Functional characterization of the mutations in *Pepper mild mottle virus* overcoming tomato *tm-1*-mediated resistance

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SUMMARY

In tomato plants, *Pepper mild mottle virus* (PMMoV) cannot replicate because the *tm-1* protein inhibits RNA replication. The resistance of tomato plants to PMMoV remains durable both in the field and under laboratory conditions. In this study, we constructed several mutant PMMoVs and analysed their abilities to replicate in tomato protoplasts and plants. We found that two mutants, PMMoV-899R,F976Y and PMMoV-899R,F976Y,D1098N, were able to replicate in tomato protoplasts, but only PMMoV-899R,F976Y,D1098N was able to multiply in tomato plants. Further analysis showed that the D1098N mutation of the replication proteins weakened the inhibitory effect of the *tm-1* protein and enhanced the replication efficiency of PMMoV-899R,F976Y,D1098N. We also observed that the infectivity of the viruses decreased in the order wild-type PMMoV > PMMoV-899R,F976Y > PMMoV-899R,F976Y,D1098N in original host plants, pepper and tobacco plants. On the contrary, the single mutation D1098N abolished PMMoV replication in tobacco protoplasts. On the basis of these observations, it is likely that the deleterious side-effects of mutations in replication proteins prevent the emergence of PMMoV mutants that can overcome *tm-1*-mediated resistance.

Keywords: 130 K protein, nonhost, *Pepper mild mottle virus*, *Tobamovirus*, tomato, *tm-1*.

INTRODUCTION

Successful virus propagation in a plant requires a number of interactions between viral and host factors (Ishibashi *et al.*, 2010; Mine and Okuno, 2012; Nagy and Richardson, 2012). Viruses must counteract the resistance response of the host to establish infection. Resistance (*R*) gene-mediated resistance, often associated

with the hypersensitive response, is triggered by either direct or indirect interactions between the *R* gene-encoded protein and avirulence effectors encoded by the virus (Kang *et al.*, 2005; Moffett, 2009). In addition, nonfunctional alleles of the plant genes encoding essential proteins for viral infection and a plant gene encoding for an inhibitor of viral multiplication also confer resistance to viral infections (Bhat *et al.*, 2013; Ishibashi *et al.*, 2007, 2009; Wang and Krishnaswamy, 2012). In contrast, viruses often overcome resistance through mutations in virus-encoded proteins that expand their host range (Elena *et al.*, 2011; Fraser, 1992).

The genus *Tobamovirus* of the family *Virgaviridae* constitutes a group of plant viruses that form rod-shaped virions of approximately 300 nm in length and contain a 6.4-kb single-stranded, positive-sense RNA genome (Adams *et al.*, 2009). The tobamovirus genomic RNA encodes 130- and 180-kDa replication proteins (130 K and 180 K proteins) that share the same first initiation codon. The 130 K protein contains methyltransferase and helicase domains separated by a nonconserved region (Hirashima and Watanabe, 2001; Nishikiori *et al.*, 2011, 2012). The 180 K read-through protein contains an additional polymerase domain (Buck, 1996). These are multifunctional proteins, involved not only in virus replication (Nishikiori *et al.*, 2006), but also in the suppression of post-transcriptional gene silencing (Csorba *et al.*, 2007; Ishibashi *et al.*, 2011; Kubota *et al.*, 2003) and viral cell-to-cell movement (Hirashima and Watanabe, 2001; Mizumoto *et al.*, 2010; Rabindran *et al.*, 2005). A 30-kDa movement protein (MP) and a 17-kDa coat protein (CP) are produced from 3'-co-terminal subgenomic RNAs that are generated during replication (Deom *et al.*, 1987; Meshi *et al.*, 1987; Takamatsu *et al.*, 1987).

Tomato mosaic virus (ToMV), *Tobacco mild green mosaic virus* (TMGMV), and *Pepper mild mottle virus* (PMMoV) are members of the tobamoviruses that infect solanaceous plants. In solanaceous plants, several monogenic genes, such as the *N* and *N'* genes in tobacco (*Nicotiana tabacum* L.), the allelic *L* genes in pepper (*Capsicum annuum* L.) and the *Tm-1*, *Tm-2* and *tm-1* genes in tomato (*Solanum lycopersicum* L.), that confer resistance to tobamovirus infections, have been identified (Erickson *et al.*, 1999; Ishibashi *et al.*, 2007, 2009; Lanfermeijer *et al.*, 2005; Meshi *et al.*, 1987; Sekine *et al.*, 2012; Tomita *et al.*, 2011; Whitham

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et al., 1994). Several mutant tobamoviruses that overcome the resistance mediated by the monogenic traits have been isolated under field conditions (Genda *et al.*, 2007; Hamada *et al.*, 2007; Strasser and Pfitzner, 2007; Tsuda *et al.*, 1998).

