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ORIGINAL ARTICLE

Activation of *Tm-2²* resistance is mediated by a conserved cysteine essential for tobacco mosaic virus movement

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Abstract

The tomato *Tm-2²* gene was considered to be one of the most durable resistance genes in agriculture, protecting against viruses of the *Tobamovirus* genus, such as tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV). However, an emerging tobamovirus, tomato brown rugose fruit virus (ToBRFV), has overcome *Tm-2²*, damaging tomato production worldwide. *Tm-2²* encodes a nucleotide-binding leucine-rich repeat (NLR) class immune receptor that recognizes its effector, the tobamovirus movement protein (MP). Previously, we found that ToBRFV MP (MP^ToBRFV) enabled the virus to overcome *Tm-2²*-mediated resistance. Yet, it was unknown how *Tm-2²* remained durable against other tobamoviruses, such as TMV and ToMV, for over 60 years. Here, we show that a conserved cysteine (C68) in the MP of TMV (MP^TMV) plays a dual role in *Tm-2²* activation and viral movement. Substitution of MP^ToBRFV amino acid H67 with the corresponding amino acid in MP^TMV (C68) activated *Tm-2²*-mediated resistance. However, replacement of C68 in TMV and ToMV disabled the infectivity of both viruses. Phylogenetic and structural prediction analysis revealed that C68 is conserved among all Solanaceae-infecting tobamoviruses except ToBRFV and localizes to a predicted jelly-roll fold common to various MPs. Cell-to-cell and subcellular movement analysis showed that C68 is required for the movement of TMV by regulating the MP interaction with the endoplasmic reticulum and targeting it to plasmodesmata. The dual role of C68 in viral movement and *Tm-2²* immune activation could explain how TMV was unable to overcome this resistance for such a long period.

KEYWORDS
movement protein, plasmodesmata, *Tm-2²*, TMV, *Tobamovirus*, ToBRFV

1 | INTRODUCTION

Plant viruses cause diseases that severely restrict crop production. Some of the most harmful plant viruses are members of the *Tobamovirus* genus, such as tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV). Viral symptoms include severe plant yellowing and stunting, and reductions in yield and fruit quality, and these viruses are highly transmissible by mechanical contact (Broadbent, 1976). The tobamovirus genome consists of a single-stranded, positive-sense RNA of about 6.4 kb that encodes two subunits of an RNA-dependent RNA polymerase (RdRp), a movement protein (MP) and a coat protein (CP) (Goelet et al., 1982). Successful infection depends on the MP, which

enables the virus to move from cell to cell (Navarro et al., 2019). MP binds to the viral RNA, targets it to plasmodesmata (PD), membrane-lined channels interconnecting adjacent cells, and increases the PD size exclusion limit in a process termed gating (Reagan & Burch-Smith, 2020). These processes depend on various MP attributes, including its specific subcellular targeting, interaction with cellular factors (e.g., endoplasmic reticulum [ER] and microtubules), and the binding of multiple host proteins (Pitzalis et al., 2018).

Dominant resistance (R) genes in plants are pivotal factors for the control of plant pathogens in agriculture. Most R genes encode the nucleotide-binding leucine-rich repeat (NLR) class of immune receptors that confer resistance by recognizing specific pathogen-derived molecules named effectors (Adachi et al., 2019; Jones & Dangl, 2006). On effector recognition, NLRs oligomerize into a structure named the resistosome to form a calcium-permeable plasma membrane channel (Bi et al., 2021; Martin et al., 2020; Wang et al., 2019). The formed calcium influx then induces programmed cell death of the infected plant tissue that limits pathogen infection in a process termed the hypersensitive response (HR) (Meier et al., 2019). The tomato Tm-2^2 gene encodes an NLR that confers protection against viruses of the $Tobamovirus$ genus, including TMV and ToMV. Tm-2^2 recognizes and associates with the viral MP, the effector, to trigger the resistance response against tobamoviruses (Chen et al., 2017; Weber et al., 1993). This recognition results in the self-association of Tm-2^2, which activates HR-mediated immunity against the virus (Wang et al., 2020).

Until recently, Tm-2^2 was considered one of the most durable R genes in agriculture (Hall, 1980). However, a newly emerged to-bamovirus named tomato brown rugose fruit virus (ToBRFV) has overcome all tobamovirus resistance genes, including Tm-2^2, and caused substantial damage to the global tomato industry (Luria et al., 2017; Zhang et al., 2022). Recently, we found that the MP of ToBRFV (MP^{ToBRFV}) is the viral factor that enables ToBRFV to overcome Tm-2^2 resistance (Hak & Spiegelman, 2021). Expression of MP^{ToBRFV} failed to trigger Tm-2^2-mediated HR and substitution of the original MP sequence of the nonresistance-breaking virus ToMV with MP^{ToBRFV} enabled the recombinant virus to overcome Tm-2^2 resistance in tomato. Another recent study mapped the six amino acids in MP^{ToBRFV} (H67, N125, K129, A134, I147, and I168) required for ToBRFV to overcome Tm-2^2, pinpointing the precise sites that enable evasion of Tm-2^2-mediated resistance (Yan et al., 2021).

Because the MP is crucial for intercellular movement, we hypothesized that mutations that enable the MP to overcome Tm-2^2-mediated resistance may also affect cell-to-cell movement of the virus. Our recent findings indicated that MP^{ToBRFV} cell-to-cell movement was reduced compared to MP of TMV (MP^{TMV}) (Hak & Spiegelman, 2021). The potential trade-off between Tm-2^2 immune evasion and viral movement could explain the high durability of Tm-2^2 against TMV and ToMV. However, whether such a trade-off exists and the potential mechanism underlying it are currently unknown. Here, we show that the presence of a cysteine residue (C68) conserved among Solanaceae-infecting tobamovirus movement proteins activates Tm-2^2 resistance. Our results show that the C68 is required for TMV and ToMV movement by

directing MP movement on the ER and subsequent localization to PD. Interestingly, in MP^{ToBRFV}, the corresponding position is occupied by histidine (H67), but intercellular movement is sustained. The lack of tolerance to mutations in C68 can therefore explain how TMV and ToMV were unable to overcome Tm-2^2 for over 60 years.

