone no.	CP amino acid substitutions			<i>C. frutescens</i> (L^2/L^2)		<i>C. chinense</i> (L^3/L^3)	
	43	50	138	Inoc.	Upper	Inoc.	Upper
pTPW1	Thr	Asp	Met	+	Mosaic	HR	NS
pTPC43	Lys	Asp	Met	+	Mosaic	+	NS
pTPC50	Thr	Gly	Met	+	Mosaic	+	NS
pTPC4350	Lys	Gly	Met	+	Mosaic	+	Mosaic
pTPC138	Thr	Asp	Asn	+	Mosaic	+	Mosaic

^a Bold = CP amino acid substitutions; Inoc. = inoculated; NS = no symptoms; (+) = symptomless infection; HR = hypersensitive reaction.

point mutations in the PMMoV CP gene, the *Sna*BI (residue 5531)-*Bam*HI (just downstream of PMMoV-J cDNA) fragment of pTPW1 was amplified by RT-PCR. First, mutated PCR fragments were amplified with four forward primers introducing the point mutations and the reverse primer (5'-d GGTACCGGATCCTGGGCGCTACCCGCGGTTCG) with *Kpn*I (underlined) and *Bam*HI (bold) sites; next, the complete CP region was amplified with the forward primer (5'-d GAGCTCAATGGCTGACAGATTAC**GTAA**) with *Sac*I (underlined) and *Sna*BI (bold) sites and the point-mutated PCR fragments from the first reaction as the reverse primers; and last, the final products were cloned between the *Sac*I and *Kpn*I sites of pBluescriptSK(+). The four forward primers introducing point mutations in the first PCR reaction were designed as follows: TPC43, a 5804 to 5823 nucleotide sequence with a C to A substitution at 5815; TPC50, consisting of nucleotide 5825 to 5844 sequence with an A to G substitution at 5836; and TPC4350, corresponding to nucleotides 5810 to 5844 with substitutions of C to A at 5815 and A to G at 5836. The last primer, TPC138, corresponding to nucleotides 6091 to 6111 with substitutions of a T to A at 6101 and a G to T at 6102, was constructed based on the sequence of PMMoV-I CP, which has the ability to overcome the L^3 resistant gene (Berzal-Herranz et al. 1995). PCR-amplified sequences were confirmed by DNA sequencing, after cloning into *E. coli* (JM109). The mutated DNA fragments were digested with

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (A) and Western blot (immunoblot) (B) analysis for systemic infections of each mutant on *Capsicum frutescens* carrying the L^2 resistant gene and *C. chinense* with the L^3 gene. Lane 1, healthy *C. frutescens*; lanes 2 and 9, pTPW1; lanes 3 and 10, pTPC43; lanes 4 and 11, pTPC50; lanes 5 and 12, pTPC4350; lanes 6 and 13, pTPC138 as positive control from the report of Berzal-Herranz et al. (1995); lane 7, low molecular marker; lane 8, purified PMMoV-J; and lane 14, healthy *C. chinense*.

*Sna*BI and *Bam*HI, gel purified, and ligated into the corresponding regions of pTPW1. Mutant PMMoV RNAs were transcribed by T7 RNA polymerase from the respective mutagenized cDNA clones of PMMoV-J and propagated in *N. benthamiana*. Five volumes of RNase-free 10 mM phosphate buffer (pH 7.0) were added to the reaction tubes and used to inoculate plants by rubbing onto leaves sprinkled with Carborundum, which served as an abrasive. Ten days later, sap from systemic mosaic leaves was used to inoculate two pepper species, *C. frutescens* and *C. chinense*, carrying L^2 or L^3 resistance genes, respectively. The upper leaves of inoculated pepper plants were observed for 30 days after inoculation and symptoms noted.

