Identification of genetic determinants of tomato brown rugose fruit virus that enable infection of plants harbouring the Tm-2^2 resistance gene

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Abstract
Tomato cultivars containing the Tm-2^2 resistance gene have been widely known to resist tobacco mosaic virus (TMV) and tomato mosaic virus. Tomato brown rugose fruit virus (ToBRFV), a new emerging tobamovirus, can infect tomato plants carrying the Tm-2^2 gene. However, the virulence determinant of ToBRFV that overcomes the resistance conferred by the Tm-2^2 gene remains unclear. In this study, we substituted the movement protein (MP) encoding sequences between ToBRFV and TMV infectious clones and conducted infectivity assays. The results showed that MP was the virulence determinant for ToBRFV to infect Tm-2^2 transgenic Nicotiana benthamiana plants and Tm-2^2-carrying tomato plants. A TMV MP chimera with amino acid residues 60–186 of ToBRFV MP failed to induce hypersensitive cell death in the leaves of Tm-2^2 transgenic N. benthamiana plants. Chimeric TMV containing residues 60–186 of ToBRFV MP could, but chimeric ToBRFV containing 61–187 residues of TMV MP failed to infect Tm-2^2 transgenic N. benthamiana plants, indicating that 60–186 residues of MP were important for ToBRFV to overcome Tm-2^2 gene-mediated resistance. Further analysis showed that six amino acid residues, H^67, N^125, K^129, A^134, I^147, and I^168 of ToBRFV MP, were critical in overcoming Tm-2^2-mediated resistance in transgenic N. benthamiana plants and tomato plants. These results increase our understanding of the mechanism by which ToBRFV overcomes Tm-2^2-mediated resistance.

KEYWORDS
movement protein, overcoming resistance, Tm-2^2, tomato brown rugose fruit virus
1 | INTRODUCTION

Plant viruses are important pathogens that cause huge economic losses in crop production. Breeding and planting resistant cultivars are the most efficient strategies for controlling plant viruses (Nicaise, 2014). Long-term coevolution between plants and viruses has caused the evolution of specific resistance (R) genes in host plants. The products of R genes recognize the avirulence protein (Avr) of the virus and activate resistance responses, which are often accompanied by a hypersensitive response (HR) characterized by cell death at the virus infection sites. For example, the N protein in tobacco plants recognizes the 50 kDa helicase domain of tobacco mosaic virus (TMV) replicase to elicit HR (Erickson et al., 1999) and the coat protein (CP) of potato virus X is recognized by the Rx protein in potatoes, which elicits a resistance response (Bendahmane et al., 1999).

Tm-2^2, the product of the Tm-2^2 resistance gene, can recognize movement proteins (MPs) of TMV and tomato mosaic virus (ToMV) and activates a resistance response. For several decades, the Tm-2^2 gene has been introduced into numerous commercial tomato cultivars to resist ToMV and TMV (Hall, 1980; Lanfermeijer et al., 2003). However, two strains, ToMV-LIA from the Netherlands and ToMV-2^2 from the UK, can overcome Tm-2^2-mediated resistance (Calder & Palukaitis, 1992; Weber et al., 1993). Four amino acid residues, E^{130}, E^{184}, R^{238}, and E^{244} of ToMV-2^2 MP differ from the residues K^{130}, G^{184}, S^{238}, and K^{244} of ToMV-L MP, which failed to overcome Tm-2^2-mediated resistance. Furthermore, substitution of C-terminal residues S^{238} and K^{244} in ToMV-L MP to R^{238} and E^{244} or deleting 30 residues of the C-terminal (retaining residues 1–234) in ToMV-L can overcome Tm-2^2 resistance in tomato (Weber & Pfitzner, 1998; Weber et al., 1993). However, deletion of 77 residues from the C-terminal of ToMV MP (retaining residues 1–187) or deletion of 81 residues from the C-terminal (retaining 1–187 residues) of TMV MP can trigger Tm-2^2-mediated HR cell death (Chen et al., 2017; Weber et al., 2004). These results suggest that residues 1–187 of ToMV and TMV MPs are critical for Tm-2^2 recognition to induce resistance, and the C-terminal region may play a role in regulating the exposure of Tm-2^2 recognition region located in MP residues 1–187 (Chen et al., 2017).

