Amino acids in *Tobamovirus* coat protein controlling pepper *L*^{1a} gene-mediated resistance

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SUMMARY

In pepper plants (genus *Capsicum*), the resistance against *Tobamovirus* spp. is conferred by *L* gene alleles. The recently identified *L* variant *L*^{1a} can recognize coat proteins (CPs) of *Tobacco mild green mosaic virus* Japanese strain (TMGMV-J) and *Paprika mild mottle virus* Japanese strain (PaMMV-J), but not of *Pepper mild mottle virus* (PMMoV), as the elicitor to induce resistance at 24 °C. Interestingly, *L*^{1a} gene-mediated resistance against TMGMV-J, but not PaMMV-J, is retained at 30 °C. This observation led us to speculate that *L*^{1a} can discriminate between CPs of TMGMV-J and PaMMV-J. In this study, we aimed to determine the region(s) in CP by which *L*^{1a} distinguishes TMGMV-J from PaMMV-J. By using chimeric CPs consisting of TMGMV-J and PaMMV-J, we found that the chimeric TMGMV-J CP, whose residues in the β -sheet domain were replaced by those of PaMMV-J, lost its ability to induce *L*^{1a} gene-mediated resistance at 30 °C. In contrast, the chimeric PaMMV-J CP with the β -sheet domain replaced by TMGMV-J CP was able to induce *L*^{1a} gene-mediated resistance at 30 °C. Furthermore, viral particles were not detected in the leaves inoculated with either chimeric virus. These observations indicated that the amino acids within the β -sheet domain were involved in both the induction of *L*^{1a} gene-mediated resistance and virion formation. Further analyses using chimeric CPs of TMGMV-J and PMMoV indicated that amino acids within the β -sheet domain alone were not sufficient for the induction of *L*^{1a} gene-mediated resistance by TMGMV-J CP. These results suggest that multiple regions in *Tobamovirus* CP are implicated in the induction of *L*^{1a} gene-mediated resistance.

INTRODUCTION

Plants have evolved surveillance mechanisms to recognize pathogens and to initiate defence responses. Resistance (*R*) gene-mediated resistance is usually a highly specific event that depends

on a matching specificity between *R* gene products in plants and pathogen effectors. The largest class of *R* proteins consists of nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Chisholm *et al.*, 2006; Jones and Dangl, 2006). These proteins contain either a Toll and interleukin-1 receptor (TIR) or a coiled-coil (CC) domain at their N termini (Jones and Dangl, 2006; McHale *et al.*, 2006). Two general modes of effector recognition by NB-LRR-type *R* proteins reportedly exist: (i) direct physical interaction with the effector proteins, and (ii) indirect interaction mediated by additional plant proteins (Chisholm *et al.*, 2006; Jones and Dangl, 2006).

In pepper plants (genus *Capsicum*), resistance against *Tobamovirus* spp. is conferred by *L* gene alleles and is expressed as a local lesion that confines virus spread at the primary infection site. *L* gene alleles can be categorized into four classes, *L*¹, *L*², *L*³ and *L*⁴, based on the increasing effectiveness against different kinds of *Tobamovirus* spp. (Boukema, 1980, 1984). Map-based and homology-based cloning has revealed that *L*¹, *L*², *L*³ and *L*⁴ genes encode CC-NB-LRR-type resistance proteins (1328 amino acids) that are highly homologous to each other (97.5%–98.9% identity) (Tomita *et al.*, 2008, 2011). Meanwhile, *Tobamovirus* spp. are classified into five pathotypes, P₀, P₁, P_{1,2}, P_{1,2,3} and P_{1,2,3,4}, based on the infection patterns in pepper plants carrying different *L* gene alleles (Boukema, 1980, 1984). P₀ pathotype viruses, including *Tobacco mosaic virus*, *Tomato mosaic virus* and *Tobacco mild green mosaic virus* (TMGMV), are unable to systemically infect plants carrying any *L* gene. P₁ pathotype viruses, such as *Paprika mild mottle virus* (PaMMV), can systemically infect plants carrying the *L*¹ gene, but not the *L*²–*L*⁴ genes. Likewise, P_{1,2} pathotype viruses, such as *Pepper mild mottle virus* (PMMoV), can systemically infect plants carrying *L*¹ and *L*² genes, but not *L*³ and *L*⁴ genes. In addition, naturally occurring PMMoV variants, which break *L*³, or *L*³ and *L*⁴ gene-mediated resistance, are designated as P_{1,2,3} and P_{1,2,3,4} pathotypes, respectively (Antignus *et al.*, 2008; Berzal-Herranz *et al.*, 1995; Genda *et al.*, 2007; Hamada *et al.*, 2002, 2007; Tsuda *et al.*, 1998). It has been shown that *Tobamovirus* coat protein (CP) functions as the elicitor for *L* gene-mediated resistance and serves as the determinant in assigning a viral pathotype (Berzal-Herranz