2 | RESULTS

2.1 | The presence of C68 in the MP triggers Tm-2^2 resistance

Transient co-expression of MP^{TMV} with Tm-2^2 in $Nicotiana$ $benthamiana$ leaves results in the appearance of HR-mediated cell death due to Tm-2^2 immune activation (Figure 1a,b; Hak & Spiegelman, 2021). In contrast, co-expression of MP^{ToBRFV} with Tm-2^2 resulted in only minor cell death (Figures 1a,b and S1). To determine regions of the MP^{TMV} that activate Tm-2^2, two hybrid MP^{TMV}/MP^{ToBRFV} clones were generated. Fusion of MP^{TMV} amino acids 1 to 50 to MP^{ToBRFV} amino acids 51 to 266 (MP$^{ToBRFV\ 1–50}$/MP$^{TMV\ 217–266}$) failed to activate Tm-2^2 (Figure S1). However, MP^{TMV} amino acids 1 to 110 fused to MP^{ToBRFV} amino acids 110 to 266 (MP$^{TMV\ 1–110}$/MP$^{ToBRFV\ 111–266}$) triggered the Tm-2^2 response (Figure S1). These results suggested that elements within MP^{TMV} amino acids 51–110 are required for the activation of Tm-2^2. Among the MP^{ToBRFV} amino acids required for overcoming Tm-2^2-mediated resistance (Yan et al., 2021), histidine 67 (H67) is the only one that localizes to this region. To test if H67 and the corresponding amino acid in MP^{TMV}, cysteine 68 (C68), are determinants of the Tm-2^2 immune response, we used site-directed mutagenesis to introduce reciprocal mutations in MP^{TMV} and MP^{ToBRFV}, thereby changing both amino acids to their identity in the corresponding virus. Indeed, the replacement of MP^{ToBRFV} H67 with cysteine (MP$^{ToBRFV\ H67C}$) triggered strong HR cell death when co-expressed with Tm-2^2 (Figure 1a,b). When H67 was replaced with another amino acid, serine (MP$^{ToBRFV\ H67S}$), it failed to trigger Tm-2^2-mediated HR cell death, suggesting a specific role for cysteine in Tm-2^2 activation (Figure 1a,b). On the other hand, replacement of MP^{TMV} C68 with histidine (MP$^{TMV\ C68H}$) still triggered HR when co-expressed with Tm-2^2 (Figure 1a,b), indicating that MP^{TMV} elements other than C68 are also capable of activating the Tm-2^2 response.

2.2 | MP^{TMV} C68 is conserved in sequence and structure

C68 localizes to a region essential for protein folding (Citovsky et al., 1992) and for association with the ER (Peiro et al., 2014), suggesting its putative function in cell-to-cell movement. To determine the conservation of C68, protein sequence alignment of Solanaceae-infecting tobamoviruses was performed (Figures 2a and S2). This analysis revealed that C68 is highly conserved, while H67 is unique to MP^{ToBRFV}, suggesting a role for C68 among Solanaceae-infecting tobamoviruses.
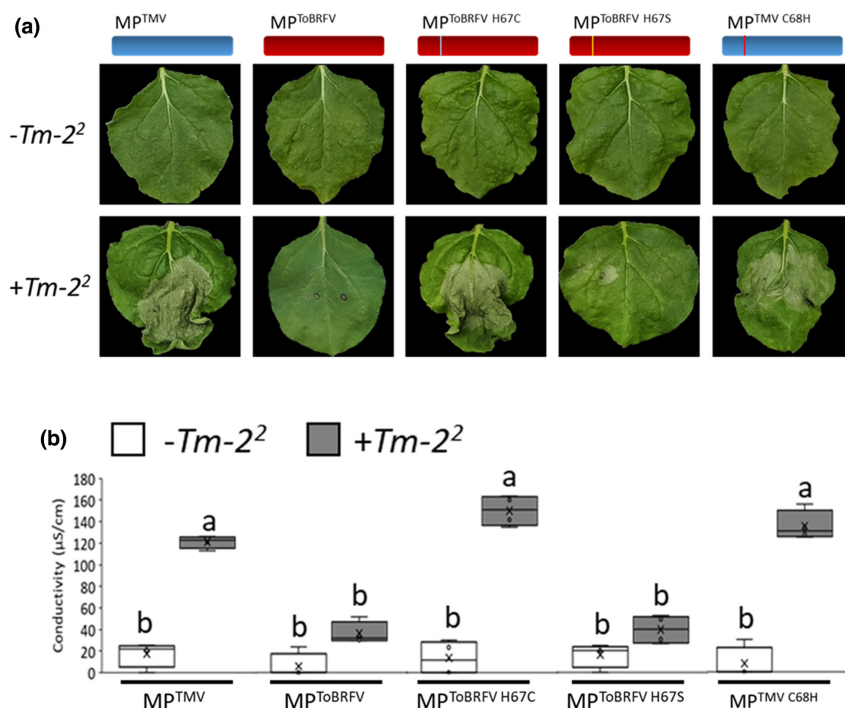
(a)

To determine the importance of H67/C68, we performed three-dimensional (3D) protein structural analysis of MP^TMV and MP^ToBRFV. As there are no existing 3D structures for any tobamovirus MPs, we used the AlphaFold2 modelling platform (Jumper et al., 2021). These analyses provided high-confidence predictions for both proteins, including at the H67/C68 position (Figures 2b,c and S3). Based on this analysis, C68/H67 localized to a jelly-roll fold, typical of various viral proteins (Krupovic et al., 2022), which contains two previously identified membrane interactions motifs (Figure 2b; Peiro et al., 2014). To explore the conservation of this fold, the structure of four other members of the 30K MP superfamily was modelled using AlphaFold2: MP of cauliflower mosaic virus (MP^CaMV), MP of cucumber mosaic virus (MP^CMV), MP of tomato bushy stunt virus (MP^TBSV), and MP of tomato spotted wilt virus (MP^TSWV) (Figure 2d). Interestingly, this analysis revealed a putative jelly-roll fold conserved among all these analysed MPs. These results suggest that C68 is localized in an MP region conserved in sequence, structure, and function, and possibly plays a dual role in both activating the *Tm-2²* immune response and cell-to-cell movement.

2.3 | C68 is essential for TMV and ToMV infection

The conservation of C68 suggests a role for this amino acid in viral infection. To test the function of C68, the C68H mutation was introduced into a ToMV infectious clone (ToMV^MP(C68H)-ToMV) (Hamamoto et al., 1993). Tomato plants homozygous for the ToMV-sensitive *tm-2* allele or the *Tm-2²* resistance allele were then inoculated with the mutant viral RNA (Figure 3a). As controls, plants were also inoculated with nonmutant ToMV and a hybrid version of ToMV harbouring MP^ToBRFV (ToMV^MP-ToBRFV) replacing its original MP (Hak & Spiegelman, 2021). Four plants from each treatment were tested 3weeks after inoculation for appearance of visual symptoms and presence of viral proteins

by immunoblot analysis. As expected, ToMV successfully infected *tm-2* plants, but not *Tm-2²* plants (Figure 3a,b). Consistent with our previous report (Hak & Spiegelman, 2021), the ToMV^MP-ToBRFV also infected both *tm-2* and *Tm-2²* plants (Figure 3a,b). However, ToMV^MP(C68H)-ToMV could not infect both genotypes (Figure 3a,b).