Tomato brown rugose fruit virus (ToBRFV), a newly emerging to-bamovirus, can infect tomato plants containing the Tm-2^2 resistance gene (Luria et al., 2017; Yan et al., 2021b). ToBRFV was first reported in Israel and Jordan, and is currently found in dozens of countries in Africa, America, Asia, and Europe (Alfaro-Fernández et al., 2021; Alkowni et al., 2019; Amer & Mahmoud, 2020; Beris et al., 2020; Camacho-Beltrán et al., 2019; Fidan et al., 2019; Ling et al., 2019; Luria et al., 2017; Menzel et al., 2019; Panno et al., 2019; Salem et al., 2016; Skelton et al., 2019; van de Vossenberg et al., 2020; Yan et al., 2019). Additionally, ToBRFV infection causes severe loss of commercial value, and thus presents a serious threat to tomato production. However, the mechanism by which ToBRFV escapes Tm-2^2 resistance remains unclear. Here, we report that MP is a virulence determinant of ToBRFV that infects plants harbouring Tm-2^2, and six residues (I^{467}, N^{125}, K^{229}, A^{234}, I^{147}, and I^{168}) in the central region of MP are critical sites that enable ToBRFV to escape from Tm-2^2 recognition.

2 | RESULTS

2.1 | Movement protein is the virulence determinant of ToBRFV infecting Tm-2^2-transgenic N. benthamiana and Tm-2^2-carrying tomato plants

Our results showed that ToBRFV Shandong isolate (ToBRFV-SH) could infect wildtype Nicotiana benthamiana, Tm-2^2 transgenic N. benthamiana, and Tm-2^2-carrying tomato (cultivar jinpeng) plants (Yan et al., 2021b), whereas the TMV HEB2 isolate could infect wildtype N. benthamiana plants but failed to infect Tm-2^2 transgenic N. benthamiana or tomato cv. Jinpeng plants. The role of ToBRFV MP in overcoming Tm-2^2 resistance was investigated by constructing ToBRFV and TMV infectious clones based on the binary vector pCB301 and designating them as pCBToBRFV and pCBTMV, respectively. The chimeric constructs pCBToBRFV-TMV1-268 (ToBRFV-TMV1-268) and pCBTMV-To1-266 (TMV-To1-266) were obtained by exchanging the MP genes of ToBRFV and TMV (Figure 1a). Wildtype and Tm-2^2 transgenic N. benthamiana plants were separately infiltrated with Agrobacterium cultures harbouring one of the aforementioned four constructs. At 5 days postagroinfiltration (dpai), systemic leaves of all wildtype N. benthamiana plants inoculated with ToBRFV, TMV, ToBRFV-TMV1-268, or TMV-To1-266 showed mosaic and epinasty symptoms (Figure 1b, upper panel). In particular, TMV and ToBRFV-TMV1-268 failed to induce any symptoms in Tm-2^2 transgenic N. benthamiana plants, whereas ToBRFV and TMV-To1-266 induced symptoms of mosaic and epinasty (Figure 1b, lower panel). Furthermore, reverse transcription (RT)-PCR results showed the presence of viral RNA in the systemic leaves of all inoculated wildtype N. benthamiana plants (Figure 1c); however, viral RNA was detected in the systemic leaves of Tm-2^2 transgenic N. benthamiana plants inoculated with ToBRFV and TMV-To1-266, but not in those inoculated with TMV and ToBRFV-TMV1-268 (Figure 1d). We determined viral progenies in the systemic leaves of N. benthamiana and Tm-2^2 transgenic N. benthamiana plants by sequencing, and did not discover any revertants. We further inoculated the tomato cv. Jinpeng plants with ToBRFV, TMV, ToBRFV-TMV1-268, or TMV-To1-266. At 12 dpai, ToBRFV induced mosaic symptoms in systemically infected leaves of all inoculated tomato plants, and TMV-To1-266 induced mild mosaic symptoms; however, TMV and ToBRFV-TMV1-268 failed to induce any viral symptoms (Figure 1e). Moreover, RT-PCR results detected the presence of viral RNA in the systemically infected leaves of tomato plants inoculated with ToBRFV and TMV-To1-266, but not in those inoculated with TMV and ToBRFV-TMV1-268 (Figure 1f). To exclude the possibility of a reverse mutation, we verified the virus progenies in the systemically infected leaves of tomato plants. At 21 dpai, ToBRFV-TMV1-268 and TMV failed to induce any symptoms in the systemic leaves of Tm-2^2 transgenic N. benthamiana plants and tomato cv. Jinpeng plants, whereas ToBRFV and TMV-To1-266 caused serious symptoms (Figure 1g). We also detected viral genomic and CP RNA accumulation in the inoculated leaves of tomato cv. Jinpeng.
Movement protein (MP) is the virulence determinant of tomato brown rugose fruit virus (ToBRFV) to infect Tm-22 transgenic Nicotiana benthamiana plants. (a) Schematic representation of infectious clones pCBTMV, pCBToBRFV, and their chimeras. The MP gene was swapped between tobacco mosaic virus (TMV) and ToBRFV to produce chimeras. (b) Symptom and (c, d) virus accumulation in the systemically infected leaves of wildtype N. benthamiana and Tm-22 transgenic N. benthamiana plants inoculated with TMV, ToBRFV, TMV-To1-266, and ToBRFV-TMV1-268 at 5 days postagroinfiltration (dpi). The experiment was repeated three times independently. At least three N. benthamiana plants were inoculated for each construct in one experiment. (e, f) Symptom and virus accumulation in the systemically infected leaves of tomato cv. Jinpeng plants inoculated with TMV, ToBRFV, TMV-To1-266, and ToBRFV-TMV1-268 at 12 dpi. Virus accumulation was detected by reverse transcription PCR. The ubiquitin gene (UBI) was used as an internal control. The experiment was repeated three times independently. In total, 18 tomato cv. Jinpeng plants were inoculated with each construct in three independent experiments. The inoculated leaves are marked with yellow arrowheads, systemic leaves without symptoms are marked with red arrows, while systemically infected leaves showing viral symptoms are marked with white arrowheads.
plants using reverse transcription-quantitative PCR (RT-qPCR). At 3 dpai, the accumulation levels of TMV-To1-266 genomic and CP RNA were higher than those of TMV, but the genomic and CP RNA accumulation levels of ToBRFV-TMV1-268 were lower than those of ToBRFV (Figure S2).