Both PMMoV-Ij and the first mutant, pTPC4350, containing a Thr to Lys substitution at position 43 and Asp to Gly at 50, failed to produce necrotic local lesions on either *C. frutescens* or *C. chinense* but caused systemic infections in both plants. The second mutant, pTPC43, had Thr 43 substituted with Lys, and the third mutant, pTPC50, had Asp 50 changed to Gly. These mutants did not induce necrotic lesions on inoculated leaves or systemic symptoms on upper leaves of *C. chinense*. However, both showed systemic symptoms in *C. frutescens*. These results may be caused by the L^3 gene-mediated tobamoviral resistance functioning on the L^3 pathway and not directly on the L^3 gene, or by another, yet unknown resistance mechanism in *C. chinense*. Alternatively, either single mutation may hinder movement through the en-

tire plant due to an unstable CP particle. In support of this idea, the pTPC50 in lane 4 of the Western blot in Figure 2B was partially degraded. These results suggest that these mutant CPs are insufficient for viral assembly or virion stability in *C. chinense* carrying the L^3 gene. pTPC138, with a Met to Asn substitution at position 138 of the CP, was used to inoculate *C. chinense* and *C. frutescens* as a positive control (Berzal-Herranz et al. 1995). pTPC138 conferred mosaic symptom severity and progression on both *C. chinense* and *C. frutescens* similar to that conferred by pTPC4350. RT-PCR sequencing confirmed that progeny mutants pTPC43, pTPC50, pTPC4350, and pTPC138 had maintained their original mutant sequence (data not shown).

Use of the anti-PMMoV-J antibody conjugated with alkaline phosphatase after isolation as an IgG (AP-anti-PMMoV, in-house reagent; Clark and Adams 1977) in a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of inoculated and noninoculated leaves gave results consistent with mutant symptoms (data not shown). To confirm virus infection, Western blots were conducted with samples of upper, noninoculated *C. chinense* and *C. frutescens* leaves 30 days post inoculation, as described by Knecht and Dimond (1984). Blots were probed with the AP-anti-PMMoV. CP was detected only in leaves showing mosaic symptoms (Fig. 2). These results reveal that the ability to overcome the L^3 gene-mediated resistance is attributable to the double substitutions of the CP amino acid sequence, at positions 43 and

Fig. 3. Spatial location of respective amino acid residues in PMMoV coat protein (CP) that are sufficient to overcome the L^3 gene-mediated resistance. Yellow is used to show the amino acids (43 and 50) in PMMoV-Ij that are different from PMMoV-J and PMMoV-S. Blue represents residue 138, the only difference between PMMoV-I and the other PMMoV CPs that enables it to overcome L^3 resistance.

50, as demonstrated by PMMoV-Ij. Natural isolates of PMMoV-Ij overcome resistance through double substitutions at 43 and 50, but not 138, which remains unchanged. Thus, there are two distinct ways of overcoming the L^3 resistance gene: (i) by creating a double mutation in both residues 43 and 50 in PMMoV-Ij, or (ii) by making an amino acid substitution at position 138 in the natural isolate, PMMoV-I.

The first well-characterized example of a plant-virus interaction described the tobamovirus CP as the specific elicitor against the N' gene from *N. sylvestris* (Saito et al. 1987; Knorr and Dawson 1988). The three-dimensional structure of CP appears to be important in virus recognition and N' gene function (Culver et al. 1994). Amino acid substitutions that induce HR were found primarily in the surface region between two CP subunits positioned in a protohelix or disk formation (Culver et al. 1991). In addition, it was shown that the CP of PMMoV-S, and PMMoV-J, elicit HR in *C. chinense* carrying the L^3 gene. The three-dimensional structure of TMV CP was used to model mutant PMMoV CPs with the molecular graphics program Insight II (Biosym Technologies, Urayasu, Japan), at a set resolution of 2.9 Å (Namba et al. 1989). According to this program, the amino acid changes of PMMoV-Ij CP were located in the right skewed helix, and most likely interact with adjacent CP subunits (Taraporewala and Culver 1996), as shown in Figure 3. A substitution at amino acid 138 alone, which is located in the β sheet close to the C terminus of the CP of PMMoV-I, is also sufficient to break L^3 gene-mediated resistance (Berzal-Herranz et al. 1995). Based on each mutation location in this analysis, the ability to bypass resistance probably occurs through different mechanisms for each virus, considering that the unique amino acids of PMMoV-Ij hypothetically affect the assembled viral particle, whereas in PMMoV-I they may either affect virion stability or interact directly with the L^3 gene product (Taraporewala and Culver 1997).

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