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et al., 1995; de la Cruz *et al.*, 1997; Gilardi *et al.*, 1998, 2004). Despite the low similarity in amino acid sequences, CPs of *Tobamovirus* spp. have been reported to have similar tertiary structure (Namba and Stubbs, 1986; Pattanayek and Stubbs, 1992; Wang *et al.*, 1997). Thus, it has been speculated that L proteins recognize the structural configurations of *Tobamovirus* CPs (Culver, 2002). However, the domain(s) of CPs controlling the induction of L gene-mediated resistance has not yet been identified.

Plant cultivation at temperatures above 28 °C abolishes *Tobamovirus* resistance exerted by L^1 , L^2 , L^3 and L^4 genes (Boukema, 1984). We have recently identified a new allele, L^{1a} , which shows different temperature sensitivity from other L genes (Sawada *et al.*, 2004). The L^{1a} gene encodes a CC-NB-LRR-type protein with six amino acids difference relative to the L^1 protein (Tomita *et al.*, 2011). In spite of the high amino acid sequence similarity, L^{1a} is able to respond to CPs of P_0 and P_1 pathotypes to induce resistance at 24 °C (Matsumoto *et al.*, 2008; Sawada *et al.*, 2004). Interestingly, L^{1a} gene-mediated resistance against the P_0 pathotype, but not the P_1 pathotype, is retained at 30 °C (Matsumoto *et al.*, 2008; Sawada *et al.*, 2004). These observations suggest that L^{1a} discriminates between CPs of P_0 and P_1 pathotypes.

To verify this possibility, we aimed to determine the amino acid region of CP responsible for the discrimination between TMGMV Japanese strain (TMGMV-J; P_0 pathotype) and PaMMV Japanese strain (PaMMV-J; P_1 pathotype) by L^{1a} . We constructed a number of chimeric CPs consisting of TMGMV-J CP and PaMMV-J CP, and analysed their ability to induce resistance in L^{1a} homozygous *Cap-sicum annuum* plants (L^{1a} plants) at 30 °C. We found that the chimeric CP with a TMGMV-J background and amino acids located in the β -sheet structure of PaMMV-J did not induce L^{1a} gene-mediated resistance at 30 °C. In contrast, the chimeric CP with the reciprocal change in amino acids induced resistance in L^{1a} plants at 30 °C. Both chimeric CPs could induce resistance in L^{1a} plants at 24 °C. These observations indicate that L^{1a} recognizes amino acids in the β -sheet structure of CP, leading to resistance induced by the CP of TMGMV-J at 30 °C. However, the analysis of chimeric CP consisting of TMGMV-J and PMMoV ($P_{1,2}$) indicated that amino acids in the β -sheet domain of TMGMV-J CP alone were not sufficient for the induction of L^{1a} gene-mediated resistance at both 24 and 30 °C. These findings suggest that multiple regions in *Tobamovirus* CP are implicated in the induction of L^{1a} gene-mediated resistance.