Tm-1 is a semi-dominant trait of tomato and was originally introgressed from the wild tomato species *S. habrochaites*. To date, several *Tm-1*-breaking ToMV mutants, which have amino acid substitutions in the helicase domain of replication proteins, have been obtained (Hamamoto *et al.*, 1997; Ishibashi *et al.*, 2012; Meshi *et al.*, 1988; Strasser and Pfitzner, 2007). *tm-1* is a recessive allele of *Tm-1* and the amino acid sequence of the *tm-1* protein shows 97% identity with that of the *Tm-1* protein (Ishibashi *et al.*, 2012). *tm-1* confers resistance to TMGMV and PMMoV, but not to ToMV. In contrast with the case for *Tm-1* and ToMV, the *tm-1*-mediated resistance in tomato plants against TMGMV and PMMoV has remained unbreakable under field conditions. Under experimental conditions, mutant TMGMV, which can overcome *tm-1*-mediated resistance and is efficiently spread systemically in tomato plants, was obtained from transgenic tobacco plants expressing *tm-1* (Ishibashi *et al.*, 2009). However, mutant PMMoVs that can overcome *tm-1*-mediated resistance have not been obtained, even under experimental conditions.

In this study, we aimed to construct mutant PMMoVs that could overcome *tm-1*-mediated resistance in tomato plants. Based on an amino acid comparison with ToMV and *tm-1*-breaking TMGMV mutants, we constructed several mutant PMMoVs and analysed their abilities to replicate in tomato plants and protoplasts. Although some mutants, including PMMoV-899R,F976Y, were able to replicate in tomato protoplasts, none of these mutants could multiply in tomato plants. Through the analysis of the spontaneous mutant PMMoV in tomato plants, we found that a single

additional amino acid substitution, D1098N, enabled PMMoV-899R,F976Y multiplication in tomato plants. However, the single mutation D1098N abolished PMMoV replication in tobacco protoplasts. In addition, the infectivity of the viruses in the original host plants, pepper and tobacco plants decreased in the order PMMoV-J > PMMoV-899R,F976Y > PMMoV-899R,F976Y with the D1098N mutation. Based on these observations, we provide possible explanations for the high durability of *tm-1*-mediated resistance to PMMoV infection.

RESULTS

Isolation of a PMMoV mutant that can move systemically in tomato plants

To obtain PMMoV mutants that can overcome *tm-1*-mediated resistance in tomato plants, we introduced mutations in the coding regions of the 130 K and 180 K proteins in the PMMoV Japanese strain (PMMoV-J) based on an amino acid sequence comparison of the 130 K proteins among ToMV, TMGMV Japanese strain (TMGMV-J) and PMMoV-J (Fig. 1). The nucleotide sequences of the 130 K protein of TMGMV-J exhibit 65% identity and 91% similarity to those of PMMoV-J. The amino acid sequences of the ToMV 130 K protein exhibit 74% identity and 94% similarity to those of PMMoV-J. TMGMV-T894M,F970Y is a mutant TMGMV, which efficiently spreads systemically in tomato plants (Ishibashi *et al.*, 2009). This mutant TMGMV harbours two amino acid substitutions, threonine to methionine at position 894 and phenylalanine to tyrosine at position 970. Because phenylalanine at position 970 in TMGMV-J corresponded to phenylalanine at 976 in PMMoV-J, we constructed PMMoV-F976Y, in which the

Fig. 1 Partial amino acid sequence alignment of 130-kDa (130 K) proteins of *Tomato mosaic virus* (ToMV), *Tobacco mild green mosaic virus* Japanese strain (TMGMV-J) and *Pepper mild mottle virus* Japanese strain (PMMoV-J). The amino acid sequences of the PMMoV mutants used in this study are also shown; the changed amino acid residues are indicated with a red underline or triangles. Threonine-894 and phenylalanine-970 of the TMGMV 130 K protein and aspartic acid-1097 of the ToMV 130 K protein are indicated by green and blue triangles, respectively.