To explore whether the function of C68 is conserved across tobamoviruses and host species, we generated TMV-GFP recombinant virus clones (Lindbo, 2007) containing the MP^ToBRFV (TMV-GFP^MP-ToBRFV) or MP (C68H) mutant (TMV-GFP^MP(C68H)-TMV) in place of the original TMV MP (Figure 3c). Green fluorescent protein (GFP) fluorescence levels were monitored in systemic leaves (fourth leaf from the apex) using the in vivo imaging system (IVIS) (Figure 3d). TMV-GFP and TMV-GFP^MP-ToBRFV systemically infected *N. benthamiana* plants (Figure 3c,d). However, TMV-GFP^MP(C68H)-TMV was unable to infect *N. benthamiana* plants (Figure 3c,d). These results establish that the amino acid C68 in MP is essential for the systemic infection of both TMV and ToMV in *N. benthamiana* and tomato, respectively. In addition to C68H, we generated other mutations in MP^TMV that match their respective identity in MP^ToBRFV (Figure S4). These include deletion of valine 5 (ΔV5) and replacement of asparagine 169 with isoleucine (N169I), which is also required for overcoming *Tm-2²* (Yan et al., 2021). Neither mutation disabled TMV-GFP from infecting *N. benthamiana* plants (Figure S4), suggesting a unique role for C68 in TMV cell-to-cell movement and therefore infectivity.

2.4 | Intercellular movement of MP^TMV relies on C68

The loss of systemic infection in TMV and ToMV C68H mutants (Figure 3) suggests that C68 is essential for MP function. To determine the effect of C68H on MP cell-to-cell movement, we examined the

FIGURE 2 Conservation of C68 in sequence and structure. (a) Protein sequence alignment of Solanaceae-infecting tobamovirus movement proteins. C68 is highlighted by a black arrow and ToBRFV H67 is highlighted by a black square. (b–d) AlphaFold models of MP^{TMV} (b), MP^{ToBRFV} (c), and four distant members of the 30K movement protein superfamily (MP^{CaMV}, MP^{CMV}, MP^{TBSV}, and MP^{TSWV}) (d). Purple marks the conserved β-barrel fold. The white arrows in (b) show the hydrophobic endoplasmic reticulum association regions previously described by Peiro et al. (2014). The red arrow marks C68 in MP^{TMV} (b) and the yellow arrow marks H67 in MP^{ToBRFV} (c).
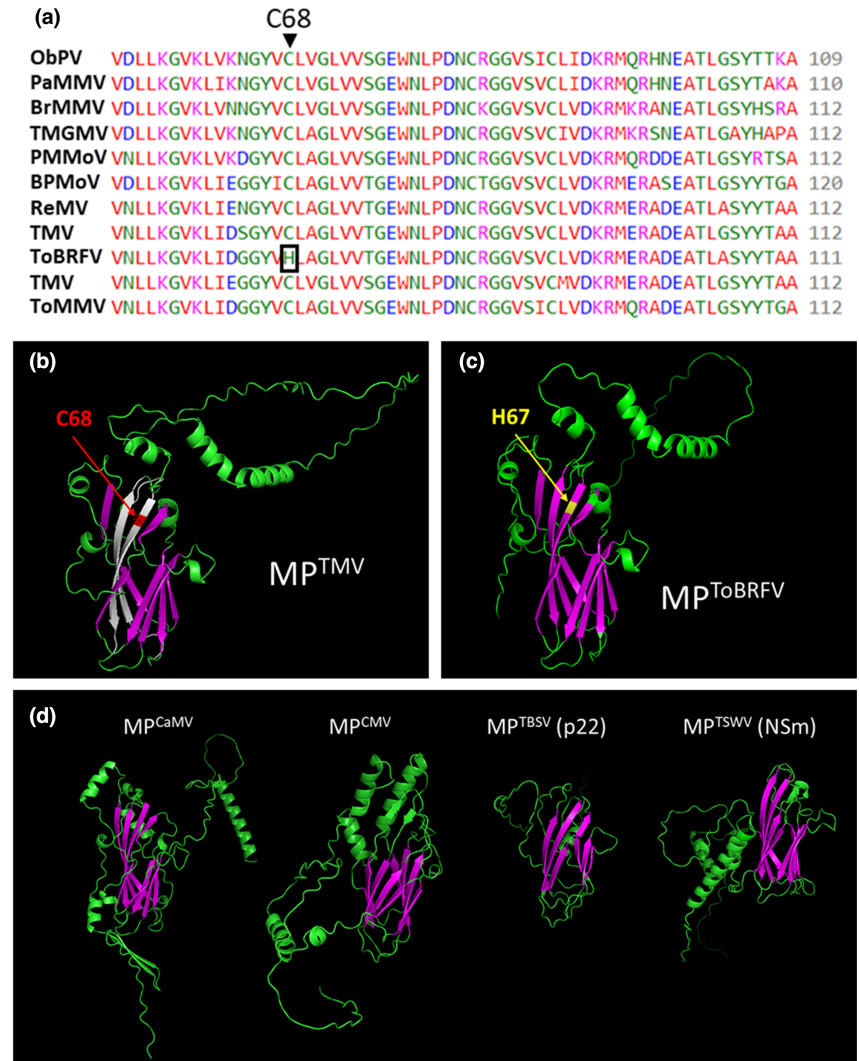
TMV-GFP inoculation sites in *N. benthamiana* leaves 5 days after inoculation (Figure 4). At this time point, the virus is already replicating and moving from cell to cell, resulting in round infection foci. The TMV-GFP infection foci size was 1.2 mm^2 (Figure 4a,d). Consistent with its reduced mobility, the TMV-GFP$^{MP-ToBRFV}$ infection foci size was 0.5 mm^2 (Figure 4b,d). Strikingly, TMV-GFP$^{MP(C68H)-TMV}$ was able to replicate in the infected cells but could not move out of proximal cells (Figure 4c,d), suggesting that C68 is required for TMV cell-to-cell movement.

To further examine if C68 is required for MP movement, *MP* open reading frames were fused to a gene encoding yellow fluorescent protein (YFP) and expressed under control of the cauliflower mosaic virus 35S promoter. ER-targeted mCherry (ER-mCherry) was expressed from the same binary plasmid as a nonmobile control marking the original cell of expression. *N. benthamiana* leaves were infiltrated with a low dilution of *Agrobacterium* to express the constructs in distinct epidermal cells as previously described (Hak & Spiegelman, 2021). We observed intercellular movement of both MP^{TMV} and MP^{ToBRFV} (Figure 5a–f,j–k). However, the C68H mutation abolished cell-to-cell movement of MP^{TMV} (Figure 5g–k). Replacement of other MP^{TMV} amino acids with the corresponding MP^{ToBRFV} amino acids required for overcoming *Tm-2²*, N135A and

N169I, did not disable intercellular movement of MP^{TMV} (Figure S5), suggesting that C68 plays an important role in controlling the movement function of MP^{TMV}.