RESULTS

Amino acids in TMGMV-J CP responsible for the induction of L^{1a} gene-mediated resistance at 30 °C

Based on the amino acid sequence alignment of the CPs from TMGMV-J, PaMMV-J and PMMoV ($P_{1,2}$), we defined five regions

Fig. 1 (A) Amino acid sequence alignment of the predicted coat proteins (CPs) of Tobacco mild green mosaic virus Japanese strain (TMGMV-J), Paprika mild mottle virus Japanese strain (PaMMV-J) and Pepper mild mottle virus [PMMoV ($P_{1,2}$)]. The numbers to the left of the sequences indicate the amino acid positions. Identical residues are shaded. The positions of the PC1–5 regions are indicated by black lines. (B) Ribbon diagram of TMGMV-J CP. The locations of PC1, PC2a, PC3, PC4 and PC5 are indicated in red and the location of PC2b is indicated in blue.

(PC1–5) with high sequence diversity (Fig. 1A). According to the three-dimensional structure of TMGMV-J CP (Pattanayek and Stubbs, 1992), regions PC1–5 are each located within the left-slewed α -helix, the right-slewed α -helix and β -sheet, the RNA-binding loop, the left radial α -helix and the C-terminal outer face, respectively (Fig. 1B). To determine the region implicated in the induction of L^{1a} gene-mediated resistance at 30 °C, five chimeric TMGMVs (TMG/PC1–PC5) were constructed by replacing each of the five regions of CP with the corresponding region of PaMMV-J CP. L^{1a} homozygous plants (*C. annuum* L. cv. MK-18-3-1; L^{1a} plants) were inoculated with RNA transcripts of the mutant viruses and incubated for 10 days at 24 or 30 °C. To assess the induction of L^{1a} gene-mediated resistance, the distribution of CPs in inoculated leaves was examined by press blot immunoassay.

All chimeric TMGMVs used in this study induced local lesions in inoculated leaves of L^{1a} plants at 24 °C (Figs 2 and S1, see Supporting Information). In addition, immunoreactive signals for CP were localized to the sites of the lesions (Figs 2 and S1). These observations indicate that the amino acid substitutions in

Fig. 2 Symptoms and distributions of coat protein (CPs) in inoculated leaves of *Capsicum annuum* L. cv. MK-18-3-1 harbouring the *L*^{1a} gene at 24 and 30 °C. Inoculated leaves were subjected to press blot immunoassay after photography. The accumulation of CP in leaves inoculated with chimeric *Tobacco mild green mosaic virus* (TMGMV), except for TMG/PaCP and TMG/Pa-PC2b, was analysed using an anti-TMGMV Japanese strain CP antibody. The accumulation of CP in leaves inoculated with TMG/PaCP and TMG/Pa-PC2b was analysed using an anti-Paprika mild mottle virus Japanese strain CP antibody.

TMGMV-J CP do not affect the elicitor activity of the CPs on *L*^{1a} gene-mediated resistance at 24 °C.

When *L*^{1a} plants were incubated at 30 °C after inoculation with wild-type TMGMV-J, they exhibited local lesions in inoculated leaves, and immunoreactive signals for CP were localized to the lesions (Fig. 2). In contrast, TMG/PaCP, a chimeric TMGMV with its entire CP gene replaced with that of PaMMV-J, did not cause local lesions in inoculated leaves or immunoreactive signals for CP diffused in the leaves (Fig. 2). When *L*^{1a} plants were inoculated with TMG/PC1, TMG/PC3, TMG/PC4 and TMG/PC5, local lesions and immunoreactive signals for CP localized to the lesions were observed in the inoculated leaves (Fig. S1). In contrast, *L*^{1a} plants inoculated with TMG/PC2 did not exhibit local lesions in inoculated leaves or immunoreactive signals for CP diffused in the leaves (Fig. 2). These results suggest that the PC2 region is responsible for *L*^{1a} gene-mediated resistance.

To further map the residues involved in *L*^{1a} gene-mediated resistance, the PC2 region of TMGMV-J CP was divided and additional chimeric viruses were constructed. When *L*^{1a} plants were inoculated with TMG/PC2a, in which the PC2a region of TMGMV-J CP was replaced with the corresponding region of PaMMV-J CP (Fig. 1A), local lesions on inoculated leaves were observed and immunoreactive signals for CP were localized to the lesions

(Fig. 2). In contrast, *L*^{1a} plants inoculated with TMG/PC2b, whose PC2b region of TMGMV-J CP was replaced with the corresponding region of PaMMV-J CP (Fig. 1A), did not exhibit local lesions in inoculated leaves or immunoreactive signals for CP diffused in the leaves (Fig. 2). According to the three-dimensional structure of TMGMV-J CP (Pattanyek and Stubbs, 1992), the PC2b region is located within the outer β -sheet structure (Fig. 1B).

