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.

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ABSTRACT

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The *Capsicum* spp. L genes (L^1 to L^4) confer resistance to tobamoviruses. Currently, the L^4 gene from *Capsicum chacoense* is the most effective resistance gene and has been used widely in breeding programs in Japan which have developed new resistant cultivars against *Pepper mild mottle virus* (PMMoV). However, in 2004, mild mosaic symptoms

began appearing on the leaves of commercial pepper plants in the field which possessed the L^4 resistance gene. Serological and biological assays on *Capsicum* spp. identified the causal virus strain as a previously unreported pathotype, $P_{1,2,3,4}$. PMMoV sequence analysis of the virus and site-directed mutagenesis using a PMMoV-J of the $P_{1,2}$ pathotype revealed that two amino acid substitutions in the coat protein, Gln to Arg at position 46 and Gly to Lys at position 85, were responsible for overcoming the L^4 resistance gene.

Additional keywords: elicitor, methyl bromide, particle assembly.

Pepper mild mottle virus (PMMoV), a member of the genus *Tobamovirus*, is one of the most serious pathogens that afflict commercial pepper production, causing considerable worldwide economic losses (1,17). PMMoV is a rod-shaped particle, ≈ 300 nm in length, which is spread epidemically between plants through mechanical contact with contaminated sources such as soil and seed (28). The PMMoV genome consists of a 6.3-kb positive-sense, linear, single-stranded RNA which encodes at least four proteins: the 130- and 180-kDa replication proteins, a 30-kDa movement protein (MP), and a 17-kDa coat protein (CP) (15).

Since *Tobacco mosaic virus* (TMV) was first reported in pepper cultivation in 1952 (16), considerable efforts have been directed at controlling and eradicating tobamoviruses in *Solanaceous* crops, including soil fumigation with methyl bromide, testing for viral accumulation soil, seed sterilization, screening for novel resistant genes, breeding programs, and vaccination with attenuated viruses (3,5,12,13,29,30).

Some cultivars, species, and accessions of the genus *Capsicum* show the hypersensitive response (HR)-mediated resistance against tobamoviruses, and are classified hierarchically into the five resistant gene groups: L^1 , L^{1a} , L^2 , L^3 , and L^4 (3,22). The resistance genes are inherited in a Mendelian nature and are allelic variants of the L locus on chromosome 11 of *Capsicum* spp. (4,9,22). The ability of the tobamovirus to break L gene-mediated resistance determines its pathotype: P_0 , P_1 , $P_{1,2}$, or $P_{1,2,3}$. Thus, viruses belonging to the P_0 pathotype are unable to infect plants carrying any L gene. Likewise, viruses belonging to the P_1 , $P_{1,2}$, and $P_{1,2,3}$ pathotypes can systemically infect plants with the: L^1 and L^{1a} genes; L^1 to L^2 genes; and L^1 to L^3 genes; respectively (4,22).

According to this classification, past PMMoV isolates found over the world have been classified as either the $P_{1,2}$ or $P_{1,2,3}$ pathotype (20).

The *Tobamovirus* CP is the elicitor of L gene-mediated resistance and is a determinate in assigning a viral pathotype (2,6–8,10,26). Site-directed mutagenesis and chimeric PMMoV assays have demonstrated that amino acid substitutions in the CP are responsible for overcoming resistance conferred by the L^3 gene. Viral inoculation of *Capsicum* spp. carrying the L^3 gene revealed that either a single (Met to Asn, position 138) or a double (Thr to Lys, position 43 and Asp to Gly, position 50) amino acid change in the PMMoV CP induced mosaic symptoms (2,26), whereas a single amino acid change (Ser to Ala, position 81) induced systemic necrosis (10).

In 2004, mild mosaic symptoms began to appear on commercial green pepper *Capsicum annuum* cv. L4 Miogi (L^4/L^+) plants growing in a production field in the Hokkaido Prefecture, Japan. We suspected that these symptoms resulted from infection by a new L^4 gene-breaking viral pathotype. We isolated the causal virus and used double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and host range studies to first identify the culprit as a PMMoV with a new pathotype: $P_{1,2,3,4}$. The results presented here show our analysis of the viral genome sequence and identified the viral factor responsible for overcoming L^4 gene-mediated resistance using an infectious cDNA clone, from the $P_{1,2}$ pathotype of PMMoV-J named pTPW1 (15).

