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The role of different innate and environmental factors in *Tm-2*^{2}-mediated resistance to tomato mottle mosaic virus

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Abstract

Tomato mottle mosaic virus (ToMMV) poses a threat to production and quality of tomato fruits. The *Tm-2*^{2} gene confers resistance to some tobamoviruses by recognizing viral movement proteins. However, *Tm-2*^{2}-mediated resistance against ToMMV is not well known. Here, we found that ToMMV could infect wild-type but not *Tm-2*^{2} transgenic *Nicotiana benthamiana* plants and could also infect tomato cultivar Moneymaker but not resistant cultivar Jili with homozygous *Tm-2*^{2}. Chimeric viral ToMMV$^{ToBRFV-MP}$ with swapped ToMMV MP to MP of tomato brown rugose fruit virus could systemically infect *Tm-2*^{2} transgenic *N. benthamiana* and tomato cultivars Jili plants. Further, transient expression of ToMMV MP in the leaves of *Tm-2*^{2} transgenic *N. benthamiana* plants induced hypersensitive response-associated cell death, suggesting that the MP of ToMMV was the avirulent factor for the *Tm-2*^{2} resistance gene. ToMMV could infect *Tm-2*^{2}-containing cultivar Jinpeng 1 but not Chaobei. Sequence analysis revealed that cultivars Chaobei and Jinpeng 1 were heterozygous, where Chaobei consists of *Tm-2*^{2} and *Tm-2* genes, while Jinpeng 1 consists of *Tm-2*^{2} and *tm-2* genes. Transient co-expression assays showed that both *Tm-2*^{2} and *Tm-2* but not *tm-2* could recognize ToMMV MP and induce hypersensitivity response-associated cell death in *N. benthamiana* leaves, suggesting that homozygous tomato harboring *Tm-2*^{2} and heterozygous tomato containing *Tm-2*^{2} and *Tm-2* may exhibit more durable resistance to ToMMV than heterozygous tomato carrying *Tm-2*^{2} and *tm-2*. Further, *Tm-2*^{2} transgenic *N. benthamiana* and tomato cultivar Jili plants with silenced *Tm-2*^{2} gene were susceptible to ToMMV. Also, silencing type-I J-domain *MIP1* gene compromised *Tm-2*^{2}-mediated resistance to ToMMV in *Tm-2*^{2} transgenic *N. benthamiana* and tomato cultivar Jili. Moreover, we found that viral RNA could accumulate in the systemic leaves of *Tm-2*^{2} transgenic *N. benthamiana* plants and tomato cultivar Jili at 35°C, but not at 20, 25, or 30°C. Altogether, our findings reveal that the *Tm-2*^{2} confers resistance to ToMMV by recognizing MP, and the resistance is regulated by the allele combinations, accumulation levels of *Tm-2*^{2}, *MIP1*, and the temperature.

Keywords Tomato mottle mosaic virus, *Tm-2*^{2}, *MIP1*, Movement protein, *Tm-2*^{2} allele composition, Temperature

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Background

Tomato (*Solanum lycopersicum*) is an economically important vegetable crop grown worldwide. However, the yield and quality of tomato fruits are seriously threatened by tobamoviruses, such as tomato mosaic virus (ToMV), tobacco mosaic virus (TMV), the emerging tomato brown rugose fruit virus (ToBRFV), and tomato mottle mosaic virus (ToMMV) (Rivarez et al. 2021).

ToMMV was firstly reported in infected greenhouse tomato plants in Mexico (Li et al. 2013) and since then it has been reported in many other countries (Webster et al. 2014; Turina et al. 2016; Ambrós et al. 2017; Sui et al. 2017; Nagai et al. 2019; Lovelock et al. 2020), including China (Li et al. 2014, 2020; Chai et al. 2018; Che et al. 2018; Zhan et al. 2018; Tettey et al. 2022).