A comparison of PC2b sequences between TMGMV-J and PaMMV-J revealed six amino acid substitutions and two amino acid insertions in PaMMV-J CP (Fig. 1A). To investigate the effect of the amino acid substitutions in the context of PaMMV-J CP on *L*^{1a} gene-mediated resistance, we constructed chimeric TMGMV (TMG/Pa-PC2b) containing mutated PaMMV-J CP with the PC2b region replaced by the corresponding region of TMGMV-J CP (Fig. 1A). *L*^{1a} plants inoculated with TMG/Pa-PC2b exhibited local lesions in inoculated leaves at 30 °C as well as at 24 °C, and immunoreactive signals for CP were localized to the lesions (Fig. 2). This result indicates that amino acid substitutions in the PC2b region allow PaMMV-J CP to acquire the ability to induce resistance in *L*^{1a} plants at 30 °C. Based on these observations, we conclude that amino acids in the PC2b region in the context of TMGMV-J CP and PaMMV-J CP are particularly important for inducing *L*^{1a} gene-mediated resistance.

Fig. 3 Symptoms and distributions of coat proteins (CPs) in inoculated leaves of *Capsicum annuum* L. cv. MK-18-3-1 harbouring the L^{1a} gene at 24 °C and 30 °C. Inoculated leaves were subjected to press blot immunoassay after photography and the viral CP was detected using an anti-Pepper mild mottle virus CP antibody.

To investigate the effect of the PC2b region of TMGMV-J CP in the context of PMMoV ($P_{1,2}$) CP on L^{1a} gene-mediated resistance, chimeric PMMoV (PMMoV-PC2b), in which the PC2b region of PMMoV ($P_{1,2}$) CP was replaced with the corresponding region of TMGMV-J CP (Fig. 1A), was constructed. When L^{1a} plants were incubated at 24 °C and 30 °C after inoculation with PMMoV-PC2b, immunoreactive signals for CP diffused in the inoculated leaves were found, similar to those observed with PMMoV ($P_{1,2}$) (Fig. 3). This result suggests that a domain(s) in CP, in addition to the PC2b region, is required for the elicitor activity of TMGMV-J CP

Fig. 4 Symptoms and distributions of coat proteins (CPs) in inoculated leaves of *Capsicum annuum* L. cv. Verbeterde Glas harbouring the L^1 gene at 24 °C. Inoculated leaves were subjected to press blot immunoassay after photography. The accumulation of CP in leaves inoculated with Tobacco mild green mosaic virus Japanese strain (TMGMV-J) and TMG/PC2b was analysed using an anti-TMGMV-J CP antibody. The accumulation of CP in leaves inoculated with TMG/PaCP and TMG/Pa-PC2b was analysed using an anti-Paprika mild mottle virus Japanese strain CP antibody.

in L^{1a} gene-mediated resistance. It should be noted that, when L^3 homozygous plants (*C. annuum* L. cv. Tosa-midori) were inoculated with PMMoV-PC2b, local lesions and localized immunoreactive signals for CP were observed at 24 °C (Fig. S2, see Supporting Information).

Involvement of the PC2b region of TMGMV-J CP and PaMMV-J CP in the induction of L^1 gene-mediated resistance

