The Tomato Brown Rugose Fruit Virus Movement Protein Overcomes *Tm-2²* Resistance in Tomato While Attenuating Viral Transport

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Tomato brown rugose fruit virus is a new virus species in the *Tobamovirus* genus, causing substantial damage to tomato crops. Reports of recent tomato brown rugose fruit virus (ToBRFV) outbreaks from around the world indicate an emerging global epidemic. ToBRFV overcomes all tobamovirus resistances in tomato, including the durable *Tm-2²* resistance gene, which had been effective against multiple tobamoviruses. Here, we show that the ToBRFV movement protein (MP^{ToBRFV}) enables the virus to evade *Tm-2²* resistance. Transient expression of MP^{ToBRFV} failed to activate the *Tm-2²* resistance response. Replacement of the original MP sequence of tomato mosaic virus (ToMV) with MP^{ToBRFV} enabled this recombinant virus to infect *Tm-2²*-resistant plants. Using hybrid protein analysis, we show that the elements required to evade *Tm-2²* are located between MP^{ToBRFV} amino acids 1 and 216 and not the C terminus, as previously assumed. Analysis of ToBRFV systemic infection in tomato revealed that ToBRFV spreads more slowly compared with ToMV. Interestingly, replacement of tobacco mosaic virus (TMV) and ToMV MPs with MP^{ToBRFV} caused an attenuation of systemic infection of both viruses. Cell-to-cell movement analysis showed that MP^{ToBRFV} moves less effectively compared with the TMV MP (MP^{TMV}). These findings suggest that overcoming *Tm-2²* is associated with attenuated MP function. This may explain the high durability of *Tm-2²* resistance, which had remained unbroken for over 60 years.

Keywords: movement protein, plant immunity, resistance gene, *Solanum lycopersicum*, *Tm-2²*, tobamovirus, ToBRFV, tomato brown rugose fruit virus

Genus *Tobamovirus* is one of the most devastating groups of plant viruses, which includes tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), and cucumber green mottle mosaic virus (Broadbent 1976; Dombrovsky et al. 2017; Rybicki 2015). Tobamoviruses form rod-shaped virions that encompass a single-stranded, sense RNA genome of about 6.4 kb. This genome

encodes four main open reading frames (ORFs), two subunits of a 126-kDa protein and its read-through 183 kDa variant forming the viral replication complex, a 30-kDa movement protein (MP) and a 17.5 kDa coat protein (CP) (Goelet et al. 1982; Ishibashi and Ishikawa 2016). Cell-to-cell movement of the virus depends on the viral-encoded MP, which binds to multiple cellular components to transport the viral RNA through plasmodesmata, membrane-lined channels interconnecting adjacent cells (Lucas 2006; Pitzalis and Heinlein 2018; Reagan and Burch-Smith 2020; Ueki and Citovsky 2011).

A recent outbreak of a new tobamovirus named tomato brown rugose fruit virus (ToBRFV) has substantially damaged the tomato industry in Israel and Jordan, leading to extensive reductions in both yield and fruit quality (Luria et al. 2017; Salem et al. 2016). Recent reports of ToBRFV outbreaks around the world indicate an emerging global epidemic (Cambrón-Crisantos et al. 2018; Fidan et al. 2019; Ling et al. 2019; Menzel et al. 2019; Panno et al. 2019; Skelton et al. 2019; Yan et al. 2019). ToBRFV overcomes all known tobamovirus resistances in tomato, including the durable *Tm-2²* resistance (*R*) gene and, therefore, poses a substantial threat for the global tomato industry (Luria et al. 2017).

Dominant *R* genes are currently the most powerful means to control plant pathogens (de Ronde et al. 2014). Most of them encode the nucleotide-binding (NB) leucine-rich repeat (NLR) class of intracellular immune receptors (DeYoung and Innes 2006). NLRs recognize specific pathogen-encoded effectors either directly or indirectly (Padmanabhan and Dinesh-Kumar 2014) and trigger an immune signaling cascade that confines the pathogen to the infection site, typically through one of two processes, either induction of programmed cell death in a process termed the hypersensitive response (HR) or extreme resistance, in which resistance is achieved apparently without cell death. Most plant NLRs contain a coiled-coil (CC) or toll-interleukin-1-receptor homology domain at their N terminus, a centrally located NB domain, and a leucine rich repeat (LRR) domain at the C terminus (Burdett et al. 2019).