To resist viral diseases, plants have evolved resistance (*R*) gene-mediated innate immune systems (Kourelis and Van Der Hoorn 2018). The proteins encoded by the *R* genes can recognize the avirulence (Avr) factors of the viruses and trigger a cascade of signals that leads to hypersensitive response (HR) or extreme resistance at the point of infection (Dangl et al. 2013). Growing crops harboring *R* genes is an economically and environmentally friend way compared to the use of chemical pesticides. For this reason, many cultivated crops adopt *R* genes against viral infection. Examples include the *N* gene of *Nicotiana glutinosa* against TMV (Dinesh-Kumar et al. 1995; Marathe et al. 2002), *L* genes of pepper against some tobamoviruses (Boukema 1980), and *Tm-1, Tm-2,* and *Tm-2*^{2} in tomato against ToMV infection (Lanfermeijer et al. 2003; Ishibashi et al. 2007).

The *Tm-2*, *Tm-2*^{2}, and *tm-2* genes belong to the coiled coil-nucleotide binding site-leucine rich repeat (CC-NB-LRR) *R* gene family. The *Tm-2* and *Tm-2*^{2} alleles confer resistance to TMV and ToMV by recognizing movement proteins (MPs), whereas the *tm-2* allele cannot (Weber and Pfitzner 1998; Lanfermeijer et al. 2003). The expression levels of *Tm-2*^{2} influence the function of *Tm-2*^{2} in resisting TMV infections (Zhang et al. 2013). Also, silencing of *NbMIP1* or *NbHsp90* compromised *Tm-2*^{2}-mediated resistance against TMV and ToMV in the *Tm-2*^{2} transgenic *Nicotiana benthamiana* plants (Du et al. 2013; Qian et al. 2018). However, the *tm-2*, *Tm-2*, or *Tm-2*^{2} cannot mount resistance to ToBRFV (Luria et al. 2017; Hak and Spiegelman 2021; Yan et al. 2021a), and the mechanism of their resistance to ToMMV is not clear.

Temperature affects *R* gene-mediated resistance against some plant viruses. TMV could infect heterozygous *Tm-2*^{2} tomato plants systemically at 30–31°C (Pilowsky et al. 1981). The *N* gene of tobacco confers resistance to TMV, but a temperature of 28°C suppressed the resistance mediated by *N* gene (Whitham et al. 1996). The *Tsw*-mediated resistance against tomato spotted wild virus (TSWV) in pepper plants is also suppressed at a continuously high temperature of 32°C (Moury et al. 1998). However, the *Rx* gene of potato conferred resistance to potato virus X (PVX) at a temperature of 32°C (Richard et al. 2020). Further, the *R-BPMV* resistance gene in *Phaseolus vulgaris* provides resistance against bean pod mottle virus (BPMV) at temperatures up to 35°C (Meziadi et al. 2021).

Here, we found that *Tm-2*^{2} displayed resistance to ToMMV by recognizing the MP. The *Tm-2*^{2}-mediated resistance was affected by *Tm-2*^{2} expression levels which may be attributed to the allele combinations, *MIP1* and temperature. This study will be insightful for breeding resistant tomato cultivars to ToMMV and designing cultivation management methods to control ToMMV.

Results

Tm-2^2 confers resistance to ToMMV

To determine the resistance of *Tm-2*^{2} to ToMMV, we inoculated the wild-type and *Tm-2*^{2} transgenic *N. benthamiana* plants with ToMMV. At 7 days post-agro-infiltration (dpai), the systemic leaves of the wild-type *N. benthamiana* plants showed mosaic and epinasty symptoms, whereas the *Tm-2*^{2} transgenic *N. benthamiana* plants showed no symptoms (Fig. 1a). Reverse transcription-PCR (RT-PCR) analysis showed the presence of ToMMV RNA in the systemic leaves of the wild-type *N. benthamiana* that was agro-infiltrated with ToMMV but not in the *Tm-2*^{2} transgenic *N. benthamiana* plants (Fig. 1b). Western blot analysis also showed a detectable level of ToMMV coat protein (CP) in the systemic leaves of the wild-type *N. benthamiana* plants but not the *Tm-2*^{2} transgenic *N. benthamiana* plants (Fig. 1c). This note was also observed in tomato cultivar Jili carrying homozygous *Tm-2*^{2} (*Tm-2*^{2}*/Tm-2*^{2}) and Moneymaker carrying homozygous *tm-2* (*tm-2/tm-2*) plants. No symptoms were observed in ToMMV-challenged Jili tomato plants, while the tomato cultivar Moneymaker showed leaf malformation and mosaic in the systemic leaves (Fig. 1d). RT-PCR analysis also showed the presence of ToMMV RNA in the systemic leaves of the tomato cultivar Moneymaker but not in the tomato cultivar Jili (Fig. 1e). Western blot analysis also showed a detectable level of ToMMV CP in the systemic leaves of tomato cultivar Moneymaker but not Jili (Fig. 1f). These results show that *Tm-2*^{2} conferred resistance to ToMMV.