2.5 | C68 is required for the localization of MP^{TMV} to the ER and PD

To examine how replacement of C68 resulted in the loss of MP^{TMV} cell-to-cell movement, we analysed the subcellular localization of MP^{TMV} C68H to PD using aniline blue callose staining (Figure 6). Plasmodesmatal localization was calculated as the ratio between the MP-YFP fluorescent signal intensity colocalized with callose at PD and the MP-YFP signal intensity along the plasma membrane (PM) at the cell perimeter (Figure 6a,e). This ratio indicated the level of MP specific localization to PD. MP^{TMV}-YFP showed the highest PD localization (Figure 6a,e). In MP^{ToBRFV}-YFP, plasmodesmatal localization was also evident, but 25% reduced as compared to MP^{TMV} (Figure 6b,e), a result consistent with its reduced cell-to-cell movement. Strikingly, the MP$^{TMV(C68H)}$-YFP mutant was not distinctly targeted to PD (Figure 6c,e). Instead, this protein localized to
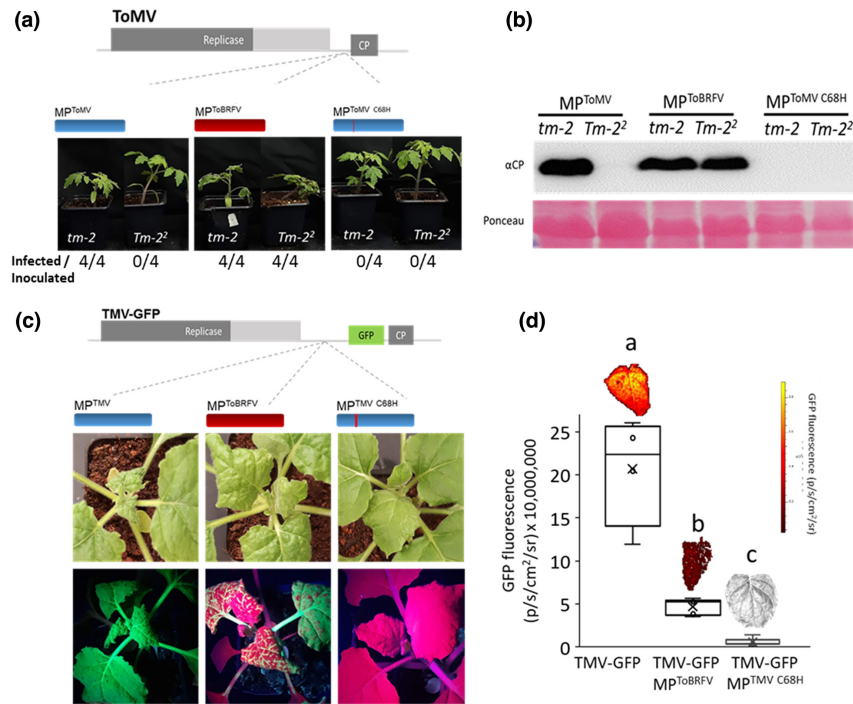
FIGURE 3 Substitution of movement protein (MP) amino acid C68 results in loss of ToMV and TMV infectivity in tomato and *Nicotiana benthamiana*. (a) Tomato plants (cv. Moneymaker) homozygous to the *tm-2* or *Tm-2²* allele infected with ToMV, ToMV whose original movement protein was replaced by MP^{ToBRFV}, and ToMV with the C68H mutation in the MP open reading frame. (b) Western blot analysis of ToMV CP in young leaves of *tm-2* and *Tm-2²* plants infected with ToMV expressing the various MPs. Images and leaf samples were taken at 13 days postinoculation (dpi). (c) White light (top) and UV light (bottom) images of *N. benthamiana* plants infected with TMV-GFP infectious clones harbouring MP^{TMV} (left), MP^{ToBRFV} (middle), or MP$^{TMV\ C68H}$ (right). (d) In vivo imaging-based quantitative analysis of green fluorescent protein (GFP) fluorescence in systemic leaves (fourth leaf from the apex) of plants infected with TMV-GFP harbouring the different movement proteins (MP^{TMV}, MP^{ToBRFV}, or MP$^{TMV\ C68H}$). Images were taken at 10 dpi. Different letters indicate significance in Tukey's HSD test ($p < 0.05$, $n \geq 4$).
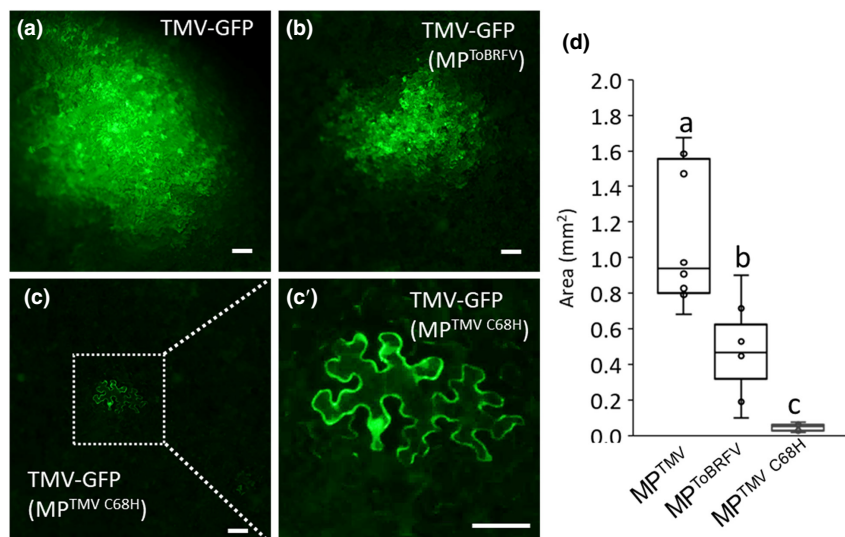

FIGURE 4 Substitution of MP^{TMV} amino acid C68 results in loss of viral cell-to-cell movement. (a–c) Cell-to-cell movement of the TMV-GFP infectious clones harbouring MP^{TMV} (a), MP^{ToBRFV} (b), or MP$^{TMV\ C68H}$ (c) at 5 days postinoculation. Magnification of the segmented section in the upper panel showing the failure of TMV-GFP MP$^{TMV\ C68H}$ to move out of the infected cell is shown in (c'). (d) Quantification of infection foci area. Different letters above bars indicate significance in Tukey's HSD test ($p < 0.05$, $n \geq 9$). Scale: 100 μm.

the plasma membrane, as observed by colocalization with the Flot1-RFP plasma membrane marker (Figure 6d,e). It is important to note that the level of PD localization did not correlate with the MP-YFP fluorescence levels at the whole cell (Figure S6), suggesting that the mislocalization of MP$^{TMV(C68H)}$-YFP was not a result of reduced protein accumulation. These results indicate that C68 is required for the MP^{TMV} intercellular transport function by targeting it to PD.