MATERIALS AND METHODS

Viral isolation and DAS-ELISA. Leaves exhibiting mild mosaic symptoms were collected from green pepper plants (*C. annuum* cv. L4 Miogi, L^4/L^+) grown in production fields in Hokkaido, Japan. A PMMoV DAS-ELISA detection kit was used to diagnose viral infection according to the manufacturer's instructions (Japan Plant Protection Association, Tokyo). Patho-

genicity was confirmed by mechanical inoculation of *C. annuum* cv. L4 Miogi with leaf sap from the infected plant. The causal virus producing positive DAS-ELISA result for PMMoV and systemic symptoms on *C. annuum* cv. L4 Miogi was tentatively named *L*⁴ resistance gene-breaking virus (L4BV). A Japanese ordinary strain of PMMoV (PMMoV-J, P_{1,2} pathotype) was used as a standard and was transcribed from plasmid pTPW1 (15) (accession no. AB000709).

Plant materials and inoculation. *C. annuum* cultivars Miogi (*L*³/*L*⁺) and L4 Miogi (*L*⁴/*L*⁺) (Nihon Horticultural Production and Research Institute, Chiba, Japan), *C. chinense* PI159236 (*L*³/*L*³), and *C. chacoense* PI260429 (*L*⁴/*L*⁴) were used in host range studies to aid in determining the viral pathotype. Plants were grown in a closed greenhouse with a light-and-dark cycle of 16 and 8 h, respectively, under metal halide lamps. The day and night temperature was maintained at 25 and 20°C, respectively. Sap was isolated from L4BV-infected leaves of *C. annuum* cv. L4 Miogi and mixed with 50 mM sodium phosphate buffer (pH 7.0). Mechanical inoculation was performed on the first to the third true leaves of *C. chinense* PI159236, and *C. annuum* cvs. Miogi and L4 Miogi, and on the fifth to the seventh true leaves of *C. chacoense* PI260429. Symptoms were observed from 4 to 15 days postinoculation (dpi). Inoculated and uninoculated upper leaves were collected at 5 and 15 dpi, respectively, and assessed by DAS-ELISA to determine the extent of viral infection.

Nucleotide sequence. Total RNA from L4BV-infected *C. annuum* cv. L4 Miogi leaves was extracted using QIAzol (Qiagen, Hilden, Germany). Reverse-transcription polymerase chain reaction (RT-PCR) was performed using ReverTra-Plus (Toyobo Ltd., Osaka, Japan) and four primer pairs based on the sequence of PMMoV-J (15) (Table 1). First-strand cDNA was synthesized at 42°C for 30 min; then the reaction was terminated by heating at 85°C for 5 min. Four cDNA fragments were amplified using denaturation at 94°C for 2 min followed by 30 cycles of 98°C for 10 s, 52°C for 30 s, and 68°C for 2 min 30 s. Amplified fragments were purified and directly sequenced with the BigDye Terminators v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and multiple primers designed to cover the full length of the genome based on available sequence information of PMMoV-J (15). Sequencing results were analyzed with SEQUENCHER (ver. 3.0 for Macintosh; Gene Codes

Corporation, Ann Arbor, MI) and GENETYX (ver. 7.0 for Windows; Genetyx Corporation, Tokyo).

Construction of CP mutants based on pTPW1. PCR-based site-directed mutagenesis was used to create PMMoV mutants with single or double amino acid changes in the CP gene using an infectious cDNA clone of PMMoV-J, pTPW1, as the template (11,15). A cDNA clone with a single Gln to Arg amino acid substitution at position 46 (Q46R) was designated as pTPC46, a Gly to Lys change at position 85 (G85K) was designated as pTPC85, and a clone with a double Q46R and G85K substitutions was designated as pTPC4685. To create pTPC46, first-round PCR was carried out using the primer pairs PY19-L4R6 and L4F9-ST74 (Table 2). The two PCR products then were used as templates for the primer pair PY19-ST74. pTPC85 was constructed similarly using the two primer pairs PY19-L4R5 and L4F10-ST74 (Table 2) for first-round PCR, and PY19-ST74 for second-round PCR. For pTPC4685, first-round PCR used three primer pairs, PY19-L4R6, L4F9-L4R5, and L4F10-ST74. Second-round PCR was performed using primers PY19 and ST74 (all primer sequences are summarized in Table 2). Amplified fragments were digested with *Sna*BI and *Bam*HI and cloned into pTPW1.