The MP of ToMMV is the avirulence factor for *Tm-2*^{2}

To determine whether the ToMMV *MP* gene is an *Avr* gene for the *Tm-2*^{2} gene, we swapped the *MP* genes of ToMMV and ToBRFV infectious clones to generate chimeric viruses ToMMV$^{MP-ToBRFV}$ and ToBRFV$^{MP-ToMMV}$, respectively (Fig. 2a). At 7 dpai, like the wild-type viruses, ToMMV$^{MP-ToBRFV}$ and ToBRFV$^{MP-ToMMV}$ induced epinastic viral symptoms in the wild-type *N. benthamiana* plants (Fig. 2b). RT-PCR detection showed the presence of viral RNA in the systemic leaves of the wild-type *N. benthamiana* plants (Fig. 2c). However, ToBRFV and ToMMV$^{MP-ToBFRV}$, but not ToMMV or ToBRFV$^{MP-ToMMV}$, induced epinasty symptoms on the *Tm-2*^{2} transgenic *N. benthamiana* plants at 7 dpai (Fig. 2d). The

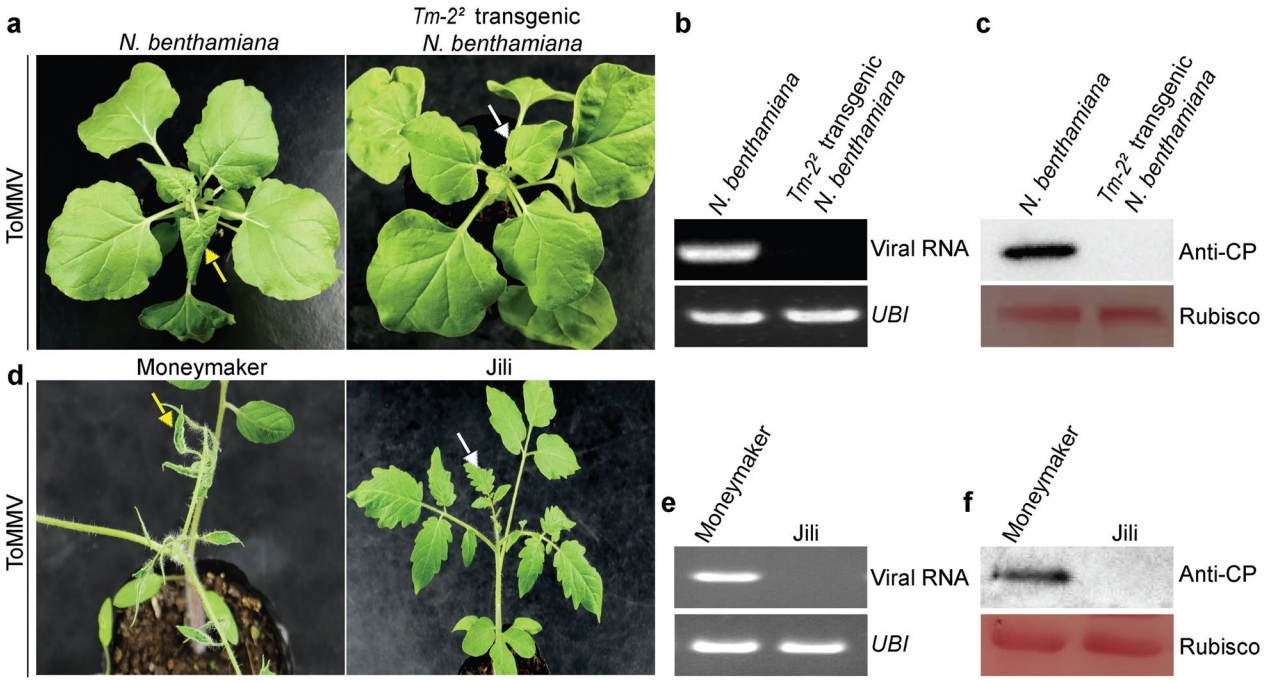

Fig. 1 *Tm-2²*-mediated resistance against ToMMV infection. **a** The symptoms of ToMMV-inoculated wild-type and *Tm-2²* transgenic *Nicotiana benthamiana* plants at 7 days post-agro-infiltration (dpai). **b** RT-PCR detection of ToMMV RNA in systemic leaves of wild-type and *Tm-2²* transgenic *N. benthamiana* plants. The *ubiquitin* (*UBI*) gene was used as an internal control. **c** Western blot analysis of ToMMV CP in the systemic leaves of the wild-type and the *Tm-2²* transgenic *N. benthamiana* plants using CP antibody (anti-CP). **d** The symptoms of ToMMV-inoculated tomato cultivars Moneymaker and Jili at 14 dpai. **e** RT-PCR detection of ToMMV RNA in systemic leaves of tomato cultivars Moneymaker and Jili. **f** Western blot analysis of ToMMV CP in the systemic leaves of tomato cultivars Moneymaker and Jili. Yellow arrows show symptomatic systemic leaves and white arrows show asymptomatic systemic leaves

RT-PCR detection showed the presence of viral RNA in the systemic leaves of ToBRFV and ToMMV$^{MP-ToBFRV}$ inoculated *Tm-2²* transgenic *N. benthamiana* plants but not in those infiltrated with ToMMV or ToBRFV$^{MP-ToMMV}$ (Fig. 2e). We also inoculated tomato cultivars Jili and Moneymaker with ToMMV, ToBRFV, ToMMV$^{MP-ToBFRV}$, and