As shown in Figure 2, C68 is part of an ER interaction motif. Because targeting of MP to PD requires its interaction with the ER network (Wright et al., 2007), we postulated that the C68H mutation may alter MP^{TMV} interaction with the ER. To address this, we used confocal microscopy to image the upper plane of *N. benthamiana* epidermal cells co-expressing ER-mCherry with either MP^{TMV}-YFP or MP$^{TMV(C68H)}$-YFP (Figure 7). In these regions, MP^{TMV}-YFP
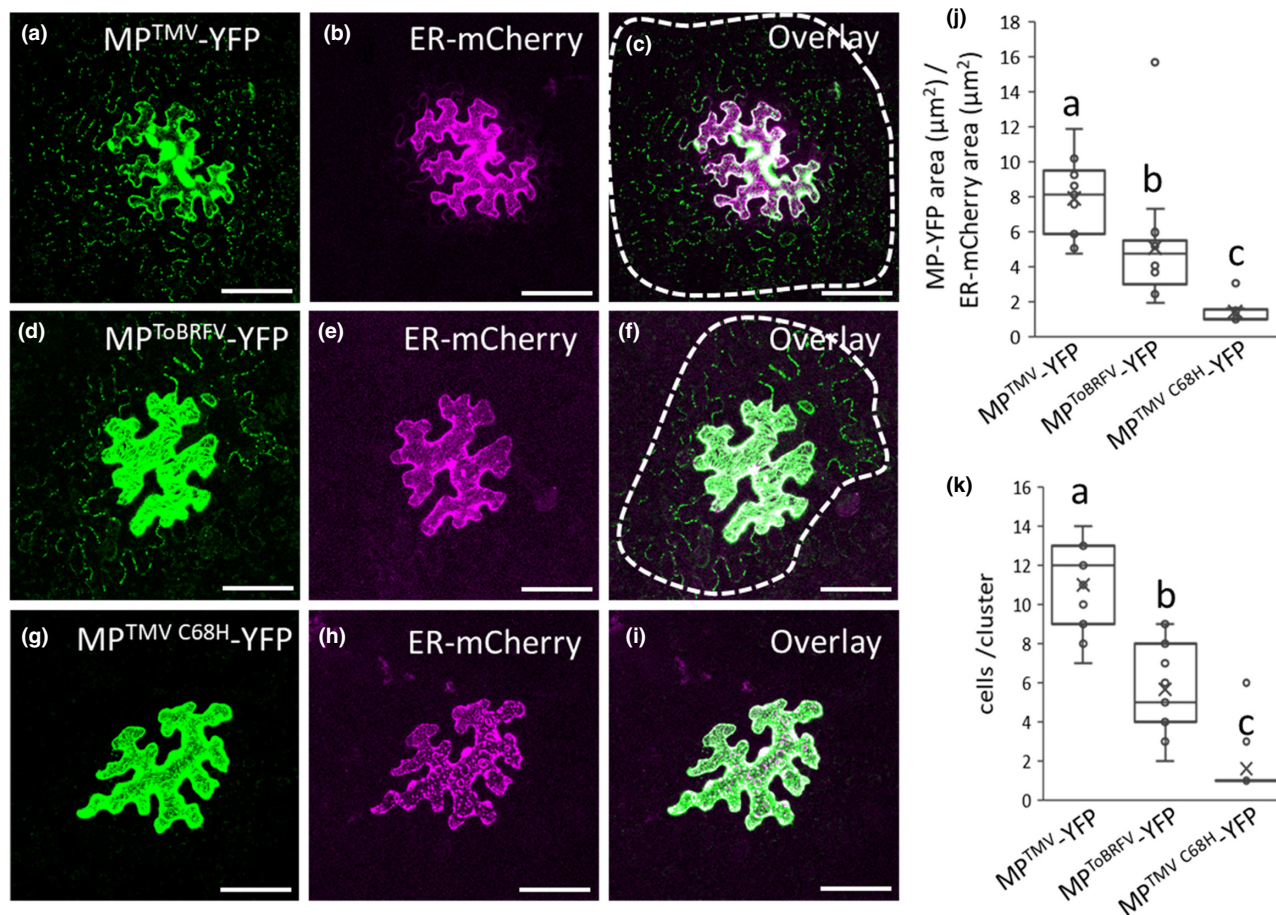
FIGURE 5 C68 is essential for MP^{TMV} intercellular movement. (a–c) Z-stack maximal projection confocal images of a *Nicotiana benthamiana* leaf epidermal cell expressing MP^{TMV}-YFP (a), the nonmobile endoplasmic reticulum (ER)-mCherry marker (b), and their overlay (c). (d–f) Z-stack maximal projection confocal images of epidermal cells expressing MP^{ToBRFV}-YFP (d), ER-mCherry (e), and their overlay (f). (g–i) Z-stack maximal projection confocal images of epidermal cells expressing MP$^{TMV\ C68H}$-YFP (g), ER-mCherry (h), and their overlay (i). (j) Quantitative analysis of the ratio between the area of MP^{TMV}-YFP, MP^{ToBRFV}-YFP, and MP$^{TMV\ C68H}$-YFP signal and the area of ER-mCherry signal. (k) Quantitative analysis of the number of cells per MP^{TMV}-YFP or MP^{ToBRFV}-YFP and MP$^{TMV\ C68H}$-YFP cluster. Different letters indicate significance in Tukey's HSD test ($p < 0.05$, $n ≥ 11$). Scale: 100 μm.

formed vesicles that were 76% colocalized with the ER (Figure 7a–c,g). On the other hand, the localization of MP$^{TMV(C68H)}$-YFP was altered to microdomain-like structures, and the colocalization with the ER was calculated at only 48% (Figure 7b–g). We further used live confocal imaging to assess the ability of the MP to traffic on the ER network. The MP^{TMV}-YFP-associated vesicles were largely mobile, with an average velocity of 1.48 μm/s (Figure 7h,I and Video S1). In marked contrast, MP$^{TMV(C68H)}$-YFP bodies were largely immobile (Figure 7h,I and Video S2). These results suggest that C68 is required for MP^{TMV} targeting to PD by regulating its association with the ER.

3 | DISCUSSION

The co-evolution of *R* genes and their effectors (also known as *Avr* factors) is a major driving force in the arms race between plants and pathogens. The durability of *R* genes relies on the importance