These results indicate that MP is a virulence determinant of ToBRFV that infects Tm-2² transgenic N. benthamiana and Tm-2² carrying tomato plants.

### 2.2 | The central region of ToBRFV MP is essential to avoid recognition by Tm-2²

To determine the region of ToBRFV MP that helps evade Tm-2² recognition, we constructed pCamGFP-ToBRFVMP and pCamGFP-TMVMP, which can separately express the fusion of ToBRFV MP or TMV MP with green fluorescent protein (GFP) at their N-terminus (Figure 2a). Agrobacterium cultures harbouring constructs pCamGFP, pCamGFP-ToBRFVMP (the expressed protein was named GFP-ToBRFVMP), or pCamGFP-TMVMP (GFP-TMVMP) were individually infiltrated into fully expanded leaves of Tm-2² transgenic N. benthamiana plants. At 4 dpai, only the infiltrated leaf patches expressing TMVMP, but not ToBRFVMP or GFP, displayed HR cell death under white light (Figure 2b, left), which was confirmed using trypan blue staining (Figure 2b, right).

We constructed vectors to transiently express chimeric MPs (Figure 2a). The nucleotide sequences encoding residues 1–187 and residues 188–268 of TMV MP in pCamGFP-TMVMP were substituted with the sequences encoding residues 1–187 and 187–266 of ToBRFV MP to produce pCamGFP-TMVMP-To1-186 (GFP-TMVMP-To1-186) and pCamGFP-TMVMP-To187-266 (GFP-TMVMP-To187-266), respectively. Agrobacterium cultures carrying pCamGFP-TMVMP, pCamGFP-ToBRFVMP, or either of these chimeric constructs were infiltrated into fully expanded leaves of Tm-2² transgenic N. benthamiana plants. At 4 dpai, leaf patches expressing mutant GFP-TMVMP-To187-266 displayed HR cell death, which was not observed in GFP-TMVMP-To1-186 (Figure 2c), indicating that MP residues 1–186 in ToBRFV were critical in evading Tm-2² recognition.