Resistance against tobamoviruses in tomato was achieved by the introgression of the *R* genes *Tm-1*, *Tm-2*, and its allele *Tm-2²* from wild tomato species (Ishibashi et al. 2007; Lanfermeijer et al. 2003; Meshi et al. 1989). In recent decades, resistance-breaking strains have emerged for *Tm-1* and *Tm-2*, leaving *Tm-2²* as the key gene for control of tobamovirus in tomato for the last 60 years (Meshi et al. 1989; Strasser and Pfitzner 2007). *Tm-2²* encodes a CC-NLR, which associates with its effector, tobamovirus MP, on the plasma membrane to activate an immune response (Chen et al. 2017; Weber et al. 1993). This association confers plant resistance through the induction of either HR or extreme resistance (Hall 1980), an outcome that depends on the specific level of *Tm-2²* expression (Zhang et al. 2013). Recently,

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it was shown that, upon activation by the MP, *Tm-2*² proteins self-associate on the plasma membrane to form an oligomer that relays the immune signal in a process that depends on both its CC and NB domains (Wang et al. 2020). Previous studies concluded that the C-terminal part of MP is essential for recognition by *Tm-2*² (Weber and Pfltzner 1998; Weber et al. 1993); two amino-acid substitutions at the ToMV MP (MP^{ToMV}) C terminus of the resistance-breaking ToMV isolate ToMV-2², S238R and K244E, were found to overcome *Tm-2*² resistance (Weber et al. 1993). Moreover, a premature stop codon in MP^{ToMV}, causing truncation of the MP C-terminal 30 amino acids, enabled ToMV to infect plants harboring *Tm-2*² (Weber and Pfltzner 1998).

ToBRFV is currently the only known tobamovirus that overcomes *Tm-2*² resistance (Luria et al. 2017). Previous sequence analysis showed that ToBRFV contains 21 amino acids potentially required to evade *Tm-2*². Of those, 12 mutations are located in the ToBRFV MP (MP^{ToBRFV}) and likely contribute to evading *Tm-2*² (Maayan et al. 2018). These include mutations that potentially change protein features such as secondary structures, phosphorylation sites and disulfide bonds. However, so far, there was no experimental evidence for the role of MP^{ToBRFV} in *Tm-2*² avoidance.

Here, we show that MP^{ToBRFV} overcomes *Tm-2*² resistance, independent of its C-terminal part. In addition, we show that this function is associated with reduced systemic and cell-to-cell movement, suggesting that the elements required for *Tm-2*² evasion have negative effects on MP^{ToBRFV} function. These findings may

explain the high durability of *Tm-2*² against other tobamoviruses such as TMV and ToMV.

RESULTS

MP^{ToBRFV} does not trigger *Tm-2*²-mediated cell death.

Activation of NLR receptors by their effectors can be monitored by the appearance of HR-mediated cell death when the NLR is coexpressed with its effector (Moffett et al. 2002; Whitham et al. 1996). Multiple studies showed that coexpression of MP^{TMV} and *Tm-2*² can trigger HR in *Nicotiana benthamiana* (Chen et al. 2017; Wang et al. 2020; Zhang et al. 2013). To test if *Tm-2*² is triggered by MP^{ToBRFV}, the coding sequences of MP^{ToBRFV} and MP^{TMV} were cloned under the control of the 35S promoter (*p35S:MP^{ToBRFV}* and *p35S:MP^{TMV}*, respectively). Clones were then transiently expressed in leaflets of tomato cv. Ikram (a *Tm-2*²/*tm-2* heterozygote) using agroinfiltration (Fig. 1A to C). When *p35S:MP^{TMV}* was expressed, cell death was observed in infiltrated leaflets as a result of immune response activation (Fig. 1A). On the other hand, leaflets infiltrated with *p35S:MP^{ToBRFV}* showed only marginal necrosis (Fig. 1B), similar to that of the empty-vector control treatment (Fig. 1C). To confirm that these findings are a direct result of *Tm-2*² activity, a similar experiment was performed with *N. benthamiana*. Here again, transient coexpression of *p35S:MP^{TMV}* with *p35S:Tm-2*² caused marked lesions and electrolyte leakage consistent with the establishment of HR

Fig. 1. *Tm-2*²-mediated cell death in tomato and *Nicotiana benthamiana* in response to tobacco mosaic virus and tomato brown rugose fruit virus movement proteins (MP^{TMV} and MP^{ToBRFV}, respectively). **A to C**, Transient expression of MP^{TMV} (A), MP^{ToBRFV} (B), and empty vector (C) in tomato cv. Ikram containing the *Tm-2*² resistance gene. **D to H**, Transient expression of MP^{TMV} with *Tm-2*² (D), MP^{ToBRFV} with *Tm-2*² (E), *Tm-2*² (F), MP^{TMV} (G), and MP^{ToBRFV} (H) in *N. benthamiana* leaves. **I**, Electrolyte leakage assay of *N. benthamiana* leaves expressing the various constructs (asterisks [***] indicate $P < 0.001$, Student's *t* test, $n \geq 4$). N/D = not detected.