Wild-type TMGMV-J induced local lesions on inoculated leaves of L^1 homozygous plants (*C. annuum* L. cv. Verbeterde Glas; L^1 plants) at 24 °C (Fig. 4). In contrast, TMG/PaCP did not induce local lesions in L^1 plants at 24 °C or immunoreactive signals for CP diffused in the inoculated leaves (Fig. 4). To investigate whether the PC2b region of TMGMV-J CP and PaMMV-J CP was also implicated in L^1 gene-mediated resistance at 24 °C, L^1 plants were

inoculated with TMG/PC2b or TMG/Pa-PC2b and incubated at 24 °C for 10 days. Interestingly, in contrast with *L*^{1a} plants incubated at 30 °C, both TMG/PC2b and TMG/Pa-PC2b induced chlorotic lesions in inoculated leaves of *L*¹ plants at 24 °C, with localized immunoreactive signals for CP observed at the sites of the chlorotic lesions (Fig. 4). These observations indicate that the effect of the PC2b region in TMGMV-J and PaMMV-J CPs is significantly different between *L*^{1a}-mediated resistance at 30 °C and *L*¹-mediated resistance at 24 °C.

Involvement of the PC2b region of TMGMV-J and PaMMV-J CPs in virion formation

Although CP accumulation in inoculated leaves was observed in *L*¹ plants inoculated with TMG/PC2b and TMG/Pa-PC2b at 24 °C, no immunoreactive signals for CP were detected in uninoculated upper leaves at 10 days post-inoculation. In addition, immunoreactive signals for CP were not detected in uninoculated upper leaves of *L*^{1a} plants inoculated with TMG/PC2b at 30 °C, although immunoreactive signals for CP diffused in the inoculated leaves (Fig. 2). It has been reported that the defect in systemic infection is associated with the defect in viral particle assembly (Dawson *et al.*, 1988; Saito *et al.*, 1990). To determine whether this was the case for TMG/PC2b and TMG/Pa-PC2b, we analysed the virion accumulation in inoculated leaves of *Nicotiana benthamiana*. Viral particles were easily detected in the homogenate of leaves inoculated with wild-type TMGMV-J and TMG/PaCP (Fig. 5). However, viral particles were not detected in the homogenate of leaves inoculated with TMG/PC2b and TMG/Pa-PC2b (Fig. 5), although CP accumulation was observed (Fig. S3, see Supporting Information). In addition, viral particles were not detected in the homogenate of *L*^{1a} plant leaves inoculated with TMG/PC2b at 30 °C. These observations suggest that, in addition to *L*¹- and *L*^{1a}-mediated resistance, the PC2b region of TMGMV-J CP and PaMMV-J CP is involved in virion formation.

DISCUSSION

Tobamovirus CPs share similar tertiary structures (Namba and Stubbs, 1986; Pattanayek and Stubbs, 1992; Wang *et al.*, 1997). The central core of CP consists of four α -helices (left and right slewed and left and right radial) connected by two inner loops and an outer β -sheet. Both N and C termini are located along the outer surface of the virion. It has been reported that amino acid residues of *Tobacco mosaic virus* CP critical for the induction of *L*¹ gene-mediated resistance are located in several parts of the CP structure, including the right face of α -helices and the outer β -sheet (Dardick *et al.*, 1999). In addition, it has been reported that amino acid residues of PMMoV (P_{1,2,3}) CP responsible for overcoming *L*³ gene-mediated resistance are distributed in several parts of the CP structure (Berzal-Herranz *et al.*, 1995; Genda *et al.*, 2007; Hamada

Fig. 5 Electron micrograph of viral particles in leaf homogenates of *Nicotiana benthamiana* inoculated with *Tobacco mild green mosaic virus* Japanese strain (TMGMV-J), TMG/PC2b, TMG/Pa-PC2b and TMG/PaCP. Bar, 500 nm.

et al., 2002, 2007; Tsuda *et al.*, 1998). In this study, we showed that mutated PaMMV-J CP with amino acids in the PC2b region (located in the outer β -sheet) replaced by TMGMV-J CP induced *L*^{1a} gene-mediated resistance at 30 °C (Fig. 2). However, TMGMV-J CP containing the PC2b region of PaMMV-J lost its ability to induce *L*^{1a} gene-mediated resistance at 30 °C, but not at 24 °C (Figs 2 and S1). These observations suggest that the residues in the PC2b region are particularly important for *L*^{1a} gene-mediated resistance induced by TMGMV-J CP and PaMMV-J CP. However, the results obtained from the analysis of mutant PMMoV (Fig. 3) indicate that the residues in the PC2b region of TMGMV-J CP are not sufficient to induce *L*^{1a} gene-mediated resistance at both 24 °C and 30 °C. It is probable that, at any temperature, multiple regions in *Tobamovirus* CP are implicated in the induction of *L*^{1a} gene-mediated resistance.