and conservation of its targeted effector (Jones & Dangl, 2006). A successful *R* gene will therefore target effector elements essential for pathogen infectivity and/or survival. However, there are only a few known examples for the mechanisms underlying such trade-offs. Previously, we have shown that overcoming *Tm-2²* by MP^{ToBRFV} was associated with an attenuation of viral movement (Hak & Spiegelman, 2021), suggesting that the elements required for evading *Tm-2²* also function in MP intercellular movement. Here we show that among the amino acids required to overcome *Tm-2²* (Yan et al., 2021), H67 localizes to a critical site for MP function, replacing a cysteine (C68) conserved among Solanaceae-infecting tobamoviruses, and placed within a structure conserved among multiple movement proteins. Substitution of MP^{ToBRFV} H67 with cysteine triggered *Tm-2²* resistance, suggesting that the presence of this amino acid in the context of the viral MP activates Tm-2². This is consistent with a previous finding that H67C substitution disabled the capacity of ToBRFV to systemically infect plants harbouring *Tm-2²* (Yan et al., 2021). Interestingly, our results show that the same
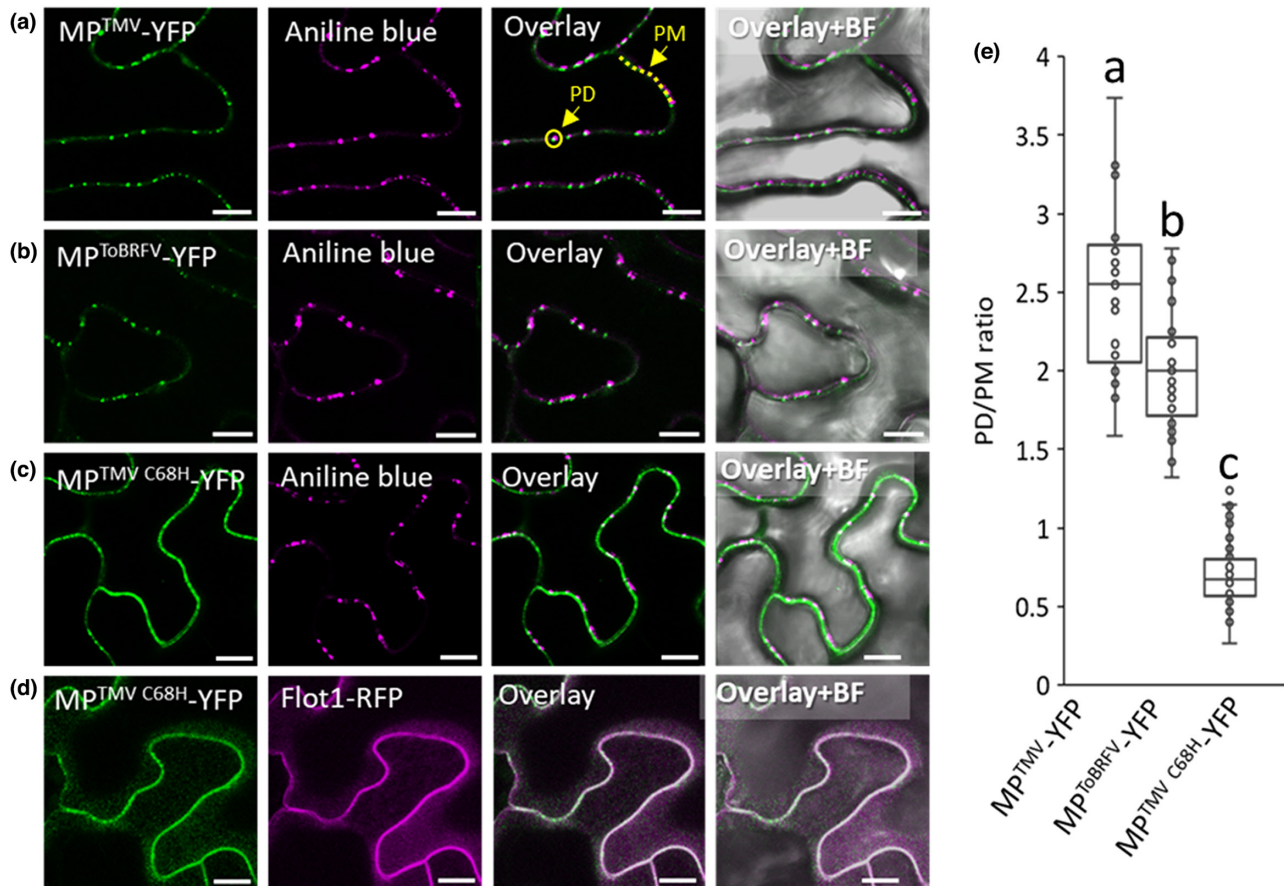
FIGURE 6 C68 is essential for MP^{TMV} plasmodesmatal localization. Localization of MP^{TMV}-YFP (a), MP^{ToBRFV}-YFP (b), and MP$^{TMV\ C68H}$-YFP (c) proteins (green) to plasmodesmata (PD) (purple). Plasmodesmata were labelled using aniline blue callose staining. Note that the MP$^{TMV\ C68H}$-YFP (c) fails to specifically be targeted to PD. (c) Quantitative analysis of the accumulation of the various MP-YFP proteins in PD. The calculation was based on the ratio between MP-YFP signal in PD and the average fluorescent signal in the plasma membrane (PM). Different letters indicate significance in Tukey's HSD test ($p < 0.05$, $n \geq 25$). Scale: 10 μm.

cysteine is essential for the function of ToMV and TMV by enabling MP subcellular targeting to PD via the ER.

The ER plays a major role in TMV intercellular transport (Pitzalis et al., 2018). Initially, TMV replication complexes are associated with the ER. Then, MP^{TMV}-associated complexes detach and move along the ER membrane to be targeted to PD (Guenoune-Gelbart et al., 2008; Sambade et al., 2008; Wright et al., 2007). The interaction of MP^{TMV} is mediated by two hydrophobic regions, which associate, but do not integrate into the ER membrane. These regions span amino acids 61–80 and 148–167. Although sequentially distant, our AlphaFold-based homology model predicted these two sections to be topologically interacting and take part in a putative jelly-roll fold common to various MPs (Figure 2b-d). These results suggest that Tm-2^2 targets the viral MP in an element crucial for its function.

There are only few examples in which resistance-breaking mutations cause penalties in viral infectivity or competition (García-Arenal & Fraile, 2013). Mutations in the potato virus Y NIa protein that enable it to overcome the *Ry*-mediated resistance also caused loss of its protease activity (Mestre et al., 2003). Mutations in turnip mosaic virus that overcome *TuRB01* and *TuRB04* resistances in *Brassica napus* impaired the virus's ability to compete with a

nonresistance-breaking viral strain on a susceptible host (Jenner et al., 2002). *Tm-1* resistance-breaking mutations in the ToMV replicase delay viral replication in tobacco protoplasts (Ishibashi et al., 2012). Mutations in pepper mild mottle virus CP that break *L*-mediated resistance in pepper were associated with high fitness penalties (Fraile et al., 2011). However, recently it was shown that the effect of resistance-breaking mutations is more complex and depends on the specific mutation, the host genotype, and the infecting isolate (Moreno-Pérez et al., 2016).