To further delimit the essential region, we constructed five chimeric transient expression vectors. The nucleotide sequences encoding residues 1–59, 1–126, 60–126, 60–186, and 127–186 of ToBRFV MP were substituted for the corresponding sequences of TMV MP in pCamGFP-TMVMP to produce pCamGFP-TMVMP-To1-59 (GFP-TMVMP-To1-59), pCamGFP-TMVMP-To126 (GFP-TMVMP-To126), pCamGFP-TMVMP-To187-266 (GFP-TMVMP-To187-266), pCamGFP-TMVMP-To126 (GFP-TMVMP-To126), and pCamGFP-TMVMP-To187-266.
pCamGFP-TMVMP-To60-186 (GFP-TMVMP-To60-186), and pCamGFP-TMVMP-To127-186 (GFP-TMVMP-To127-186), respectively (Figure 2a). At 4 dpi, the results of the transient expression experiment showed that GFP-TMVMP-To1-59, GFP-TMVMP-To1-126, GFP-TMVMP-To60-126, and GFP-TMVMP-To127-186 induced HR cell death, whereas GFP-TMVMP-To60-186 failed to induce distinct HR cell death in leaves of Tm-22 transgenic N. benthamiana plants (Figure 2c). Moreover, GFP-TMVMP-To127-186 induced indistinct cell death, which was confirmed by the trypan blue staining (Figure 2c). We further agroinfiltrated these transient constructs into the leaves of N. benthamiana plants. Western blot results showed that the constructs expressed MP at high levels, and the existence of HR cell death was unrelated to the expression levels of chimeric MP (Figure S3). These results indicate that residues 60–186 in the central region of ToBRFV MP are necessary to evade recognition by Tm-22.

2.3 | Residues 60–186 of MP are critical for ToBRFV to escape Tm-22-mediated resistance

The MP domains of ToBRFV were mapped to infect Tm-22 transgenic N. benthamiana plants by substituting the nucleotide sequences encoding residues 1–186, 60–186, 127–186, and 60–266 of ToBRFV MP for the corresponding sequences of TMV MP, producing vectors pCBTMV-To1-186 (TMV-To1-186), pCBTMV-To60-186 (TMV-To60-186), pCBTMV-To127-186 (TMV-To127-186), and pCBTMV-To60-266 (TMV-To60-266), respectively (Figure 3a). Wildtype and Tm-22 transgenic N. benthamiana plants were separately agroinfiltrated with the aforementioned plasmids. At 5 dpi, all chimeric viruses induced mosaic and epinasty symptoms on the systemically infected leaves of wildtype N. benthamiana plants (Figure 3b). Viral RNA was detected in the systemically infected leaves of wildtype N. benthamiana plants inoculated with the above mutants (Figure 3c). Mutants TMV-To60-186, TMV-To1-186, and TMV-To60-266 induced mosaic and epinasty symptoms, identical to TMV-To1-266, in systemic leaves of all Tm-22 transgenic N. benthamiana plants (Figure 3b). Viral RNA was detected in the systemic leaves of the Tm-22 transgenic N. benthamiana plants inoculated with TMV-To60-186, TMV-To1-186, and TMV-To60-266 (Figure 3d). However, no distinct viral symptoms or viral RNA accumulation was observed in the systemic leaves of the Tm-22 transgenic N. benthamiana plants inoculated with TMV-To127-186 (Figure 3b,d). These results suggest that residues 60–186 of ToBRFV MP play a critical role in overcoming Tm-22-mediated resistance.

To further confirm the role of residues 60–186 of ToBRFV MP in the infection of Tm-22 transgenic N. benthamiana plants, we substituted the nucleotide sequences encoding residues 1–60, 61–187, and 188–268 of TMV MP with the corresponding sequences of ToBRFV MP in pCBToBRFV, producing pCBToBRFV-TMV1-60 (ToBRFV-TMV1-60), pCBToBRFV-TMV127-186 (ToBRFV-TMV1-187), pCBToBRFV-TMV188-268, and (ToBRFV-TMV188-268), respectively (Figure 4a). Wildtype and Tm-22 transgenic N. benthamiana plants were separately inoculated with ToBRFV and one of these three chimeras. At 5 dpai, ToBRFV or any one of the chimeras induced mosaic symptoms (Figure 4b, upper panel) and accumulated viral RNA (Figure 4c) in the systemic leaves of all wildtype N. benthamiana plants; in contrast, only ToBRFV-TMV1-60 and ToBRFV-TMV188-268, and not ToBRFV-TMV61-187, induced systemic mosaic symptoms identical to that of ToBRFV (Figure 4b, lower panel) and accumulated viral RNA (Figure 4d) in systemically infected leaves of all Tm-22 transgenic N. benthamiana plants. No reversion virus was observed in the systemic leaves.