Resistance proteins can function either by direct interaction with the corresponding effector proteins (the receptor–ligand model) (Keen, 1990) or by sensing specific alterations in plants that result from the action of the effector proteins (the guard model and the decoy model) (van der Hoorn and Kamoun, 2008; Van der Biezen and Jones, 1998). The most detailed studies on direct association have been carried out in the flax (*Linum usitatissimum*)–flax rust (*Melampsora lini*) pathosystem (Ellis *et al.*, 2007a). More than 30 closely linked TIR-NB-LRR genes on multiple loci (*K*, *L*, *M*, *N* and *P*) of flax recognize approximately 30

flax rust effectors (Ellis *et al.*, 2007a). It has been reported that the recognition of AvrL567 by L6 and AvrL11 by L11 is exclusively controlled by the LRR domain (Ellis *et al.*, 2007b). In addition, the structural and mutagenesis analyses of AvrL567 have shown that binding between the flax L proteins and avirulence (Avr) proteins takes place in multiple contact points across a large surface area of the flax L protein and the Avr protein (Wang *et al.*, 2007). Recently, we have shown that all pepper *L* gene alleles reported to date encode CC-NB-LRR-type resistance proteins (Tomita *et al.*, 2011). In addition, the analysis of chimeras consisting of five *L* gene alleles (L^1 , L^2 , L^{2b} , L^3 and L^4) has shown that the combination of amino acids at different positions of the LRR domain determines the recognition spectrum of L proteins (Tomita *et al.*, 2011). LRR is a protein–ligand interaction domain found in numerous proteins across all taxonomic kingdoms, including immune system receptors in plants and animals (Ausubel, 2005). LRR domains have an arc-shaped structure with a higher surface-to-volume ratio compared with globular proteins (Kobe and Kajava, 2001). Although it is not known whether pepper L proteins recognize *Tobamovirus* CP directly or indirectly, previous and present findings suggest that multiple parts of the LRR domain of pepper L protein may interact directly with multiple parts of *Tobamovirus* CP.

In this study, we observed that the effect of amino acid substitutions in the PC2b region of the TMGMV-J CP during the induction of L^{1a} gene-mediated resistance at 30 °C differed significantly from that of L^1 gene-mediated resistance at 24 °C (Figs 2 and 4). This may imply that L^{1a} and L^1 proteins recognize different parts of TMGMV-J CP. Among the six amino acid differences between L^{1a} and L^1 proteins, three were located in the NB domain and three were found in the LRR domain. Our preliminary observation suggested that two amino acid polymorphisms in the LRR domain may be implicated in the induction of resistance by TMGMV-J CP at 30 °C and PaMMV-J CP at 24 °C (H. Mizumoto and Y. Hikichi, unpublished). The structure-based analysis of the interaction between these two amino acids in the LRR domain of the L^{1a} protein and the PC2b region in TMGMV-J CP will provide essential insights into the mechanisms underlying the recognition of *Tobamovirus* CP by L proteins.

Tobamovirus CP plays a pivotal role in systemic virus movement by assembling virus RNA (Dawson *et al.*, 1988; Saito *et al.*, 1990). In this study, we observed that amino acid substitutions in the PC2b region of TMGMV-J and PaMMV-J CPs resulted in the loss of the systemic movement of the viruses. The accumulation of virions was not observed in plants inoculated with mutant viruses (TMG/PC2b and TMG/Pa-PC2b) (Fig. 5), indicating that amino acids in the PC2b region of TMGMV-J and PaMMV-J CPs are also involved in virion formation. It is noteworthy that mutant TMGMV-J breaking any *L* gene resistance, and mutant PaMMV-J breaking L^{1a} to L^4 gene resistance, have not been obtained from the natural environment. It is possible that the domains in CP of P_0 and P_1 pathotype *Tobamovirus* spp. required for L^1 and L^4 gene-mediated

resistance are also essential for virion formation, thus making it difficult for P_0 and P_1 pathotype *Tobamovirus* spp. to break the resistance.