Fig. 2 Viral RNA accumulation in tomato protoplasts inoculated with *Pepper mild mottle virus* Japanese strain (PMMoV-J) or PMMoV mutants (PMMoV-899R, PMMoV-R1TG, PMMoV-F976Y, PMMoV-899R,F976Y and PMMoV-R1TG,F976Y). Viral genomic RNA (G) and coat protein subgenomic RNA (CPsg) were detected by Northern blot hybridization analysis. Ethidium bromide (EtBr) staining of the ribosomal RNA is shown as a loading control. The assays were performed at least three times and representative accumulation patterns of viral RNAs are shown.

phenylalanine at position 976 was substituted with tyrosine (Fig. 1). In contrast, the amino acid sequences around the threonine at position 894 in TMGMV-J are not conserved between TMGMV-J and PMMoV-J (Fig. 1). Thus, we constructed a mutant PMMoV, named PMMoV-R1TG, in which six amino acid residues (893–898) in PMMoV-J were substituted with the corresponding residues found in TMGMV-T894M,F970Y (Fig. 1). On the other hand, the amino acid sequences around the threonine at position 894 in TMGMV-J are relatively conserved between PMMoV-J and ToMV, except for an arginine at position 897 in ToMV (Fig. 1). Because ToMV replicates efficiently in tomato plants (genotype *tm-1/tm-1*), we constructed another mutant, named PMMoV-899R, in which an arginine was inserted between the proline at position 898 and the cysteine at position 899 in the PMMoV-J 130 K and 180 K proteins (Fig. 1). In addition, two mutants, named PMMoV-R1TG,F976Y and PMMoV-899R,F976Y, which contained double mutations, were constructed.

We prepared protoplasts from a suspension-cultured cell line derived from *S. lycopersicum* cv. GCR26 (genotype *tm-1/tm-1*) and inoculated them with RNA transcripts of PMMoV-J and the five mutant PMMoVs (PMMoV-899R, PMMoV-R1TG, PMMoV-F976Y, PMMoV-R1TG,F976Y and PMMoV-899R,F976Y). Protoplasts were incubated for 20 h and viral RNA accumulation was analysed by Northern blot hybridization. PMMoV-F976Y, PMMoV-R1TG,F976Y and PMMoV-899R,F976Y accumulated viral RNAs, whereas PMMoV-899R, PMMoV-R1TG and PMMoV-J did not (Fig. 2). These results indicate that the F976Y mutation enables PMMoV-J to replicate in tomato protoplasts.

We then examined the infectivity of PMMoV-F976Y, PMMoV-R1TG,F976Y and PMMoV-899R,F976Y in tomato plants

(*S. lycopersicum* cv. Ohgata-Fukuju, genotype *tm-1/tm-1*). Tomato plants were inoculated with RNA transcripts and incubated for 14 days. Inoculated leaves and uninoculated upper leaves were homogenized, and the accumulation of PMMoV CP was assessed by Western blotting. CP accumulation was not observed, even in the inoculated leaves, in any of the plants inoculated with PMMoV-F976Y and PMMoV-R1TG,F976Y. Only one of the 10 tomato plants inoculated with PMMoV-899R,F976Y showed CP accumulation in the inoculated leaves (data not shown). It is possible that a spontaneous mutant PMMoV, which can replicate in tomato plants, has emerged in the tomato plant inoculated with PMMoV-899R,F976Y.

To assess this possibility, the virus from the tomato homogenate was propagated in pepper plants (*C. annuum* cv. MK-18-2-1), and the viral RNA extracted from the pepper plants was inoculated into eight tomato plants. Interestingly, CP accumulated in all the inoculated leaves and in the uninoculated upper leaves of two of eight plants at 14 days post-inoculation (dpi) (Fig. S1, see Supporting Information). Nucleotide sequence analysis of entire 130 K and 180 K protein coding regions confirmed the 899R and F976Y mutations in the viral RNA extracted from the pepper plants. In addition, a single nucleotide substitution at nucleotide 3361 (G→A), causing an amino acid substitution from aspartic acid to asparagine at position 1098, was found (Fig. 1). No other mutations were observed in the coding regions of the 130 K and 180 K proteins.