(Fig. 1D and I), while coexpression of *p35S:MP^{ToBRFV}* with *p35S:Tm-2²* resulted in minimal cell death (Fig. 1E) and reduced electrolyte leakage (Fig. 1I). Individual expression of *Tm-2²*, *MP^{ToBRFV}*, and *MP^{TMV}* resulted in no cell death or electrolyte leakage (Fig. 1F to I). The marginal immune activation by *MP^{ToBRFV}* indicated that this protein was able to evade *Tm-2²* resistance.

A recombinant clone of ToMV harboring MP^{ToBRFV} infects *Tm-2²* resistant tomato plants.

To test the ability of *MP^{ToBRFV}* to overcome *Tm-2²* resistance in tomato, a hybrid clone was generated, based on an existing infectious clone of ToMV (Hamamoto et al. 1993), in which the original MP sequence of ToMV was replaced with *MP^{ToBRFV}* (*ToMV^{MP-ToBRFV}*) (Fig. 2A). This clone was then used to infect tomato plants homozygous for the ToMV-sensitive *tm-2* allele or the *Tm-2²* allele (Fig. 2B). Viral RNA was quantified in inoculated and systemic leaves (Fig. 2C and D), using quantitative reverse

transcription PCR (qRT-PCR). As expected, inoculation of *tm-2* plants with ToMV resulted in severe viral symptoms (Fig. 2B) and high levels of viral accumulation in both inoculated and systemic leaves (Fig. 2C to E), while *Tm-2²* plants were completely immune to ToMV (Fig. 2B to E). In marked contrast, *ToMV^{MP-ToBRFV}* induced symptoms in both *tm-2* and *Tm-2²* plants (Fig. 2B and E) and viral accumulation was detected in plants harboring both alleles (Fig. 2C to E). It is important to note that, in both *tm-2* and *Tm-2²* *ToMV^{MP-ToBRFV}*, symptoms were milder than those of ToMV-infected *tm-2* plants (Fig. 2B), suggesting that *MP^{ToBRFV}* reduced ToMV pathogenicity. Together, these results established that *MP^{ToBRFV}* is sufficient to overcome *Tm-2²* resistance.

The MP^{ToBRFV} C terminus is dispensable for overcoming *Tm-2²*.

To characterize the *MP^{ToBRFV}* elements potentially required to evade *Tm-2²*, a protein sequence alignment was performed on *MP^{ToBRFV}*, *MP^{TMV}*, *MP^{ToMV}*, and the MP of the *Tm-2²* resistance–

Fig. 2. Replacement of tomato mosaic virus and tomato brown rugose fruit virus movement proteins (*MP^{ToMV}* and *MP^{ToBRFV}*, respectively) is sufficient to overcome *Tm-2²* resistance in tomato. **A**, Schematic illustration of ToMV and *ToMV^{MP-ToBRFV}* viruses. Segmented lines indicate the replacement of *MP^{ToMV}* (blue) with the *MP^{ToBRFV}* (red) sequence. **B**, Tomato plants (cv. Moneymaker) (upper panel) and leaves (lower panel) homozygous to the *tm-2* or *Tm-2²* allele infected with ToMV and *ToMV^{MP-ToBRFV}*. **C** and **D**, Quantitative reverse transcription PCR (RT-PCR) analysis for ToMV coat protein (CP) transcripts in inoculated (**C**) and systemic (**D**) leaves of *tm-2* and *Tm-2²* tomato plants infected with ToMV and *ToMV^{MP-ToBRFV}*. **E**, Number of plants showing systemic symptoms of ToMV infection and results of virus detection by RT-PCR assay (one asterisk [*] indicates *P* < 0.05, two [**] *P* < 0.01, Student's *t* test, *n* ≥ 4). N/D = not detected. Scale bar = 2 cm.

breaking ToMV isolate ToMV-2² (MP^{ToMV-22}) (Fig. 3A). At the whole-protein level, MP^{ToBRFV} was 81% identical to MP^{ToMV} and 78% identical MP^{ToMV}. However, at the C-terminal 50 amino acids (216 to 266) (Fig. 3A, broken line), the sequence was more variable (41 and 42% identity with MP^{ToMV} and MP^{ToMV}, respectively). Importantly, this region includes the C-terminal 30 amino acids and MP^{ToMV} amino acids S238 and K244, previously shown to be essential for recognition by *Tm-2*² (Fig. 3A, yellow) (Weber and Pfitzner 1998; Weber et al. 1993). This suggested that the MP^{ToBRFV} C terminus is the factor required to evade *Tm-2*².