EXPERIMENTAL PROCEDURES

Plasmid construction

The oligonucleotide primers used in this study are listed in Table S1 (see Supporting Information). The plasmids described below were constructed by recombining DNA fragments obtained from pTGK16 (Morishima *et al.*, 2003), pPAJ (Hamada *et al.*, 2003) and pTPW (Tsuda *et al.*, 1998), from which the infectious viral RNA of TMGMV-J, PaMMV-J and PMMoV, respectively, were transcribed *in vitro*. Polymerase chain reaction (PCR) products were verified by sequencing.

pTMG/PaCP

Two DNA fragments were amplified by PCR from pTGK16 using two primer sets: TMG4784-F and TMG-PaCP5'-R, and PaCP3'-F/TMG and TMG6311-R. A DNA fragment was amplified by PCR from pPAJ using primers TMG/PaCP5'-F and PaCP3'-R/TMG. These three DNA fragments were denatured and annealed, and used as the template for PCR using primers TMG4784-F and TMG6311-R. The amplified 1.4-kb product was digested with *Bsp*T104I and cloned into pTGK16 using the corresponding enzyme sites.

pTMG/PC2

Two DNA fragments were amplified by PCR from pTGK16 using two primer sets: TMG4784-F and TMG/PaCP (R2)5'-R, and PaCP (R2)3'/TMG-F and TMG6311-R. A DNA fragment was amplified by PCR from pPAJ using primers TMG/PaCP (R2)5'-F and PaCP (R2)3'/TMG-R. The three DNA fragments were denatured and annealed, and used as a template for PCR using primers TMG4784-F and TMG6311-R. The amplified 1.4-kb product was digested with *Bsp*T104I and cloned into pTGK16 using the corresponding enzyme sites.

pTMG/PC1, pTMG/PC3, pTMG/PC4 and pTMG/PC5

pTGK16 was used as a template for each PCR. The primer pairs used were TMG4784-F and each of the following: TMGPC1-R for pTMG/PC1, TMGPC3-R for pTMG/PC3, TMGPC4-R for pTMG/PC4 and TMGPC5-R for pTMG/PC5. Another primer, TMG6311-R, was used with each of the following: TMGPC1-F for pTMG/PC1, TMGPC3-F for pTMG/PC3, TMGPC4-F for pTMG/PC4 and TMGPC5-F for pTMG/PC5. PCR products corresponding to each mutant were mixed, denatured and annealed, and used as a template for PCR using primers TMG4784-F and TMG6311-R. The amplified 1.4-kb products were digested with *Bsp*T104I and cloned into pTGK16 using the corresponding enzyme sites.

pTMG/Pa-PC2b

Two DNA fragments were amplified by PCR from pTMG/PaCP using two primer sets: TMG4784-F and PC/TC2b-RV, and PC/TC2b-F and TMG6311-R. The two products were mixed, denatured and annealed, and used as a template for PCR using primers TMG4784-F and TMG6311-R. The

amplified 1.4-kb product was digested with *Bsp*T104I and cloned into pTGK16 using the corresponding enzyme sites.

pTMG/PC2a and pTMG/PC2b

pTGK16 was used as a template for each PCR. The primer pairs used were TMG4784-F and each of the following: PC2a-R for pTMG/PC2a and PC2b-R for pTMG/PC2b. Another primer, TMG6311-R, was used together with each of the following: PC2a-F for pTMG/PC2a and PC2b-F for pTMG/PC2b. PCR products corresponding to each mutant were mixed, denatured and annealed, and used as a template for PCR using primers TMG4784-F and TMG6311-R. The amplified 1.4-kb products were digested with *Bsp*T104I and cloned into pTGK16 using the corresponding enzyme sites.

pPMMoV-PC2b

Two DNA fragments were amplified by PCR from pTPW using two primer sets: PM+4610 and PMTC2b-R, and PMTC2b-F and PMMoV-3'. The two DNA fragments were mixed, denatured and annealed, and used as a template for PCR using primers PM+4610 and PMMoV-3'. The amplified 1.8-kb product was digested with *Nco*I/*Sac*II and cloned into pTPW using the corresponding enzyme sites.