To assess the importance of the D1098N mutation, PMMoV-899R,F976Y,D1098N was generated by introducing the G3361A mutation in PMMoV-899R,F976Y. Four tomato plants were inoculated mechanically with *in vitro* transcribed RNA, and CP accumulation in their leaves was examined at 14 dpi. CP did not accumulate in plants inoculated with PMMoV-J and PMMoV-899R,F976Y (Fig. 3 and data not shown). Conversely, the CP of PMMoV-899R,F976Y,D1098N accumulated in the inoculated leaves of all plants and the uninoculated upper leaves of one of the inoculated plants (Fig. 3). It should be noted that PMMoV-899R,F976Y,D1098N had a low ability to move systemically in tomato plants, as did the mutant virus obtained from pepper plants (Fig. 3 and Fig. S1), and tomato plants systemically infected with PMMoV-899R,F976Y,D1098N did not show any symptoms (data not shown). These observations suggest that the spontaneous D1098N mutation, when fixed in tomato or pepper plants, enables PMMoV-899R,F976Y to move systemically.

Effect of the D1098N mutation in PMMoV-J on *tm-1*-mediated resistance

To examine the effect of the D1098N mutation on PMMoV RNA replication, we compared the replication activity of PMMoV-899R,F976Y,D1098N with that of PMMoV-899R,F976Y in tomato protoplasts. Tomato protoplasts were inoculated with *in vitro* tran-

Fig. 3 Coat protein (CP) accumulation in leaves of tomato plants inoculated with *Pepper mild mottle virus* Japanese strain (PMMoV-J) or PMMoV-899R,F976Y,D1098N. At 14 days post-inoculation, total proteins were extracted from whole leaves and CP was detected by Western blotting. Coomassie Brilliant Blue (CBB) staining is shown as a loading control.

scribed RNA, and viral RNA accumulation in protoplasts at 0, 6, 12 and 20 h post-inoculation (hpi) was analysed by Northern blot hybridization. At 12 hpi, the accumulation of PMMoV-899R,F976Y,D1098N genomic RNA was significantly higher than that of PMMoV-899R,F976Y (Fig. 4). However, the accumulation of the genomic RNA of both mutant viruses eventually reached similar levels at 20 hpi (Fig. 4). These observations indicate that the D1098N mutation enhances the replication efficiency of PMMoV-899R,F976Y in tomato cells.

We next examined the interaction of the mutant 130 K protein with the tm-1 protein by co-immunoprecipitation. The myc-tagged 130 K (130K-myc) protein of PMMoV-J co-precipitated with FLAG-tagged tm-1 (tm-1-FLAG). Conversely, only trace amounts of 130K-myc bearing the 899R,F976Y or 899R,F976Y,D1098N mutation co-precipitated with tm-1-FLAG (Fig. 5A). No notable difference was observed in the amount of co-precipitated 130 K protein between PMMoV-899R,F976Y and PMMoV-899R,F976Y,D1098N (Fig. 5A). These observations indicate that the 899R and F976Y mutations in the PMMoV 130 K protein decrease the affinity for the tm-1 protein.

To determine the role of the D1098N mutation in escaping from the inhibitory effect of tm-1, we examined the replication activity of PMMoV-899R,F976Y and PMMoV-899R,F976Y,D1098N in transgenic tobacco BY-2 protoplasts, which express *tm-1* under the control of the strong constitutive promoter, cauliflower mosaic virus 35S (Ishibashi *et al.*, 2012). The inoculation of nontransgenic

Fig. 4 Time course of viral RNA accumulation in tomato protoplasts inoculated with the *Pepper mild mottle virus* (PMMoV) mutants PMMoV-899R,F976Y and PMMoV-899R,F976Y,D1098N. Total RNA was extracted from protoplasts at 0 h (immediately after inoculation) and at 6, 12 and 20 h post-inoculation (hpi). The relative accumulation level of viral genomic RNA (the level of PMMoV-899R,F976Y,D1098N genomic RNA at 20 hpi was set to 100%) was calculated from four independent experiments. Error bars represent the standard deviation. Representative accumulation patterns of viral genomic RNA (G) and coat protein subgenomic RNA (CPsg) are shown at the bottom. Ethidium bromide (EtBr) staining of the ribosomal RNA is shown as a loading control.