To test if the MP^{ToBRFV} C terminus overcomes *Tm-2*², two hybrid MP clones were generated (Fig. 3B), the first containing MP^{ToBRFV} amino acids 1 to 216 fused to MP^{ToMV} amino acids 217 to 266

(MP^{ToBRFV} 1-216/MP^{ToMV} 217-266) and the second containing MP^{ToMV} amino acids 1 to 216 fused to the MP^{ToBRFV} C-terminal 50 amino acids 217 to 266 (MP^{ToMV} 1-216/MP^{ToBRFV} 217-266) (Fig. 3B). Coexpression of *p35S::Tm-2*² with MP^{ToBRFV} 1-216/MP^{ToMV} 217-266 did not trigger cell death, indicating that the MP^{ToMV} C terminus was insufficient to activate *Tm-2*² (Fig. 3B and C). In addition, MP^{ToMV} 1-216/MP^{ToBRFV} 217-266 induced strong HR when coexpressed with *Tm-2*² (Fig. 3B and C). To verify that the MP^{ToMV} C-terminal region is not required for *Tm-2*² recognition, a clone with a truncated version of MP^{ToMV} containing a stop codon in position 238 was generated (MP^{ToMV}(Δ30)). Here again, a marked HR was observed when MP^{ToMV}(Δ30) was coexpressed with *Tm-2*², suggesting that the MP^{ToMV} C terminus is dispensable for *Tm-2*² recognition (Fig. 3B and C).

Fig. 3. Overcoming *Tm-2*² resistance is not due to the tomato brown rugose fruit virus movement protein (MP^{ToBRFV}) C terminus. **A**, Amino acid sequence alignment of MP^{ToBRFV} with tobacco mosaic virus and tomato mosaic virus MP (MP^{ToMV} and MP^{ToMV}) and the resistance-breaking MP^{ToMV-22}. A total of 33 suspected resistance-breaking mutations are outlined in black. The MP^{ToMV} amino acids Ser238 and Lys244, previously found to be required for *Tm-2*² recognition, are not conserved in all three MPs (yellow arrows). The black bar underlines the variable 50 C-terminal amino acids. **B**, Transient expression of MP^{ToMV}, MP^{ToBRFV}, the hybrids MP^{ToBRFV} 1-216/MP^{ToMV} 217-266 and MP^{ToMV} 1-216/MP^{ToBRFV} 217-266, and the truncated MP^{ToMV}(Δ30) in *Nicotiana benthamiana* leaves. Clones were expressed alone (-*Tm-2*²) or coexpressed with *Tm-2*² (+*Tm-2*²). **C**, Electrolyte leakage assay of *N. benthamiana* leaves expressing the various constructs. **D**, Schematic illustration of the ToMV-MP-ToBRFV virus expressing a hybrid movement protein with MP^{ToBRFV} amino acids 1 to 216 fused to MP^{ToMV} amino acids 217 to 266. **E**, Numbers of systemically infected *tm-2* and *Tm-2*² tomato plants inoculated with ToMV or ToMV^{MP-ToBRFV} 1-216. **F** and **G**, Quantitative reverse transcription PCR analysis for ToMV coat protein (CP) transcripts in inoculated (F) and systemic (G) leaves of *tm-2* and *Tm-2*² tomato plants infected with ToMV or ToMV^{MP-ToBRFV} 1-216. Different letters indicate significant in Tukey's honest significant difference test ($P < 0.05, n \geq 4$). One asterisk (*) indicates $P < 0.05$, Student's *t* test, $n \geq 5$; n.s. = not significant; N/D = not detected.

To confirm these results in tomato, a hybrid ToMV clone was generated with MP^{ToBRFV} amino acids 1 to 216 fused to MP^{ToMV} amino acids 217 to 266 (Fig. 3D). Viral transcripts were detected in inoculated and systemic leaves of both *tm-2* and *Tm-2²* plants 3 weeks after inoculation (Fig. 3E to G), establishing that this virus was able to overcome *Tm-2²* resistance regardless of the MP^{ToBRFV} C terminus.

MP^{ToBRFV} attenuates systemic viral infection of TMV-GFP (green fluorescent protein) and ToMV.