RNA preparation

RNA transcripts were synthesized *in vitro* from the linearized plasmids by T7 RNA polymerase using a RiboMAX Large Scale RNA Production System (Promega, Madison, WI, USA). pTPW and pPMMoV-PC2b were linearized using *Bam*HI and the other plasmids were linearized using *Sal*I. Purification and validation of the integrity of the transcribed RNA were performed as described by Mizumoto *et al.* (2003). RNA transcripts were named for their parent plasmids with the 'p' prefix removed.

Plant growth, virus inoculation and infection assay

Capsicum annuum L. cvs. Verbeterde Glas (*L*^{1/L}¹), MK-18-3-1 (*L*^{1a/L}^{1a}) and Tosa-midori (*L*^{3/L}³) were grown in pots containing a commercial soil mix (Sumitomo Forestry, Aichi, Japan) in a growth room at 24 °C with 16 h illumination per day. *Nicotiana benthamiana* was grown in pots containing a mixture of vermiculite and peat moss (3:1) as described above. Leaves of 3–4-week-old plants were inoculated mechanically with transcripts (625 ng/μL) as described by Xiong and Lommel (1991). Inoculated plants were grown in plant growth chambers (NK System, Osaka, Japan) at 24 or 30 °C with 16 h illumination per day. Inoculated leaves were collected at 10 days post-inoculation. The extent of virus movement in inoculated leaves was assayed by press blot using antibodies against TMGMV (Sawada *et al.*, 2004), PaMMV-J (Hamada *et al.*, 2003) and PMMoV (Japan Plant Protection Association, Ushiku, Ibaraki, Japan), as described previously (Hamada *et al.*, 2003).

Electron microscopy

Nicotiana benthamiana leaves inoculated with chimeric TMGMV were observed by serologically specific electron microscopy with some modifications (Genda *et al.*, 2007). Leaf samples were homogenated with 10 volumes of phosphate-buffered saline (PBS) (Sambrook *et al.*, 1989).

Carbon-reinforced collodion film grids were coated with antiserum specific to TMGMV-J CP (Sawada *et al.*, 2004) or PaMMV-J CP (Hamada *et al.*, 2003). The prepared grids were floated on homogenates for 60 min at room temperature and then washed with PBS. The grids were negatively stained with 2% phosphotungstic acid (pH 6.5) and observed under a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Symptoms and distribution of coat proteins (CPs) in inoculated leaves of *Capsicum annuum* L. cv. MK-18-3-1 harbouring the L^{1a} gene at 24 and 30 °C. Inoculated leaves were subjected to press blot immunoassay after photography. The accumulation of CPs in leaves inoculated with chimeric *Tobacco mild green mosaic virus* (TMGMV) was analysed using an anti-TMGMV Japanese strain CP antibody.

Fig. S2 Symptoms and distribution of coat proteins (CPs) in inoculated leaves of *Capsicum annuum* L. cv. Tosa-midori harbouring the L^3 gene at 24 °C. Inoculated leaves were subjected to press blot immunoassay after photography, and the viral CP was detected using an anti-*Pepper mild mottle virus* (anti-PMMoV) CP antibody.

Fig. S3 Accumulation of coat protein (CP) in leaf homogenates of *Nicotiana benthamiana* inoculated with *Tobacco mild green mosaic virus* Japanese strain (TMGMV-J), TMG/PC2b, TMG/Pa-PC2b and TMG/PaCP used in Fig. 5. Total proteins were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membrane was treated with the primary antibodies against TMGMV-J and *Paprika mild mottle virus* Japanese strain (PaMMV-J) CPs and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA, USA). Immunoreactive bands were visualized using CDP-Star according to the manufacturer's protocol (Roche, Basle, Switzerland). Coomassie Brilliant Blue (CBB) staining for the loading control is shown.

Table S1 Sequences of the oligonucleotide primers used for site-directed mutagenesis.

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