BY-2 protoplasts with PMMoV-J, PMMoV-899R,F976Y or PMMoV-899R,F976Y,D1098N resulted in similar viral RNA levels at 20 hpi (Fig. 5B). However, in the transgenic BY-2 protoplasts, viral RNA accumulation was observed only in PMMoV-899R,F976Y,D1098N-inoculated protoplasts at 20 hpi (Fig. 5B). These results suggest that PMMoV-899R,F976Y is more sensitive than PMMoV-899R,F976Y,D1098N to the inhibitory effect of the tm-1 protein. It should be noted that the D1098N mutation site coincides with the mutation found in the ToMV mutants ToMV1-2 (D1097V) and LT1^{D1097Y} (D1097Y), which overcome *Tm-1*-mediated resistance (Fig. 1) (Ishibashi *et al.*, 2012; Strasser and Pfitzner, 2007).

Infectivity of mutant PMMoVs in pepper and tobacco plants

We examined the infectivity of PMMoV-J, PMMoV-899R,F976Y and PMMoV-899R,F976Y,D1098N in pepper and tobacco plants,

Fig. 5 (A) Interaction between the 130-kDa (130 K) protein and *tm-1* *in vitro*. mRNAs coding for 130K-myc and *tm-1*-FLAG were translated in a membrane-depleted evacuated BY-2 protoplast lysate (mdBYL). The 130K-myc translation product was mixed with mock (-) or *tm-1*-FLAG translation products and immunoprecipitated using anti-FLAG antibody-conjugated agarose beads. Immunoprecipitates were analysed by Western blotting. (B) Viral RNA accumulation in nontransgenic tobacco BY-2 protoplasts (BY2) and *tm-1*-expressing tobacco BY-2 protoplasts (*tm-1*) inoculated with *Pepper mild mottle virus* Japanese strain (PMMoV-J), PMMoV-899R,F976Y or PMMoV-899R,F976Y,D1098N. The assays were performed at least three times and representative accumulation patterns of viral genomic RNA (G) and coat protein subgenomic RNA (CPsg) are shown. Ethidium bromide (EtBr) staining of the ribosomal RNA is shown as a loading control.

the original host plants of PMMoV-J. Inoculated plants were incubated for 7 days, and the accumulation levels of CP in inoculated and uninoculated upper leaves were analysed by Western blotting. In inoculated leaves of tobacco plants (*N. tabacum* cv. Samsun), which do not have any known tobamovirus resistance genes, PMMoV-899R,F976Y showed significantly lower CP accumulation levels than PMMoV-J ($P < 0.05$) (Fig. 6A). Interestingly, CP accumulation further decreased significantly in plants inoculated with PMMoV-899R,F976Y,D1098N ($P < 0.05$) (Fig. 6A). In uninoculated

Fig. 6 Accumulation of coat protein (CP) in inoculated tobacco plants (A) and pepper plants (B). Plants inoculated with *Pepper mild mottle virus* Japanese strain (PMMoV-J), PMMoV-899R,F976Y or PMMoV-899R,F976Y,D1098N were incubated for 7 days. Total protein extraction followed by Western blotting analysis showed CP accumulation. The relative accumulation level of CP (the level of PMMoV-J CP was set to 100%) was calculated from three independent experiments. The averages plus standard deviations (error bars) are shown on the graph. The data were analysed by Student's *t*-test and *P* values are indicated. Coomassie Brilliant Blue (CBB) staining is shown as a loading control.

upper leaves of tobacco plants, although a statistically significant difference was not observed, accumulation levels of CP tended to decrease in the order of wild-type PMMoV-J > PMMoV-899R,F976Y > PMMoV-899R,F976Y,D1098N (Fig. 6A). Similar results were observed in pepper plants (*C. annuum* cv. syosuke), which do not have any tobamovirus resistance genes (Fig. 6B). It should be noted that apparent symptoms were not observed in inoculated pepper and tobacco plants at 7 dpi. These observations indicate that the adaptation of PMMoV to tomato plants results in a decrease in its ability to infect the original host plants.