Since MP^{ToBRFV}, which overcomes *Tm-2²* resistance, also caused reduced symptoms in *tm-2* tomato plants (Fig. 3B), we hypothesized that the function of MP^{ToBRFV} is reduced as compared with other MPs. To test this, a TMV-GFP clone (Lindbo 2007) harboring MP^{ToBRFV} instead of its original MP (TMV-GFP^{MP-ToBRFV}) was generated. Local and systemic infection were determined 7 days after inoculation (dpi) of *N. benthamiana* plants, using GFP in vivo imaging analysis (IVIS) (Fig. 4). While TMV-GFP showed strong fluorescence in both inoculated and systemic leaves (Fig. 4A and D to F), the TMV-GFP^{MP-ToBRFV} hybrid showed weaker fluorescent signals (Fig. 4B and D to F). To confirm that the reduced infection was due to the attenuated function of MP^{ToBRFV}, a rescue assay was performed by co-agroinfiltrating TMV-GFP^{MP-ToBRFV} with *p35S:MP^{TMV}*. Interestingly, MP^{TMV} partially rescued the effect of MP^{ToBRFV} on systemic spread of

the virus (Fig. 4C and D), leading to a twofold increase in local infection and a fourfold increase in systemic accumulation of the virus (Fig. 4E and F). These results suggested that MP^{ToBRFV} is less effective than MP^{TMV} in promoting viral spread, likely due to its reduced function in cell-to-cell transport of the virus.

We further monitored the effect of MP^{ToBRFV} on systemic spread in *tm-2* tomato plants by Western blot analysis of CP accumulation (Fig. 5). Time course analysis of virus accumulation was performed in systemic tomato leaves 4, 8, and 12 dpi of plants infected with ToMV, ToMV^{MP-ToBRFV}, and the wild-type ToBRFV (Fig. 5A). ToMV was first detected in systemic leaves at 4 dpi, while the viruses harboring MP^{ToBRFV}, ToMV^{MP-ToBRFV}, and ToBRFV were barely detected. At 8 dpi, ToBRFV and ToMV^{MP-ToBRFV} could be detected in systemic leaves; however, their signal was significantly weaker than ToMV (Fig. 5A and B). At 12 dpi, there were no significant differences in accumulation among all three viruses. These results suggest that, while MP^{ToBRFV} confers the ability to overcome *Tm-2²*, it also reduces the rate of systemic infection compared with MP^{TMV} and MP^{ToMV}.

Reduced cell-to-cell transport of MP^{ToBRFV} compared with MP^{TMV}.

Since the role of a MP is to enable intercellular transport of the viral RNA, we speculated that the reduction of systemic infection caused by MP^{ToBRFV} is a result of a decrease in cell-to-cell

Fig. 4. Replacement of the tobacco mosaic virus movement protein (MP^{TMV}) with tomato brown rugose fruit virus MP (MP^{ToBRFV}) causes a reduction in tobacco mosaic virus green fluorescent protein (TMV-GFP) systemic infection in *Nicotiana benthamiana*. **A** to **C**, UV images of *N. benthamiana* plants 7 days after infection with TMV-GFP (**A**), TMV-GFP^{MP-ToBRFV} (**B**), and TMV-GFP^{MP-ToBRFV} coexpressed with *p35S:MP^{TMV}* (**C**). **D**, In vivo imaging analysis of inoculated (left panel) and systemic leaves 1 to 4 from the apex (right panel) of infected plants. **E** and **F**, Quantitative analysis of GFP fluorescence in inoculated (**E**) and systemic (**F**) leaves of plants expressing TMV-GFP, TMV-GFP^{MP-ToBRFV}, and TMV-GFP^{MP-ToBRFV} coexpressed with *p35S:MP^{TMV}* (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$, Student's *t* test, $n \geq 5$).

movement. To monitor cell-to-cell movement, the ORFs of both MP^{TMV} and MP^{ToBRFV} were fused to *YFP* and were expressed under the 35S promoter (Fig. 6A and D) in *N. benthamiana* leaves. As an immobile control, endoplasmic reticulum (ER)-mCherry was expressed from the same binary plasmid, to mark the original cell of expression (Fig. 6B and E). Low dilution (optical density at 600 nm $[OD_{600}] = 0.0001$) of agrobacterium was used to express the constructs in individual *N. benthamiana* epidermal cells and Z-stack confocal images were taken for each cell. The result was cell clusters in which the central cell expressed the MP-YFP and the proximal cells contained the trafficked MP-YFP (Fig. 6C and F). The number of adjacent cells containing the trafficked MP^{TMV} -YFP and MP^{ToBRFV} -YFP was used to quantify the level of intercellular movement. Interestingly, while MP^{TMV} -YFP moved to an average of 9.28 ± 0.38 cells ($n = 64$), MP^{ToBRFV} -YFP moved to an average of 6.8 ± 0.38 cells ($n = 51$) (Fig. 6G). In addition, movement to ≥ 10 cells was evident in 48.4% of the MP^{TMV} -YFP cell clusters but only in 13.2% of the MP^{ToBRFV} -YFP cell clusters. Consistently, movement to four or fewer cells was detected in 25.5% of MP^{ToBRFV} -YFP but only 6.25% of MP^{TMV} -YFP (Fig. 6H). Interestingly, MP^{TMV} -YFP localized predominantly to aggregates, plasmodesmata, and the cell periphery, while MP^{ToBRFV} -YFP distribution was more even and localized mostly to microtubules. These results

show that MP^{ToBRFV} intercellular movement is reduced as compared with that of MP^{TMV} .