The names of the viruses derived from in vitro-transcribed RNAs were based on the corresponding plasmids without the “p” prefix. For example, virus TPC4685 was derived from plasmid pTPC4685.

In vitro transcription, inoculation, and infection assay. RNA transcripts of PMMoV CP mutants were synthesized from *Bam*HI-digested recombinant cDNA clones using RiboMAX Large-Scale RNA Production Systems according to the manufacturer’s instructions (Promega Corp., Madison, WI). Five volumes of RNase-free 50 mM sodium phosphate buffer (pH 7.0) were added to the reaction tubes and mechanically inoculated onto leaves of *Nicotiana benthamiana* and *C. chacoense* PI260429 (*L*⁴/*L*⁴). Inoculated and uninoculated upper leaves were collected at 5 and 15 dpi, respectively, and tested using DAS-ELISA as described above.

Electron microscopy. *N. benthamiana* leaves inoculated with PMMoV CP mutant transcripts were observed by serologically specific electron microscopy (SSEM) (14) with some modifications. Leaf samples were homogenated with 100 volumes of phosphate-buffered saline (PBS) (3.8 mM NaH₂PO₄, 16 mM Na₂HPO₄, and 150 mM NaCl, pH 7.4) containing 0.1% mercaptoacetic acid.

TABLE 1. Primer pairs used for reverse-transcription polymerase chain reaction primary amplification of *Pepper mild mottle virus* (PMMoV) genome

Primer	Sequence	Position ^a
PY1a	5'-GTAAATTTTTCACAATTTAA-3'	1–20
PG15	5'-GCTGTGCTTTGATTATCTT-3'	2,270–2,251
PY6	5'-GACTTTTTTCTTGCAGACCA-3'	1,431–1,450
PG10	5'-ACAACCCCTACTAATTCGGG-3'	3,770–3,751
PY11	5'-TCAACTCGAGGTCGATGCTG-3'	2,931–2,950
PG5	5'-AATTTGAAGGCAAATCGTTT-3'	5,270–5,251
PY16	5'-CACGATCATTATTGCTGCAT-3'	4,421–4,450
ST74a	5'-TCTAGAGGATCCTGGGCCGCTACCCGCGGTTTC-3' ^b	6,357–6,338

^a Refer to the nucleotide sequence of PMMoV-J (15).

^b Underline indicates the *Bam*HI recognition site.

TABLE 2. Primers for introducing point mutations to coat protein of the Japanese strain of *Pepper mild mottle virus* (PMMoV)

Primer	Sequence	Position ^a	Amplification ^b
PY19	5'-TAATATTAGAGGTGTTGCCA-3'	5,331–5,350	X, Y, Z
ST74	5'-TCTAGAGGATCCTGGGCCGCTACCCGCGGTTTC-3' ^c	6,357–6,338	X, Y, Z
L4F9	5'-GAACTACGGTTCAACGGCAGTTCTCTGATGT-3'	5,809–5,839	X, Z
L4R6	5'-ACATCAGAGAACTGCCGTTGAACCGTAGTTTC-3'	5,839–5,809	X, Z
L4F10	5'-GTGTCGGCACTTCTCAAAGCCTTGATACTAG-3'	5,925–5,956	Y, Z
L4R5	5'-CTAGTATCAAAGGCTTTGAGAAGTGCCGACAC-3'	5,956–5,925	Y, Z

^a Refer to the nucleotide sequence of PMMoV-J (15).

^b Primers for amplification of TPC46 (X), TPC85 (Y), and TPC4685 (Z).

^c Underline indicates the *Bam*HI recognition site.