Hall (1980) suggested that the durability of *Tm-2^2*-mediated resistance against TMV and ToMV was a result of the reduced capacity of these viruses to evolve to overcome *Tm-2^2*, and predicted that this mutation would probably alter fundamental viral characteristics. Our finding that overcoming *Tm-2^2* requires the substitution of C68, which is essential for viral movement, supports this hypothesis. In light of these findings, how was ToBRFV able to overcome this resistance, whereas TMV and ToMV failed to do so? Previous lineage analysis predicted that ToBRFV probably evolved on an unknown host other than tomato, and invaded tomato due to a host-shifting event (Maayan et al., 2018). This allowed MP^{ToBRFV} to diverge from MP^{TMV} and MP^{ToMV} in multiple elements, including C68/H67, while
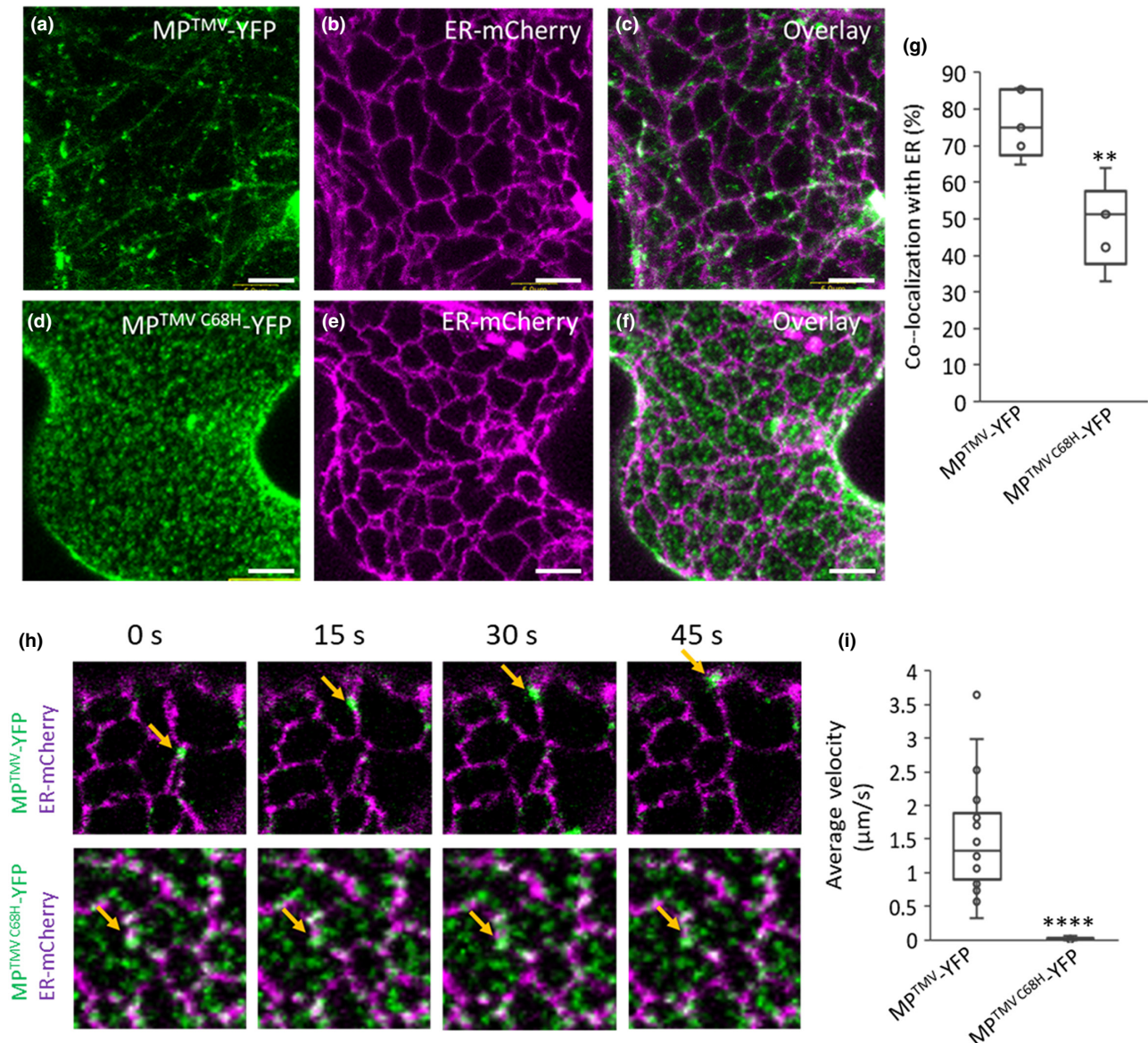
FIGURE 7 C68 is required for MP^{TMV} interaction with the endoplasmic reticulum (ER) and intracellular movement. Surface imaging of upper *Nicotiana benthamiana* leaf epidermal cell planes. (a–c) Expression of MP^{TMV}-YFP (a), ER-mCherry (b), and an overlay of both proteins (c). (d–f) expression of MP$^{TMV\ C68H}$-YFP (d), ER-mCherry (e), and an overlay of both proteins (f). (g) Quantitative analysis of MP^{TMV}-YFP and MP$^{TMV\ C68H}$-YFP colocalization with ER-mCherry. (h) Dynamics of MP^{TMV}-YFP (top) and MP$^{TMV\ C68H}$-YFP (bottom) on the ER network. Note that while MP^{TMV}-YFP are mobile on the ER, MP$^{TMV\ C68H}$-YFP are immobile (yellow arrow). (i) Quantification of MP^{TMV}-YFP and MP$^{TMV\ C68H}$-YFP vesicle velocity. Scale = 5 μm (**$p < 0.01$, ****$p < 0.0001$ in Student's t test, $n \geq 24$).

sustaining viral movement. On the other hand, in TMV and ToMV, loss of C68 would require additional compensatory mutations for MP to retain functionality. The requirement of multiple mutations to overcome *Tm-2²* combined with high levels of fitness penalties would be highly unlikely or rare and uncompetitive.

Collectively, we find that the conversion of cysteine at position 67 (H67) in MP^{ToBRFV} is crucial for evading *Tm-2²* resistance. However, in MP^{TMV}, substitution of cysteine in the corresponding position (C68) results in the abolition of cell-to-cell movement. We conclude that this strong trade-off between viral movement and breaking host resistance could be one of the primary reasons for the high durability of *Tm-2²* against TMV and ToMV for over 60 years.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials

Tomato (*Solanum lycopersicum*) seeds (cv. Moneymaker) homozygous for the *tm-2* allele (LA2706) or the *Tm-2²* allele (LA3310) were obtained from the Tomato Genome Research Center, University of California, Davis. *N. benthamiana* and tomato plants were grown in soil in a light- and temperature-controlled chamber at 25°C in a 16-h photoperiod. Five-week-old *N. benthamiana* plants were used for agroinfiltration and 3-week-old tomato plants were used for mechanical inoculation of ToMV.

4.2 | Plasmid construction

Plasmids p35S:Tm-2^2, p35S:MP^{TMV}, p35S:MP^{ToBRFV}, p35S:MP^{TMV}-YFP+ER-$mCherry$ and p35S:MP^{ToBRFV}-YFP+ER-$mCherry$ were assembled by Golden Gate cloning using the MoClo tool kit for plants (Addgene) (Weber et al., 2011) as described by Hak and Spiegelman (2021). Generation of the vectors TMV-GFP$^{MP\text{-}ToBRFV}$ and ToMV$^{MP\text{-}ToBRFV}$, based on the pJL24 (Lindbo, 2007) and pTLW3 (Hamamoto et al., 1993), were described previously (Hak & Spiegelman, 2021). Hybrid movement proteins were generated using fusion PCR. All the constructs that contain point mutations were made using site-directed mutagenesis (Ho et al., 1989) and confirmed by sequencing.