DISCUSSION

ToBRFV is a significant threat to tomato production worldwide. This highly infectious virus can be easily transmitted by seed coats, working hands, tools, soil, and beneficial pollinators (Klap et al. 2020a; Levitzky et al. 2019; Smith and Dombrovsky 2019). In addition, interaction of ToBRFV with other viruses may increase their disease severity (Klap et al. 2020b; Luria et al. 2018). The main cause for the global ToBRFV outbreak is that this novel virus overcame the long-standing tobamovirus resistance *Tm-2*². Since *Tm-2*²-based resistance remained unbroken for decades, the question of which factors caused ToBRFV to evade this resistance is of agricultural and scientific importance. Previous studies established that the viral MP is recognized by *Tm-2*² to trigger resistance against the virus. Therefore, in the current study, we investigated the role of MP^{ToBRFV} in overcoming *Tm-2*². Our findings establish that *Tm-2*² is not activated by MP^{ToBRFV} (Fig. 1) and that MP^{ToBRFV} is sufficient to overcome *Tm-2*² (Fig. 2). These findings are consistent with previous findings on the role of the viral MP as the target of *Tm-2*² resistance and establish MP^{ToBRFV} as the factor that enables ToBRFV to infect *Tm-2*²-resistant plants (Meshi et al. 1989; Weber et al. 1993).

While it has been shown that the C-terminal part of MP is essential for *Tm-2*² activation (Weber and Pfizner 1998; Weber et al. 1993), later studies suggested that the C-terminal part of the MP is dispensable for this process. Expression of a truncated version of MP^{ToMV} consisting of only the N-terminal 188 amino acids was sufficient to trigger the *Tm-2*² immune response, while expression of the C-terminal 76 amino acids alone did not activate *Tm-2*² resistance (Weber et al. 2004). In addition, a TMV MP (MP^{TMV}) variant with a C-terminal 81-amino acid deletion still activated *Tm-2*² resistance in *N. benthamiana* (Chen et al. 2017). Here, we showed that the MP^{ToBRFV} C-terminal part does not determine *Tm-2*² recognition or avoidance. Instead, it seemed that elements within MP^{ToBRFV} amino acids 1 to 216 overcome this resistance. Computational analysis of MP^{ToBRFV} revealed 12 potential amino acids that are not conserved with MP^{TMV} and MP^{ToMV} and can alter the protein charge, structure, or phosphorylation, many of which are located between amino acids 1 and 216 (Maayan et al. 2018). Our study provides a platform for further elucidating the precise MP^{ToBRFV} amino acids are required to overcome *Tm-2*².

Here, we show that overcoming *Tm-2*² resistance by MP^{ToBRFV} is associated with delayed viral spread and cell-to-cell movement (Figs. 4 to 6). Previous reports suggested that ToMV mutations required to break *Tm-2*² might impair viral movement or survival and that existing *Tm-2*² resistance-breaking ToMV isolates display reduced transmissibility (Hall 1980). Indeed, MP^{ToBRFV} amino acids 1 to 216 include elements essential for MP function (Maayan et al. 2018), including the plasmodesmal localization signal (Yuan et al. 2016), a region essential for increasing plasmodesmal size exclusion limit (Waigmann et al. 1994), the ER interaction domain (Peiró et al. 2014), and RNA binding domains (Citovsky et al. 1992). In addition, the MP C-terminal 50 amino acids, which are dispensable for *Tm-2*² avoidance (Fig. 3), are also nonessential for viral movement (Berna et al. 1991). The finding that MP^{ToBRFV} activity is reduced compared with $MP^{TMV/ToMV}$ supports the hypothesis that *Tm-2*² targets MP sequences essential for its function.

Collectively, our results establish MP^{ToBRFV} is the factor required to evade *Tm-2*². Based on our systemic and cell-to-cell movement assays, we propose that the amino acid polymorphisms required to overcome *Tm-2*² may also affect MP function. This may explain the high durability of *Tm-2*² against TMV and ToMV. We hypothesize that, in these viruses, resistance-breaking mutations would likely impair MP function leading to insufficient viral movement.