Carbon-reinforced collodion film grids were coated with anti-PMMoV CP rabbit antiserum (Japan Plant Protection Association) for 60 min at room temperature. The prepared grids were floated for 60 min on the homogenates, then washed with PBS and distilled water. The grids were negatively stained with 2% phosphotungstic acid and observed under a JEM-1230 electron microscope (JEOL Co. Ltd., Tokyo). The average number of PMMoV particles in five 132.25- μm^2 squares per grid was counted.

RESULTS

Symptoms induced by PMMoV-L4BV on indicator plants and pathotype determination. L4BV was able to systemically infect each *Capsicum* spp. used in this study. By 7 dpi, upper leaves of infected green pepper *C. annuum* cv. Miogi (L^3/L^+) showed vein clearing from the base of the petiole, followed later by mild mosaic symptoms. Infected *C. chinense* PI159236 (L^3/L^3) also became stunted and mosaic symptoms appeared on the upper leaves. The green pepper *C. annuum* cv. L4 Miogi (L^4/L^+) did not show obvious mosaic symptoms; however, stunting of the plant and yellowing of the upper leaves followed by mild mosaic symptoms appeared by 7 dpi. *C. chacoense* PI260429 (L^4/L^4) developed stem necrosis by 7 dpi and systemic necrosis by 15 dpi. Newly emerged leaves on the lateral shoots of *C. chacoense* PI260429 also showed mild mosaic symptoms at a later stage (Fig. 1E; Table 3). All plants were confirmed positive for viral infection by DAS-ELISA (data not shown). These results indicate that the L4BV strain is a L^4 resistance gene-breaking PMMoV and is the first report of a $P_{1,2,3,4}$ pathotype.

Nucleotide sequence of L4BV. The complete sequence of the L4BV consists of 6,356 nucleotides (accession no. AB276030), which encode four open reading frames (ORFs), at least, and resembles that of other PMMoV strains (1,15). In comparison with the nucleotide sequence of PMMoV-J, the L4BV strain has one nucleotide deletion in the 3' noncoding region, and 144 synonymous and 17 nonsynonymous nucleotide substitutions (data not shown). A comparison of the predicted amino acid sequence reveals the L4BV to have four amino acid substitutions in

the 130-kDa protein, four in the read-through region of the 180-kDa protein, five in the MP, and two in the CP.

In comparison with the entire nucleotide sequence of the Korean strain of PMMoV (PMMoV-Kr; accession no. AB126003) (31), the L4BV has 19 synonymous and seven nonsynonymous nucleotide substitutions (data not shown). An amino acid sequence comparison reveals the L4BV to have two amino acid substitutions in the 130-kDa protein, one in the read-through region of the 180-kDa protein, one in the MP, and two in the CP. Phylogenetic RNA analysis between L4BV and other PMMoV shows that L4BV and PMMoV-Kr fall within the same node (data not shown), suggesting that L4BV is more closely related to PMMoV-Kr than PMMoV-J.

Identification of the viral factor responsible for the L^4 resistance. In previous studies, the PMMoV CP has been identified as the viral factor involved in overcoming the L^3 gene-mediated resistance in *Capsicum* spp. Two types of amino acid substitutions seen in the CP of PMMoV-I (2) or PMMoV-Ij (26) of the $P_{1,2,3}$ pathotype group have been shown to be responsible for such resistance. In the present study, two CP amino acid substitutions, Q46R and G85K, were detected between L4BV and the $P_{1,2}$ pathotype group PMMoV-J and PMMoV-Kr (Fig. 2).

In *C. chacoense*, TPC4685 infection caused stem necrosis at 6 dpi, despite asymptomatic inoculated leaves, and later gave rise to systemic necrosis, then mild mosaic on newly emerged leaves of the lateral shoots with symptoms identical to L4BV. Neither

TABLE 3. Comparison of symptoms induced by *Pepper mild mottle virus* (PMMoV)-L4BV and PMMoV-J on test plants^a

Host	PMMoV-L4BV	PMMoV-J
<i>Capsicum annuum</i> cv. Miogi (L^3/L^+)	-/M	NLL/-
<i>C. chinense</i> PI159236 (L^3/L^3)	-/M	NLL/-
<i>C. annuum</i> cv. L4-Miogi (L^4/L^+)	-/Y, S, M	NLL/-
<i>C. chacoense</i> PI260429 (L^4/L^4)	-/N ^b	NLL/-
<i>Nicotiana tabacum</i> cv. Xanthi nc	NLL/-	NLL/-

^a Local/systemic reactions: M = mosaic, N = necrosis, NLL = necrotic local lesion, S = stunt, Y = yellowing, and - = no symptoms.