Effects of the D1098N mutation in PMMoV-J replication

To analyse the replication activity of mutant PMMoVs in tobacco protoplasts, we prepared protoplasts from a suspension-cultured cell line derived from nontransgenic tobacco BY-2 and inoculated them with RNA transcripts of PMMoV-J and PMMoV mutants having single (PMMoV-899R, PMMoV-F976Y and PMMoV-D1098N), double (PMMoV-899R,F976Y, PMMoV-899R,D1098N and PMMoV-F976Y,D1098N) and triple (PMMoV-899R,F976Y,D1098N) mutations. The accumulation of viral RNAs was observed when inoculated with PMMoV-J or any of the PMMoV mutants, except for PMMoV-D1098N, which has the D1098N mutation alone, at 20 hpi (Fig. 7A). These observations indicate that the single mutation of D1098N abolishes the ability of PMMoV to replicate in tobacco protoplasts. In addition, viral RNAs accumulated in tobacco protoplasts inoculated with PMMoV-899R, D1098N and PMMoV-F976Y,D1098N (Fig. 7A), indicating that the 899R and F976Y mutations compensate for the deleterious effect of D1098N on PMMoV replication. Interestingly, the accumulation of viral RNAs was not observed in tomato protoplasts inoculated with PMMoV-899R,D1098N or in those inoculated with PMMoV-D1098N, PMMoV-899R and PMMoV-J (Fig. 7B). Based on these observations, it is possible that the effects of the D1098N mutation on PMMoV replication in tomato protoplasts may functionally differ from those of the F976Y mutation.

DISCUSSION

Owing to the lack of proofreading activity in viral polymerase, mutation rates of RNA viruses are generally very high (Drake and Holland, 1999; Elena and Sanjuán, 2005; Sanjuán, 2012). Mutability is one of the key driving forces of viral evolution. Another aspect of mutability is its negative effect on viral fitness, which may drive a viral population to extinction (Bull *et al.*, 2007; Graci *et al.*, 2012). In addition, mutations that are beneficial in one host might reduce fitness to the original hosts (Elena *et al.*, 2009; Fraser, 1992).

In this study, we have shown that PMMoV-899R,F976Y gains the ability to replicate in tomato protoplasts (Fig. 2), but, at the same time, exhibits reduced infectivity in pepper and tobacco

Fig. 7 Viral RNA accumulation in tobacco BY-2 protoplasts (A) and tomato protoplasts (B) inoculated with *Pepper mild mottle virus* (PMMoV) mutants having single (PMMoV-899R, PMMoV-F976Y and PMMoV-D1098N), double (PMMoV-899R,F976Y, PMMoV-899R,D1098N and PMMoV-F976Y,D1098N) and triple (PMMoV-899R,F976Y,D1098N) mutations. The assays were performed at least three times and representative accumulation patterns of viral genomic RNA (G) and coat protein subgenomic RNA (CPsg) are shown. Ethidium bromide (EtBr) staining of the ribosomal RNA is shown as a loading control.

plants (Fig. 6). We have also shown that an additional D1098N mutation enables PMMoV-899R,F976Y to systemically spread in tomato plants (Fig. 3). However, this D1098N mutation further reduces the fitness of the virus in pepper and tobacco plants (Fig. 6). In addition, the single mutation D1098N abolishes PMMoV replication in tobacco protoplasts (Fig. 7). We have reported previously that two amino acid substitutions (T894M and F970Y) in the 130 K and 180 K proteins of TMGMV-J result in a reduced affinity to the tm-1 protein, thereby enabling TMGMV-J to propagate in tomato plants (Ishibashi *et al.*, 2009). However, these two mutations impair the virulence of TMGMV-J in *N. benthamiana* plants (Ishibashi *et al.*, 2011). Therefore, these observations indicate that amino acid changes in the 130 K and 180 K proteins, responsible for the escape from the inhibitory effect of the tm-1 protein, have negative effects on the infectivity of PMMoV-J and TMGMV-J.

It has been reported that some mutations enabling viruses to overcome resistance show deleterious side-effects (Carrasco

et al., 2007; Jenner *et al.*, 2002). *L* gene-mediated resistance against TMGMV-J in pepper plants has remained durable under field conditions. In a previous study, mutant TMGMV that can break *L*^{1a}-mediated resistance was obtained by introducing mutations in the CP, but this mutant TMGMV lost the ability to form virions (Mizumoto *et al.*, 2012). The pepper *Pvr4* gene confers durable resistance to *Potyvirus* spp. under field conditions. A single amino acid substitution in the NIb protein (RNA-dependent RNA polymerase) of *Potato virus Y* enables the virus to move systemically and to induce symptoms in plants having the *Pvr4* gene (Janzac *et al.*, 2010). However, this mutant virus shows decreased competitiveness against the parental virus in susceptible pepper plants (Janzac *et al.*, 2010). This growing evidence, including ours, indicates that these negative side-effects may restrict the virus in its ability to overcome host resistance, probably because the small genomic RNAs contain overlapping genes and code for multifunctional proteins.