These results show that residues 60–186 in the central region of ToBRFV MP are necessary for ToBRFV to infect Tm-22 transgenic N. benthamiana plants.

2.4 | Residues H67, N125, K129, A134, I147, and I168 of MP are critical for ToBRFV to evade Tm-22-mediated resistance

Comparing the sequence of residues 60–186 of ToBRFV MP with those of TMV MP revealed variations in 15 residues (Figure S4). The critical residue(s) of ToBRFV MP involved in overcoming Tm-22-mediated resistance were mapped by individually substituting the codons coding G63, H67, V73, A105, N125, K129, E132, A134, I147, A148, Y151, K166, I168, S179, and T181 of ToBRFV MP in pCBToBRFV by the codons coding S64, C68, I74, G100, A126, Q130, M133, N135, M142, S149, E152, R167, N169, N180, and R182 of TMV MP to produce pCBToBRFV-G63S (the produced virus was named ToBRFV-G63S), pCBToBRFV-H67C pCBToBRFV-V73I, pCBToBRFV-A105G, pCBToBRFV-N125A, pCBToBRFV-K129Q, pCBToBRFV-E132M, pCBToBRFV-A134N, pCBToBRFV-I147M, pCBToBRFV-A148S, pCBToBRFV-Y151F, pCBToBRFV-K166R, pCBToBRFV-I168N, pCBToBRFV-S179N, and pCBToBRFV-T181R, respectively. Wildtype and Tm-22 transgenic N. benthamiana plants were separately inoculated with ToBRFV or one of these 15 mutants. At 5 dpai, all 15 mutant viruses infected all N. benthamiana plants, similar to wildtype ToBRFV (Figure 5a, upper panel; Figure 5b). ToBRFV-G63S, ToBRFV-V73I, ToBRFV-A105G, ToBRFV-E132M, ToBRFV-A134N, ToBRFV-I147M, ToBRFV-A148S, ToBRFV-S179N, ToBRFV-K166R, ToBRFV-I168N, ToBRFV-S179N, and ToBRFV-T181R were all normal, while those of tombus plants carrying ToBRFV became narrow, while those of tomato plants inoculated with the six mutant viruses grew normally (Figure 6a). Furthermore, RT-PCR
analysis detected no viral RNA in the systemic leaves of tomato plants inoculated with these six mutants (Figure 6b).

These results show that six residues, H^{67}, N^{125}, K^{129}, A^{134}, I^{147}, and I^{168}, in the central region of ToBRFV MP are critical for ToBRFV-mediated infection in Tm-2^2 transgenic N. benthamiana plants and Tm-2^2 carrying tomato plants.