^b Mosaic symptoms on lateral shoots.

Fig. 1. Symptoms induced by *Pepper mild mottle virus* (PMMoV)-L4BV on *Capsicum* spp. **A to D**, The plant on the right was inoculated with L4BV and the plant on the left was mock inoculated. **A**, *Capsicum annuum* cv. Miogi (L^3/L^+); **B**, *C. chinense* PI159236 (L^3/L^3); **C**, *C. annuum* cv. L4 Miogi (L^4/L^+); **D**, *C. chacoense* PI260429 (L^4/L^4); **E**, newly emerged leaves on the lateral shoots of *C. chacoense* PI260429 showing mosaic symptoms after systemic necrosis.

TPC46 nor TPC85 caused any visible symptoms on upper systemic leaves, throughout the observation periods up to 15 dpi, although necrotic local lesions appeared on inoculated leaves at 4 dpi in TPC46 and chlorotic local lesions leading to necrotic local lesions, in some, on leaves were seen in response to TPC85 (Table 4). This apparent lack of systemic infection was confirmed by RT-PCR. TPW1, as PMMoV-J, infection led to necrotic local lesions on inoculated leaves at 4 dpi, but was unable to cause systemic symptoms and was not detected using RT-PCR. This suggests that the *L*⁴ gene provides antiviral protection (Table 4).

In *N. benthamiana*, leaves inoculated with all viruses showed no symptoms despite testing positive by DAS-ELISA (Table 4). When sap from *N. benthamiana* leaves inoculated with TPC46 and TPC85 was mechanically inoculated onto leaves of *N. tabacum*

cv. Xanthi nc to confirm pathogenicity, necrotic local lesions failed to develop. Although this might suggest that the virus has lost its infectivity, the virus titer by ELISA was similar to that of TPC4685 and TPW1, producing numerous necrotic local lesions on *N. tabacum* cv. Xanthi nc leaves (Table 4). At 10 dpi, apical leaf curling leading to yellowing occurred on upper leaves of *N. benthamiana* inoculated with TPC46 and TPC85, which was similar to that seen in TPC4685 and TPW1. Systemic infection of *N. benthamiana* with progeny viruses of TPC46 and TPC85, which exhibited curling and yellowing, was caused by systemic movement of intact but mutated or revertant tobamoviruses. This was confirmed by a recovery test with *N. tabacum* cv. Xanthi nc followed by CP gene sequencing. The observation that the progeny viruses of TPC 46 and TPC 85 possess the capability for local invasion into *N. benthamiana*, but are unable to induce local lesions upon mechanical passage, suggests interruption of virion assembly in the primary inoculated leaf.

Our results indicate that the PMMoV CP mutants we generated were able to invade inoculated leaves of both *C. chacoense* and *N. benthamiana*, but only TPC4685 was systemic in *C. chacoense*. Because sap from *N. benthamiana* leaves inoculated with TPC46 and TPC85 lost the ability to induce local infections on *N. tabacum* cv. Xanthi nc leaves (Table 4), the Q46R or G85K amino acid substitution may be related to virion stability. Previous studies have suggested a correlation between CP amino acid mutations and virion stability (23,26). To address the question of virion stability, we attempted to visualize mutant PMMoV CP precursors by electron microscopy.