Fig. 5. Delayed systemic infection by tomato mosaic virus (ToMV) in which the original movement protein (MP) sequence was replaced with tomato brown rugose fruit virus (ToBRFV) MP ($MP^{MP-ToBRFV}$) and ToBRFV in tomato plants. **A**, Western blot analysis of ToMV, $MP^{MP-ToBRFV}$, and ToBRFV and coat protein (CP) in systemic leaves 4, 8, and 12 days postinoculation (dpi). **B**, Quantitative analysis of ToMV, $MP^{MP-ToBRFV}$, and ToBRFV CPs in systemic leaves. Note that while ToMV CPs are detected in systemic leaves at 4 dpi, $MP^{MP-ToBRFV}$ and ToBRFV CPs accumulate later, at 8 dpi. Different letters indicate significantly different values ($P < 0.05$, Tukey's honest significant difference test, $n \geq 4$).

ToBRFV, on the other hand, likely evolved on an unknown host (Maayan et al. 2018), allowing its MP to diverge from MP^{TMV} and MP^{ToMV}, in a manner that will enable it to evade *Tm-2*², yet sustain sufficient levels of movement. Therefore, we conclude that MP^{ToBRFV} balances between *Tm-2*² avoidance and viral movement.

MATERIALS AND METHODS

Plant materials.

Tomato (*Solanum lycopersicum* L.) seeds (cv. Moneymaker) homozygous for the *tm-2* allele (LA2706) or the *Tm-2*² allele (LA3310) were obtained from the Tomato Genome Research Center, University of California, Davis. Cultivar Ikram tomato plants were obtained from Syngenta Co. *Nicotiana benthamiana* and tomato plants were grown in soil in a light- and temperature-controlled chamber at 25°C with a 16-h light and 8-h dark regime. Five-week-old *N. benthamiana* plants were used for agroinfiltration of TMV-GFP and three-week-old tomato plants were used for mechanical inoculation of ToMV.

Plasmid construction.

The various MP^{TMV}/MP^{ToBRFV} hybrids were generated using fusion PCR. The following binary plasmids were assembled by Golden Gate cloning using the MoClo tool kit for plants (Addgene) (Weber et al. 2011). *p35S:Tm-2*², *p35S:MP^{TMV}*, and *p35S:MP^{ToBRFV}* were generated by cloning the *Tm-2*² (AF536201), MP^{TMV}, and MP^{ToBRFV} ORFs into pICH486988, a binary level 2 Golden Gate vector that contains an expression cassette under the cauliflower mosaic virus 35S promoter. To allow Golden Gate assembly, internal *BsaI* sites were removed by one-step cloning. Plasmids containing the double expression cassettes *p35S:MP^{TMV}-YFP*, *ER-mCherry* and *p35S:MP^{ToBRFV}-YFP*, *ER-mCherry* were assembled using the level 2 vector pAGM4673. The ER-mCherry sequences were amplified from the *ER-rk CD3-959* plasmid (Nelson et al. 2007), containing the mCherry ORF with the *Arabidopsis thaliana* wall-associated kinase 2 signal peptide at the N terminus of the ER His-Asp-Glu-Leu retention signal at its C-terminus.

pTLW3 is a complementary DNA (cDNA) vector harboring the full ToMV sequence under the T7 promoter (Hamamoto et al.

Fig. 6. Reduced cell-to-cell movement of tomato brown rugose fruit virus movement protein (MP^{ToBRFV}) compared with tobacco mosaic virus MP (MP^{TMV}). **A to C**, Z-stack maximal projection confocal image of MP^{TMV}-YFP (yellow fluorescent protein) (**A**) and the nonmobile endoplasmic reticulum (ER)-mCherry (**B**) in *Nicotiana benthamiana* leaf epidermal cells. **C**, Overlay of MP^{TMV}-YFP and ER-mCherry. **D and E**, Z-stack maximal projection confocal image of tomato brown rugose fruit virus MP (MP^{ToBRFV})-YFP (**D**) and the nonmobile ER-mCherry (**E**) in *N. benthamiana* leaf epidermal cells. **F**, Overlay of MP^{ToBRFV}-YFP and ER-mCherry. Broken white lines circle the cluster of cells to which the MPs moved. **G**, Quantitative analysis of the number of cells per MP^{TMV}-YFP or MP^{ToBRFV}-YFP cluster. **H**, Fractionation of MP^{TMV}-YFP and MP^{ToBRFV}-YFP clusters: one to four cells (green), five to nine cells (yellow), and ≥10 (purple). (asterisks [***] indicate $P < 0.001$, Student's *t* test $n \geq 51$). Scale bar = 100 μ m.