3 | DISCUSSION

In this study, we found that MP was the virulence determinant for ToBRFV-mediated infection in plants harbouring the Tm-2^2 gene, and six residues in the central region of MP (residues 60–186) were critical in enabling ToBRFV to evade Tm-2^2-mediated resistance.
Virulence determinants play an essential role in viral infections. The coevolution of viruses with their hosts leads to the development of specific resistance proteins in host plants that recognize certain viral proteins and activate resistance responses. Potato cultivars carrying Ny or Nc resistance genes activated HR after infection with potato virus Y (PVY)-O and PVY-C isolates, respectively, in which the helper component-proteinase (HC-Pro) of PVY-O and PVY-C isolates has been identified as avirulence determinants (Moury et al., 2011; Tian & Valkonen, 2013, 2015). MP of ToMV and TMV can elicit HR in plants carrying the Tm-2 gene (Chen et al., 2017; Zhao et al., 2013).
Mutations that change one or more amino acid residues in viral avirulence determinants can convert them into virulence determinants on host-carrying resistance genes. Indeed, the MP of the natural mutant ToMV-2 isolate or HC-Pro of PVY-N and PVY-O isolates is essential for the viruses to overcome Tm-22- and Nc-mediated resistance, respectively (Moury et al., 2011; Tian & Valkonen, 2013, 2015; Weber et al., 1993). Recently, by replacing the MP of ToMV with ToBRFV MP, Hak and Spiegelman (2021) showed that ToBRFV MP enabled the recombinant ToMV to infect Tm-22-carrying tomato plants. Here, we further studied the mechanism of ToBRFV infecting Tm-22-carrying plants using infectious clones of both ToBRFV and TMV. The MP of ToBRFV-SD was 78.8% identical to that of TMV-HEB2 at the amino acid level. However, ToBRFV-SD could infect Tm-22 transgenic N. benthamiana plants and tomato cultivar Jinpeng (Figure 1). We noticed that TMV and ToBRFV-TMV1-268 failed to induce distinct necrotic lesions in the inoculated leaves of Tm-22 transgenic N. benthamiana plants (Figure 1b), while transiently expressing TMV MP could (Figure 2b). A similar phenomenon has been reported previously (Du et al., 2013; Zhao et al., 2013). This may be because extreme resistance (ER) could not block avirulence determinant expression under the 35S CaMV promoter, leading to HR (Bendahmane et al., 1999). Exchanging the MP genes between these two viruses revealed that MP was essential for ToBRFV to evade Tm-22-mediated resistance (Figure 1). These results indicated that the virulence determinant MP in ToBRFV assisted in infecting plants carrying the Tm-22 gene.

The central region of the MP is necessary to overcome Tm-22-mediated resistance. Two residues, R238 and E244, and 30 residues of MP in the C-terminal may regulate the exposure of the Tm-22-recognition region (Chen et al., 2017; Weber et al., 2004). Residues 1–187 of ToMV/TMV MP are critical during the recognition between MP and Tm-22, while the C-terminal region may regulate the exposure of the Tm-22-recognition region (Chen et al., 2017; Weber et al., 2004).

FIGURE 5 Tomato brown rugose fruit virus (ToBRFV) mutants with substitution of residues H67, N125, K129, A134, I147, or I168 of ToBRFV movement protein (MP) to the corresponding residues of tobacco mosaic virus (TMV) MP cannot infect Tm-22 transgenic Nicotiana benthamiana plants. (a) Symptom and (b, c) virus accumulation in the systemically infected leaves of wildtype N. benthamiana and Tm-22 transgenic N. benthamiana plants inoculated with ToBRFV or one of 15 mutants at 5 days postagroinfiltration. Virus accumulation was detected by reverse transcription PCR. The ubiquitin gene (UBI) was used as an internal control. At least nine plants were infiltrated for each mutant in three independent experiments. The systemic leaves without symptoms are marked with red arrows, while systemically infected leaves showing viral symptoms are marked with white arrowheads.
YAN et al. (2017). Hak and Spiegelman (2021) reported that the critical region of ToBRFV MP to escape Tm-2 recognition is mapped in residues 1–216 of MP. Here, we found that the N-terminal 1–59 residues and C-terminal 187–266 residues of ToBRFV MP were not involved, while the central 60–186 residues of ToBRFV MP were involved in escaping recognition by Tm-2 (Figure 2c). Further analysis of chimeric viruses showed that 60–186 residues in the central region of MP were necessary for ToBRFV to evade Tm-2-mediated resistance (Figures 3 and 4).

Six residues located in the central region of the MP are critical. Maayan et al. performed a comprehensive genomic comparison of different tobamoviruses and predicted 12 potential Tm-2 resistance-breaking amino acid sites in ToBRFV MP (Maayan et al., 2018). Among these 12 sites, deletion of V4 is predicted to be critical, and nonsynonymous substitution mutations for the other 11 sites (D46, H57, N125, A134, A148, I168, S197, S219, E222, K243, and R245) are considered important for Tm-2 resistance-breaking (Maayan et al., 2018). However, experimental evidence of their role in resistance breaking is lacking. Here, we demonstrated that six residues (H67, N125, K129, A134, I147, and I168) of ToBRFV MP were important in enabling ToBRFV to evade Tm-2-mediated resistance (Figures 5 and 6). A comparison with the analysis results of Maayan et al. showed that the roles of four residues (H47, N125, A134, and I168) of 12 sites in Tm-2 resistance-breaking were confirmed by reverse genetics; residues K129 and I168 were novel sites responsible for overcoming Tm-2 resistance. Studies on TMV CP or PVY HC-Pro have indicated the importance of the three-dimensional structure for recognizing resistance proteins N’, Ny, and Nc (Culver et al., 1994; Taraporewala & Culver, 1996; Tian & Valkonen, 2013, 2015). Identifying the three-dimensional structure of TMV MP, ToBRFV MP, and Tm-2 will provide additional details on the escape mechanism of ToBRFV MP.