Observation of virus particles. TPC4685 and TPW1 viral particles were easily detected by SSEM in a dip preparation of inoculated leaves, and were indistinguishable from original PMMoV-J particles. The average detection efficiency of TPC4685 and TPW1 viral particles in five 132.25-μm² fields was shown to be 35 to 64 times more sensitive than the usual dip preparation method without antiserum treatment of grids (Table 5). However, we were unable to detect TPC46 and TPC85 particles by SSEM

	10	20	30	40
PMMoV-L4BV	AYTVSSANQL VYLGSVWADP	LELQNLCTSA	LGNQFQTQQA	
PMMoV-I	---T---			
PMMoV-Ij	-----			
PMMoV-J	-----			
PMMoV-Kr	-----			
	50	60	70	80
PMMoV-L4BV	RTTVQRQFSD VWKTIPTATV	RFPATGFKVF	RYNAVLDSL	V
PMMoV-I	---Q---			
PMMoV-Ij	-K-Q-G-			
PMMoV-J	---Q---			
PMMoV-Kr	---Q---			
	90	100	110	120
PMMoV-L4BV	SALLKAFDTR NRIIEVENPQ	NPTTAETLDA	TRRVDDATVA	
PMMoV-I	---G---			
PMMoV-Ij	---G---			
PMMoV-J	---G---			
PMMoV-Kr	---G---			
	130	140	150	
PMMoV-L4BV	IRASISNLMN ELVRGTGMYN	QALFESASGL	TWATTP	
PMMoV-I	-----	---N---	---V---	
PMMoV-Ij	-----			
PMMoV-J	-----			
PMMoV-Kr	-----			

Fig. 2. Comparison of predicted coat protein (CP) amino acid sequences among *Pepper mild mottle viruses* (PMMoV). PMMoV-L4BV is an *L*⁴ gene-breaking strain of the P_{1,2,3,4} pathotype. PMMoV-I (2) and PMMoV-Ij (26) are P_{1,2,3} pathotypes and PMMoV-J (15) and PMMoV-Kr (31) are P_{1,2} pathotypes. Numerals on the top of sequences indicate amino acid residue numbers of the CP.

TABLE 5. Number of *Pepper mild mottle virus* (PMMoV) particles detected in inoculated leaves of *Nicotiana benthamiana*

PMMoV CP mutant ^a	Number of particles (per μm ²) ^b	
	SSEM ^c	Dip
TPC46	0.00	0.00
TPC85	0.00	0.00
TPC4685	2.55	0.04
TPW1	1.41	0.04
Mock	0.00	0.00

^a CP = coat protein.
^b Average particle number estimated from five fields of 132.25 μm²; 0.00 = not detected.
^c Serologically specific electron microscopy.

TABLE 4. Comparison of symptoms induced by *Pepper mild mottle virus* (PMMoV) coat protein (CP) mutants on *Capsicum chacoense* and *Nicotiana benthamiana*

PMMoV CP mutant	Amino acid residue of corresponding CP position		<i>C. chacoense</i> (<i>L</i> ⁴ / <i>L</i> ⁴) ^a		<i>N. benthamiana</i> ^a	
	46	85	Inoc.	Upper	Inoc.	Upper
TPC46	R	G	NLL	—	+ (—) ^b	C, Y (NLL) ^{b,c}
TPC85	Q	K	NLL ^d	—	+ (—)	C, Y (NLL) ^c
TPC4685	R	K	+	N ^e	+ (NLL)	C, Y (NLL)
TPW1	Q	G	NLL	—	+ (NLL)	C, Y (NLL)
Mock	...	...	—	—	— (—)	— (—)

^a Inoc. = inoculated leaves, Upper = noninoculated upper leaves, NLL = necrotic local lesion, + = symptom-free infection confirmed by double-antibody sandwich enzyme-linked immunosorbent assay, and — = no infection confirmed reverse-transcription polymerase chain reaction, C = curling, N = necrosis, Y = yellowing.
^b Results of infectivity tests transferred from *N. benthamiana* to *N. tabacum* cv. Xanthi nc.
^c CP sequence of progeny virus recovered from inoculated *N. tabacum* cv. Xanthi nc leaves was confirmed to have mutations.
^d Chlorotic local lesions appearing at 4 days postinoculation; various local lesions may turn necrotic.
^e Mosaic symptoms on lateral shoots.

(Table 5), suggesting that both TPC46 and TPC85 are defective in viral particle assembly.