4.3 | Transient expression and agroinfiltration

Agrobacterium tumefaciens EHA105 harbouring a binary vector was grown overnight at 28°C. Cell cultures were resuspended in 2-morpholinoethane sulfonic acid (MES) buffer (10 mM MgCl$_2$, 10 mM MES, 150 μM acetosyringone, pH 5.6) to OD$_{600}$ = 0.5. For MP-YFP and TMV-GFP cell-to-cell movement assays, OD$_{600}$ = 0.0001 and OD$_{600}$ = 5 e^{-6} were used, respectively. *Agrobacterium* solutions were infiltrated using a needleless syringe into the abaxial side of the fourth or fifth leaf of *N. benthamiana* plants.

4.4 | Electrolyte leakage assay

p35S:MP constructs were agroinfiltrated into *N. benthamiana* leaves (n = 4) at OD$_{600}$ = 0.25 at a 1:1 ratio with either p35S:Tm-2^2 or an empty vector. Twenty-seven hours after agroinfiltration, four leaf discs (1 cm diameter) were cut from each leaf and washed for 10–30 min in distilled water. The leaf discs were then carefully transferred into a tube containing 4 mL of distilled water and placed on a low speed orbital shaker. Electric conductivity was measured after 6 h using a benchtop conductivity meter. Each experiment was repeated at least three times.

4.5 | TMV-GFP infection and quantification

For systemic infection experiments, *A. tumefaciens* harbouring TMV-GFP or TMV-GFP$^{MP\text{-}ToBRFV}$ vectors at OD$_{600}$ = 0.25 were infiltrated at a 1:1 ratio with the silencing suppressor p19 into the fourth leaf of 4-week-old *N. benthamiana* plants. GFP fluorescence was monitored 6 days postinfiltration. Images were acquired and analysed using an IVIS Lumina LT (PerkinElmer) equipped with a XFOV-24 lens and Living Image v. 4.3.1 software (PerkinElmer) set (excitation/emission: 420 nm/520 nm). The optical luminescent image data were displayed in pseudocolour that represents intensity in terms of radiance (photons per second per square centimetre per steradian) and were calculated as average radiance per

leaf. For cell-to-cell movement of TMV-GFP, imaging of GFP was obtained and analysed at 5 days postinoculation (dpi) using a Nikon SMZ-25 stereomicroscope equipped with a Nikon-D2 camera and NIS Elements v. 5.11 software. The number of plants for each experiment was n = 4 and experiments were repeated at least three times.

4.6 | In vitro transcription and infection of ToMV

Two microlitres of ToMV pTLW3 plasmid, ToMV$^{MP\text{-}ToBRFV}$, or ToMV$^{MP\text{-}TMV(C68H)}$ were linearized with SmaI and were prepared by a gel extraction kit (Zymo Research). In vitro transcription was performed using the mMESSAGE mMACHINE T7 kit (Invitrogen by Thermo Fisher Scientific). Two drops of 2.5 μL of transcript were used to mechanically inoculate *N. benthamiana* plants dusted with carborundum powder prior to inoculation. Leaves of infected plants were collected 5–7 dpi to serve as inoculum for 3-week-old tomato plants.

4.7 | Detection of ToMV by immunoblotting

For detection of the systemic spread of the virus in tomato, the first young leaf (10–30 mm) and apex were collected and ground, while frozen, in a microcentrifuge tube. Laemmli buffer (40 μL of 3×) (100 mM Tris, 2% SDS, 20% glycerol, 4% β-mercaptoethanol, pH 6.8) was added and mixed into the sample, followed by centrifugation for 10 min and boiling of the supernatant for 5 min. Samples were run on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Protran), and were blocked with 3% skimmed milk in Tris-buffered saline with Tween. Coat protein (CP) of the virus was detected by rabbit anti-tobamovirus CP (1:20,000; courtesy of A. Dombrovsky) and anti-rabbit horseradish peroxidase (1:20,000; Jackson Immunoresearch). Chemiluminescence was observed using Elistar Supernova as substrate (Cyanagen), and images of protein bands were acquired using the Alliance UVITEC software.

4.8 | Cell-to-cell movement analysis of MP-YFP

Experiments were performed as previously described (Hak & Spiegelman, 2021). Constructs expressing both MP-YFP and ER-mCherry were agroinfiltrated into *N. benthamiana* and visualized 36 h later. For confocal imaging, we used an Olympus IX 81 inverted laser scanning confocal microscope (Fluoview 500) equipped with an OBIS 488 and 561 nm laser lines, and a 60× 1.0 NA PlanApo water immersion objective. YFP and mCherry were excited at 488 and 561 nm and were imaged using BA505-525 nm and BA575-620 nm emission filters, respectively. The number of YFP-fluorescent cells surrounding the original cell of expression was quantified to determine the level of movement for each MP.

4.9 | Subcellular localization of MP-YFP

For ER localization, double expression plasmids harbouring MP-YFP and ER-mCherry were used (Hak & Spiegelman, 2021). For PM localization, MP-YFP was co-expressed with Flotilin1 fused to RFP under the 35S promoter (Flot1-RFP) (Pizarro et al., 2019). For plasmodesmatal localization, aniline blue solution (0.1% in water) was infiltrated into the abaxial side of the leaf and 10 min later imaged. Images were taken using an Olympus IX 81 inverted laser scanning confocal microscope (Fluoview 500). Excitation of ana-line blue at 405 nm was done using a BA430–460 filter with an UPlanSApo 60× 1.35 oil objective, 8/0.17 FN26.5. Quantification of MP-YFP accumulation intensity in PD, PM, and ER was performed using the ImageJ software (https://imagej.nih.gov/ij/) by measuring the YFP signal intensity colocalized with each of the markers.

4.10 | Multiple sequence alignment

Sequence alignment of the Solanaceae-infecting tobamoviruses MPs pepper mild mottle virus (NP_619742.1), bell pepper mottle virus (YP_001333652.1), Rehmannia mosaic virus (YP_001041891.1), TMV (NP_597748.1), ToBRFV (YP_009182170.1), ToMV (NP_078448.1), tomato mottle mosaic virus (YP_008492930.1), Obuda pepper virus (NP_620843.1), paprika mild mottle virus (NP_671720.1), Brugmansia mild mottle virus (YP_001974325.1), and tobacco mild green mosaic virus (sp|P18338|) was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/0).

4.11 | Protein structure prediction using AlphaFold2

The 3D structures of MP^{TMV} (NP_597748.1), MP^{ToBRFV} (YP_009182170.1), MP^{CaMV} (NP_056724.1), MP^{CMV} (NP_040776.1), MP^{TBSV} (NP_062900.1), and MP^{TSWV} (AEK06236.1) were predicted using the artificial Intelligence-based AlphaFold2 through the AlphaFold Colab notebook platform (Jumper et al., 2021). For the structural comparisons, we used only the predicted model with the highest confidence score computed by AlphaFold for each protein. The PyMOL Molecular Graphics System (v. 2.4, Schrödinger) was used to examine the structural models and generate images.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.