1993). ToMV^{MP-ToBRFV} was assembled by fusion PCR and the MP^{ToBRFV} ORF was inserted between the *KpnI* and *XmaI* sites of the vector.

The binary vector pJL24 (Lindbo 2007) contains the full TMV genome with the GFP gene upstream to the MP ORF and is named here TMV-GFP. The MP^{ToBRFV} ORF was amplified from RNA extracted from ToBRFV-infected plants (accession number KX619418.1) and was cloned between the *AgeI* and *PacI* sites of the pJL24 vector, using fusion PCR to create TMV-GFP^{MP-ToBRFV}.

Transient expression and agroinfiltration.

Agrobacterium tumefaciens EHA105 harboring a binary vector was grown overnight at 28°C. Cell cultures were resuspended in morpholineethane sulfonic acid (MES) buffer (10 mM MgCl₂, 10 mM MES, 150 µM acetosyringone, pH 5.6) to an OD₆₀₀ = 0.5 (or OD₆₀₀ = 0.0001 for cell-to-cell movement experiments) and were infiltrated, using a needleless syringe, into the abaxial side of the fourth or fifth leaf of *N. benthamiana* plants.

Electrolyte leakage assay.

p35S:Tm-2² and *p35S:MP* constructs were agroinfiltrated at OD₆₀₀ = 0.25 individually or at a 1:1 ratio as indicated to *N. benthamiana* leaves. Twenty-seven hours later, four leaf discs (1 cm diameter) were cut out of each leaf and were washed for 10 to 30 min in distilled water. The leaf discs were then carefully transferred into a tube containing 4 ml of distilled water and were placed in a growth chamber. Electric conductivity was measured after 6 h, using a benchtop conductivity meter.

TMV-GFP infection and quantification.

A. tumefaciens harboring TMV-GFP or TMV-GFP^{MP-ToBRFV} vectors was infiltrated into the fourth leaf of 4-week-old *N. benthamiana* plants. GFP fluorescence was monitored 6 days postinfiltration. Images were acquired and analyzed using an IVIS Lumina LT (PerkinElmer) equipped with a XFOV-24 lens and Living Image 4.3.1 software (PerkinElmer) set (excitation/emission: 420 nm/520 nm). The optical luminescent image data were displayed in pseudocolor that represents intensity in terms of radiance (photons per second per square centimeter per steradian) and were calculated as average radiance per leaf.

In vitro transcription and infection.

Two microliters of ToMV pTLW3 plasmid, ToMV^{MP-ToBRFV}, or ToMV^{MP-ToBRFV(1-216)} were linearized with *SmaI* and were cleaned by a gel extraction kit (Zymo Research). In vitro transcription using the mMESSAGE mMACHINE T7 kit (Invitrogen by Thermo Fisher Scientific) was performed according to manufacturer instructions. Two drops of 2.5 µl of transcript were used to mechanically inoculate *N. benthamiana* plants that were dusted with Carborundum powder prior to inoculation. Leaves of the infected plants were collected 5 to 7 dpi to serve as inoculum for tomato plants.

Evaluation of ToMV levels by qRT-PCR analysis.

Total RNA was extracted from 50 to 100 mg of leaf tissue by a Plant Total RNA mini kit (Geneaid), which includes a DNase treatment. RNA (500 ng) served as a template for cDNA synthesis (PCR BIO). qRT-PCR was performed using 2xSyGreen Mix (PCR BIO) in a Rotor-Gene 6000 cycler (Corbett Research). ToMV levels were calculated as the expression ratio of ToMV-CP (ToMV-CP_F: 5'-CTACGGTTGCAATTCGGTCT-3'; ToMV-CP_R: 5'-AGGTCCAGACCAACCCAGACAT-3') and the gene of reference TIP41 (TIP41_F: 5'-ATGGAGTTTTTGAGTCTTC TGC-3'; TIP41_R: 5'-GCTGCGTTTCTGGCTTAGG-3') (Lacerda et al. 2015).

Evaluation of ToMV and ToBRFV levels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

For detection of the spread of the virus in tomato, the first small leaf (1 to 3 cm) and apex were collected and ground, while frozen, in a microcentrifuge tube. Laemmli buffer (40 µl of 3x) (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-PAGE acrylamide gels, were transferred to nitrocellulose membranes (Protran), and were blocked with 3% skimmed milk in Tris buffer saline-Tween. 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 and quantified using the Alliance UVITEC software.

Cell-to-cell movement analysis.

Constructs expressing MP-YFP and ER-mCherry were agroinfiltrated into *N. benthamiana* and were 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 60x 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.

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