These results show that both CP amino acid substitutions, Q46R and G85K, are necessary to overcome the L^4 gene-mediated resistance. Furthermore, the presence of either single amino acid substitution causes a deficiency in the systemic infection of *C. chacoense*, suggesting a disruption in virion formation.

DISCUSSION

Mechanical inoculation of L4BV causes yellowing, mild mosaic symptoms, and stunting in *C. annuum* cv. L4 Miogi (L^4/L^+); however, interestingly, it causes systemic necrosis and mosaic symptoms on lateral shoots of *C. chacoense* (L^4/L^4). This phenomenon was reproducible in inoculation tests of *C. annuum* cv. L4 Miogi and *C. chacoense* using symptomatic shoots from L4BV-infected *C. chacoense* as an inoculum (data not shown). We present two explanations for the observed biological differences in symptoms between the two hosts. Our first suggested explanation may be due to dosage-dependant differences between a plant homozygous versus heterozygous for the L^4 gene. Our results suggest that the difference in symptoms of *C. annuum* cv. L4 Miogi and *C. chacoense* is more attributable to species differences (genetic background) than dosage of the L^4 resistance gene because the *C. annuum* breeding line (L^4/L^4) derived from crossings of *C. chacoense* (L^4/L^4) and *C. annuum* (L^3/L^3) did not show systemic necrosis (data not shown). Therefore, we propose this second, more plausible possibility; specific genetic differences between plant species pertaining to the L^4 gene-mediated resistant pathway lead to diverging symptoms. Unknown important genetic factors related to systemic necrosis symptoms after L4BV infection may have been bred out of *C. chacoense*. Alternatively, genes such as *HSR203*, whose product is a suppressor of the programmed cell death pathway in tobacco, may have been maintained in *C. chacoense* (25). Further investigation is necessary to elucidate the controlling factor or factors of L4BV-induced systemic necrosis in *C. chacoense*.

Previous studies have shown that tobamovirus CPs function as an elicitor of L^1 , L^2 , L^3 , and L^4 gene-mediated resistance, leading to activation of the HR and protection of *Capsicum* spp. from tobamovirus invasion. TMV CP mutants of the P_0 pathotype elicit

a resistance response from pepper plants carrying the L^1 gene (6). The *Potato virus X*-based expression system and CPs of the *Paprika mild mottle virus* (P_1 pathotype) elicit an HR from pepper plants carrying the L^2 resistance gene, the S strain of PMMoV (PMMoV-S; $P_{1,2}$ pathotype) elicits the L^3 gene, and PMMoV-I ($P_{1,2,3}$ pathotype) elicits response from the L^4 gene (7,8). Conclusive evidence also was provided for elicitor activity to a pepper carrying the L^3 gene by point mutational analysis of PMMoV CPs (2,10,26). Although we have identified numerous amino acid differences between the sequences of L4BV and PMMoV-J, the fact that only amino acids unique to the L4BV CP are responsible for conversion of the PMMoV-J pathotype from $P_{1,2}$ to $P_{1,2,3,4}$ indicates that L^4 gene-mediated resistance also depends on tobamoviral CPs, consistent with the L^1 , L^2 , and L^3 genes.

The three-dimensional structure of all tobamoviral CPs is an α -helical bundle composed of four α -helices termed the left and right slewed (LS and RS) and left and right radial (LR and RR) (18,19,27). Based on this structure, the two amino acids Q46R and G85K are located on the RS and RR, respectively (Fig. 3). These α -helices organize the right face of the α -helical bundle. In previous studies, amino acids responsible for overcoming L^3 gene-mediated resistance have been identified that were located on the RS and C terminus region of the CP (2,26). Dardick et al. analyzed the elicitor activities of the TMV CP and revealed the importance of the right face of the α -helical bundle and the proximity of the C terminus in L^1 gene recognition by the CP (6). Our result supports the hypothesis that the right face of the CP α -helical bundle, composed of the RS and RR, plays an important role in eliciting L gene-mediated resistance.