We observed that both PMMoV-899R,F976Y and PMMoV-899R,F976Y,D1098N are able to replicate in tomato protoplasts (Fig. 4), but only PMMoV-899R,F976Y,D1098N is able to spread systemically in tomato plants (Fig. 3 and data not shown). Further analyses showed that the D1098N mutation weakens the inhibitory effect of the *tm-1* protein on PMMoV-899R,F976Y and enhances the replication efficiency of the mutant virus (Figs 4 and 5). Interestingly, the accumulation of PMMoV-899R,F976Y,D1098N genomic RNA was significantly higher than that of PMMoV-899R,F976Y at 12 hpi (Fig. 4). These observations indicate that the accumulation levels of viral RNA strongly influence the subsequent viral events, including cell-to-cell movement, which probably accounts for the inability of PMMoV-899R,F976Y to propagate in tomato plants.

We also observed that PMMoV-899R,F976Y,D1098N, which escapes the inhibitory effect of the *tm-1* protein and gains the ability to multiply in tomato plants, has a low ability to move systemically in tomato plants (Fig. 3). Interestingly, our preliminary study showed that a chimeric PMMoV, with its 130 K and 180 K protein genes replaced with those of ToMV, can efficiently move systemically in tomato plants (genotype *tm-1/tm-1*) at a similar rate to ToMV (data not shown). It is possible that unknown host protein(s), other than the *tm-1* protein, may interact with the 130 K and 180 K proteins and restrict the emergence of mutant PMMoVs that can infect tomato plants. Alternatively, the replication of PMMoV-899R,F976Y,D1098N may still be restricted by the *tm-1* protein to some extent, which affects the efficient systemic movement of the virus. Therefore, additional mutation(s) to further reduce the affinity for the *tm-1* protein are required for a systemic infection. However, such mutation(s) may cause more deleterious effects on the infectivity of PMMoV.

Resistance mediated by *tm-1* against TMGMV-J and PMMoV-J in tomato plants has remained unbroken under field conditions. Under experimental conditions, mutant TMGMV, which can over-

come *tm-1*-mediated resistance and can efficiently spread systemically in tomato plants, is obtained from transgenic tobacco plants expressing *tm-1* (Ishibashi *et al.*, 2009). However, spontaneous mutants of PMMoV-J, which can overcome *tm-1*-mediated resistance, have not been obtained under the same experimental conditions (Ishibashi *et al.*, 2009). On the basis of our observations, it can be speculated that the mutations enabling the virus to escape the inhibitory effects of the *tm-1* protein cause more deleterious side-effects in PMMoV-J than in TMGMV-J, thus hampering the emergence of such viruses.

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 pTPW (Tsuda *et al.*, 1998), from which the infectious viral RNA of PMMoV-J was transcribed *in vitro*. Polymerase chain reaction (PCR) products were verified by sequencing.

pPMMoV-R1TG, pPMMoV-899R, pPMMoV-F976Y and pPMMoV-D1098N

pTPW was used as a template for each PCR. The primer pairs used were PM+2368 and each of the following: PR1TG/R for pPMMoV-R1TG, PM-899R/R for pPMMoV-899R, PM-F976Y/R for pPMMoV-F976Y and PM-G3361A-R for pPMMoV-D1098N. Another primer, PM-3517, was used with each of the following: PR1TG/F for pPMMoV-R1TG, PM-899R/F for pPMMoV-899R, PM-F976Y/F for pPMMoV-F976Y and PM-G3361A-F for pPMMoV-D1098N. The PCR product corresponding to each mutant was mixed, denatured, annealed and used as a template for PCR employing primers PM+2368 and PM-3517. The amplified 1.1-kb products were digested with *SacI*/ *SalI* and cloned into pTPW using the corresponding restriction enzyme sites.

pPMMoV-R1TG,F976Y, PMMoV-899R,F976Y and pPMMoV-F976Y,D1098N

pPMMoV-F976Y was used as a template for each PCR. The primer pairs used were PM+2368 and each of the following: PR1TG/R for pPMMoV-R1TG,F976Y, PM-899R/R for pPMMoV-899R,F976Y and PM-G3361A-R for pPMMoV-F976Y,D1098N. Another primer, PM-3517, was used with each of the following: PR1TG/F for pPMMoV-R1TG,F976Y, PM-899R/F for pPMMoV-899R,F976Y and PM-G3361A-F for pPMMoV-F976Y,D1098N. The construction steps are described above.