Overall, MP is the virulence determinant for ToBRFV, enabling infection of Tm-2 transgenic N. benthamiana and Tm-2-carrying tomato plants, and six residues (H67, N125, K129, A134, I147, and I168) of ToBRFV MP are critical for ToBRFV to evade Tm-2-mediated resistance. These results increase our understanding of the pathogenesis of ToBRFV.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials

Transgenic N. benthamiana expressing the full-length Tm-2 gene under its native promoter was kindly provided by Professor Yule Liu from Tsinghua University (Zhang et al., 2013). Tomato cv. Jinpeng, which contains the Tm-2 gene, was obtained from Xi’an Jinpeng Seedling Company Limited. Tm-2 genes present in transgenic N. benthamiana and tomato cv. Jinpeng plants were confirmed by sequencing. Wildtype and Tm-2 transgenic N. benthamiana plants and tomato plants were grown at 23 °C under a 16 hr light/8 hr dark photoperiod.

4.2 | Plasmid construction

The full-length genomic fragments of the isolates ToBRFV-SD (Yan et al., 2021b) (accession number MT018320) and TMV-HEB2 (Jiang et al., 2019) (MH595920) were separately amplified by PCR using Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech) and inserted into the binary plasmid pCB301-2×35S-Nos to generate infectious clones pCBToBRFV and pCBTMV, respectively. The MP
genes of ToBRFV and TMV were separately amplified and transferred into pCam-35S::GFP-Nos to produce pCamGFP-ToBRFVMP and pCamGFP-TMVMP, respectively. Chimeric viruses/MPs were obtained by exchanging the complete or partial MP-coding sequences between ToBRFV and TMV. The above vectors were obtained by sequence- and ligation-independent methods using a commercial kit (Applied Biological Materials). Site-directed mutagenesis was performed according to a previously reported protocol (Liu & Naismith, 2008). The primers used for plasmid construction are listed in Table S1.

### 4.3 Virus inoculation and protein transient expression

The chimeric viruses/MPs were separately transformed into Agrobacterium tumefaciens GV3101 cells. Agrobacterium cultures harbouring different plasmids were prepared as previously described (Yan et al., 2021a) and diluted to OD600 = 0.2 for virus inoculation and protein transient expression. The agroinfiltrated plants were maintained in a growth room at 23 °C under a 16 hr light/8 hr dark cycle, and photographed under white light using a digital camera (Canon 800D).

### 4.4 RNA extraction, RT-PCR, and RT-qPCR

Total RNA was extracted from leaves of wildtype or Tm-22 transgenic N. benthamiana plants using TransZol reagent (TransGen Biotech) and treated with gDNA wiper (Vazyme) to eliminate DNA contamination. Reverse transcription was performed using random primers or gene-specific primers and HiScript II reverse transcriptase (Vazyme) following the manufacturer’s instructions. Taq DNA polymerase was used to perform PCR. RT-qPCR was conducted using the ChamQ SYBR qPCR master mix (Vazyme). The primers used for amplification are listed in Table S1.

### 4.5 Trypan blue staining

N. benthamiana leaves were boiled in a mixture of ethanol and stock solution of the stain (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, 10 ml water, 20 mg trypan blue) at a ratio of 1:2 for 5 min, washed with 2.5 g/ml chloral hydrate solution, and photographed on a white light box using a digital camera (Canon 800D).

### 4.6 Western blot

Western blotting was performed as previously described (Yan et al., 2021a). Total protein was extracted from N. benthamiana leaf tissue samples. An anti-GFP antibody (Abways) was used as the primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich) was used as the secondary antibody. SuperSignal West Dura Extended Duration Substrate solution (Thermo Fisher Scientific) was used as the HRP substrate. The reaction signal was detected using a chemiluminescent imaging and analysis system (Sage).

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### DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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