The amino acid CP substitutions, Q46R and G85K, were shown to affect viral assembly and virion stability, thereby causing defective virion formation, in addition to overcoming the L^4 gene-mediated resistance. No PMMoV mutant viral particles carrying either the Q46R or G85K mutation were observed using electron microscopy, although each CP was serologically detectable in inoculated leaves of *N. benthamiana*. In addition, no changes in electrophoretic mobility or CP degradation were observed in western blot analysis of those leaves inoculated with the PMMoV CP mutants and TPW1. This implies that the biochemical properties of both CPs mutants, Q46R and G85K, apparently are equal to those of TPW1 and TPC4685 (data not shown). It is unclear

Fig. 3. Spatial position of amino acid residues in the three-dimensional structure of *Pepper mild mottle virus* (PMMoV) coat protein (CP) needed to overcome L^3 or L^4 gene-mediated resistance. Red in the right figure is used to show the amino acid substitutions (46 and 85) seen in L4BV that enable it to overcome the L^4 gene-mediated resistance. Green represents residues 43 and 50 in PMMoV-Ij (26) that overcome the L^3 gene but not the L^4 gene. The residues responsible for breaking the L^3 gene: 81 in PMMoV-Oh (10) and 138 in PMMoV-I (2) are shown in blue and purple, respectively. LS, RS, RR, and LR indicate left slewed, right slewed, right radial, and left radial, respectively, describing the structure of the α -helical bundle of CP precursors. The left figure shows an intermediate *Tobamovirus* virion. The center contains a single CP subunit, and N and C indicate the N'- and C'-termini of the CP amino acid sequence, respectively.

why such CP mutants could not form intact virions in infected plants, although they abundantly expressed CP subunits that did not differ from those of TPW1 and TPC4685 biochemically. This lack of virion assembly could affect virus infection because the tobamoviral CP is functional in an infected plant. In fact, past work demonstrated the interaction between the *N'* gene from *N. sylvestris* and the elicitor activity of TMV virions or its intermediates in infected cells (24). Taraporewala and Culver reported that TMV mutants defective in virion formation also were defective in eliciting the HR at 25 and 29°C (23). Saito et al. reported that defective viral assembly is associated with defective systemic infection of inoculated plants (21), which most likely is why intact virions of PMMoV CP mutants carrying either amino acid substitution Q46R or G85K could not be formed in plants in the present study.

L4BV emerged over a single year-long cultivation period in the green pepper production field at Hokkaido Prefecture, Japan. Viral mutation or selection must have occurred during this time as *C. annuum* cv. L4 Miogi was introduced in an area where PMMoVs were prevalent. The present study may imply some difficulties in the emergence of the *L*⁴ gene-breaking virus because additive effects for amino acid changes in the CP of PMMoV were needed for conversion of tobamovirus pathotype from P_{1,2} to P_{1,2,3,4}. However, because amino acid substitutions previously discovered as responsible for overcoming *L*³ gene-mediated resistance were not found in the CP of L4BV (2,10,26), it may be possible that the tobamovirus which belongs to the P_{1,2,3,4} pathotype arose from a tobamovirus with a random pathotype, which infects *Capsicum* spp.

Field production of pepper crops genetic resistance for controlling plant diseases is advantageous because it is economical, labor efficient, and simple to use. The disadvantage with single dominant resistant genes, such as the *L* resistance genes, is that they often are short-lived because viruses, fungi, and bacteria quickly evolve to overcome resistance. The use of genetic resistance in sustainable agriculture should be simultaneous with consideration of the evolutionary potential of the plant pathogen, including pathogen density, pathogen inflow, pathogen mutation rate, and the type of selection the crop faces throughout the year. Crops should be monitored closely and constantly improved by biological or physical techniques.

In the present study, we report the first outbreak of a new tobamovirus, pathotype P_{1,2,3,4}, which is able to overcome the resistance conferred by the *L*⁴ gene in *Capsicum* spp. previously held as the most effective genetic resource against tobamoviruses. We also identified in the L4BV CP the double amino acid change that is needed to overcome *L*⁴ gene resistance. In addition, we demonstrated that PMMoV CP mutants in inoculated *N. benthamiana* leaves with either the Q46R or G85K mutation failed to produce detectable virions by electron microscopy, even though mutant CPs were detectable by DAS-ELISA.

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