PMMoV-899R,D1098N

Two DNA fragments were amplified by PCR from pPMMoV-899R using the two primer sets, PM+2368 and PM-G3361A-R, and PM-G3361A-F and PM-3517. The construction steps are described above.

PMMoV-899R,F976Y,D1098N

Two DNA fragments were amplified by PCR from pPMMoV-899R,F976Y using the two primer sets, PM+2368 and PM-G3361A-R, and

PM-G3361A-F and PM-3517. The construction steps are described above.

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 its derivatives were linearized using *Bam*HI. The purification and validation of the integrity of the transcribed RNA were performed as described previously (Mizumoto *et al.*, 2003). RNA transcripts were named for their parent plasmids with the 'p' prefix removed.

Viral inoculation of plants and protoplasts

Solanum lycopersicum L. cv. Ohgata-Fukuju (*tm-1/tm-1*), *C. annuum* L. cv. Shosuke (*L⁺/L⁺*) and *C. annuum* L. cv. MK-18-3-1 (*L^{1a}/L^{1a}*) were grown in pots containing a commercial soil mix (Sumitomo Forestry, Aichi, Japan). *Nicotiana tabacum* L. cv. Samsun was grown in pots containing a mixture of vermiculite and peat moss (3:1). Plants were kept in a growth room at 24 °C with 16 h of illumination. Leaves were inoculated mechanically with transcripts (625 ng/μL), as described previously (Xiong and Lommel, 1991). Inoculated plants were kept in plant growth chambers (NK System, Osaka, Japan) at 24 °C with 16 h of illumination. The maintenance of tomato GCR26 and tobacco BY-2 suspension cell cultures, preparation of protoplasts and inoculation of RNA transcripts were performed as described previously (Watanabe *et al.*, 1987). Transgenic BY-2 cells that express the *tm-1* protein have been described previously (Ishibashi *et al.*, 2012).

Northern blot hybridization and Western blotting analysis

To analyse viral RNA accumulation, 250 ng of total RNA extracted from protoplasts were subjected to Northern blot hybridization analysis as described previously (Damayanti *et al.*, 2002). A digoxigenin-labelled RNA probe corresponding to the 3' untranslated region (6182–6357) of PMMoV-J RNA and alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Basel, Switzerland) were used to detect signals for viral genomic RNA (G) and CP subgenomic RNA (CPsg).

To analyse CP accumulation, total proteins of leaves and protoplasts were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore, Billerica, MA, USA). The membrane was treated with a rabbit polyclonal anti-PMMoV-J antibody (Japan Plant Protection Association, Tokyo, Japan), followed by an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA, USA).

In Northern hybridization and Western blotting analysis, immunoreactive bands were visualized with CDP-Star according to the manufacturer's protocol (Roche). Chemiluminescence signals were detected using a LAS-3000 mini image analysis system (Fujifilm Life Science, Tokyo, Japan). Exposure times were adjusted to ensure that the signal intensity did not reach saturation.

Immunoprecipitation

Templates of the PMMoV 130K-myc fusion proteins were prepared by PCR using PMMoV-J, PMMoV-899R/F976Y or PMMoV-899R/F976Y/D1091N as

the template and T7-130K and 130K-myc primers. Messenger RNA encoding the *tm-1*-FLAG fusion protein was prepared as described previously (Ishibashi *et al.*, 2007). 130K-myc RNA (1.5 μg) was translated by addition to 13 μL of a membrane-depleted evacuated BY-2 protoplast lysate (mdBYL)-based mixture (Ishibashi *et al.*, 2007) and incubation for 1 h at 25 °C. The reaction mix was added to an mdBYL *tm-1*-FLAG translation mixture (3 μg RNA in a 15-μL mixture), and immunoprecipitation was performed using the anti-FLAG antibody, as described previously (Ishibashi *et al.*, 2009). The immunoprecipitates were analysed by Western blotting using an anti-myc antibody (MBL, Nagoya, Japan) and an anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Coat protein (CP) accumulation in leaves of tomato plants inoculated with virion RNA extracted from pepper plants. At 14 days post-inoculation, total proteins were extracted from the whole leaves and CP was detected by Western blotting. Coomassie Brilliant Blue (CBB) staining is shown as a loading control.

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