ToBRFV$^{MP-ToMMV}$. At 14 dpai, the wild-type ToBRFV and ToMMV treatments induced leaf malformation and mosaic symptoms, whereas the chimeras induced milder symptoms, including leaf mosaic and malformation symptoms in tomato cultivar Moneymaker (Fig. 2f). RT-PCR detection also showed the presence of viral RNA in systemic leaves of infected tomato cultivar Moneymaker (Fig. 2g). ToBRFV or ToMMV$^{MP-ToBFRV}$ induced leaf malformation and mosaic symptoms respectively in tomato cultivar Jili, but no symptoms were observed for ToMMV or ToBRFV$^{MP-ToMMV}$ (Fig. 2h). The RT-PCR detection results showed systemic infection of ToBRFV and ToMMV$^{MP-ToBFRV}$ in tomato cultivar Jili (Fig. 2i).

To further confirm that the MP is an Avr factor for *Tm-2²*, we transiently expressed the MPs of ToMMV, ToMV, TMV, and ToBRFV in *Tm-2²* transgenic *N. benthamiana* leaves. HR was induced at the point of infiltration for the MPs of ToMMV, ToMV, and TMV, but no HR was induced for ToBRFV MP (Additional file 1: Figure S1). These results show that the MP of ToMMV is the Avr factor of *Tm-2²*.

The *Tm-2²* allele composition influenced the tomato plant resistance against ToMMV

By screening resistant tomato cultivars, we found that two cultivars, Chaobei and Jinpeng 1 which carry *Tm-2²*, displayed distinct resistance to ToMMV. At 14 dpai, cultivar Jinpeng 1 showed leaf mosaic, stunting, and necrotic spot symptoms in systemic leaves, whereas Chaobei plants did not exhibit viral disease symptoms when challenged with ToMMV (Fig. 3a upper row). However, ToBRFV induced mosaic and deformed leaf symptoms in the systemic leaves of both Jinpeng 1 and Chaobei plants at 14 dpai (Fig. 3a lower row). RT-PCR assays using the systemic leaves showed the presence of ToMMV RNA in cultivar Jinpeng 1 but not in cultivar Chaobei at 14 dpai (Fig. 3b). However, ToBRFV RNA was detected in the systemic leaves of both Jinpeng 1 and Chaobei at 14 dpai (Fig. 3c).

To determine whether the distinct resistance observed in tomato cultivars Jinpeng 1 and Chaobei is due to

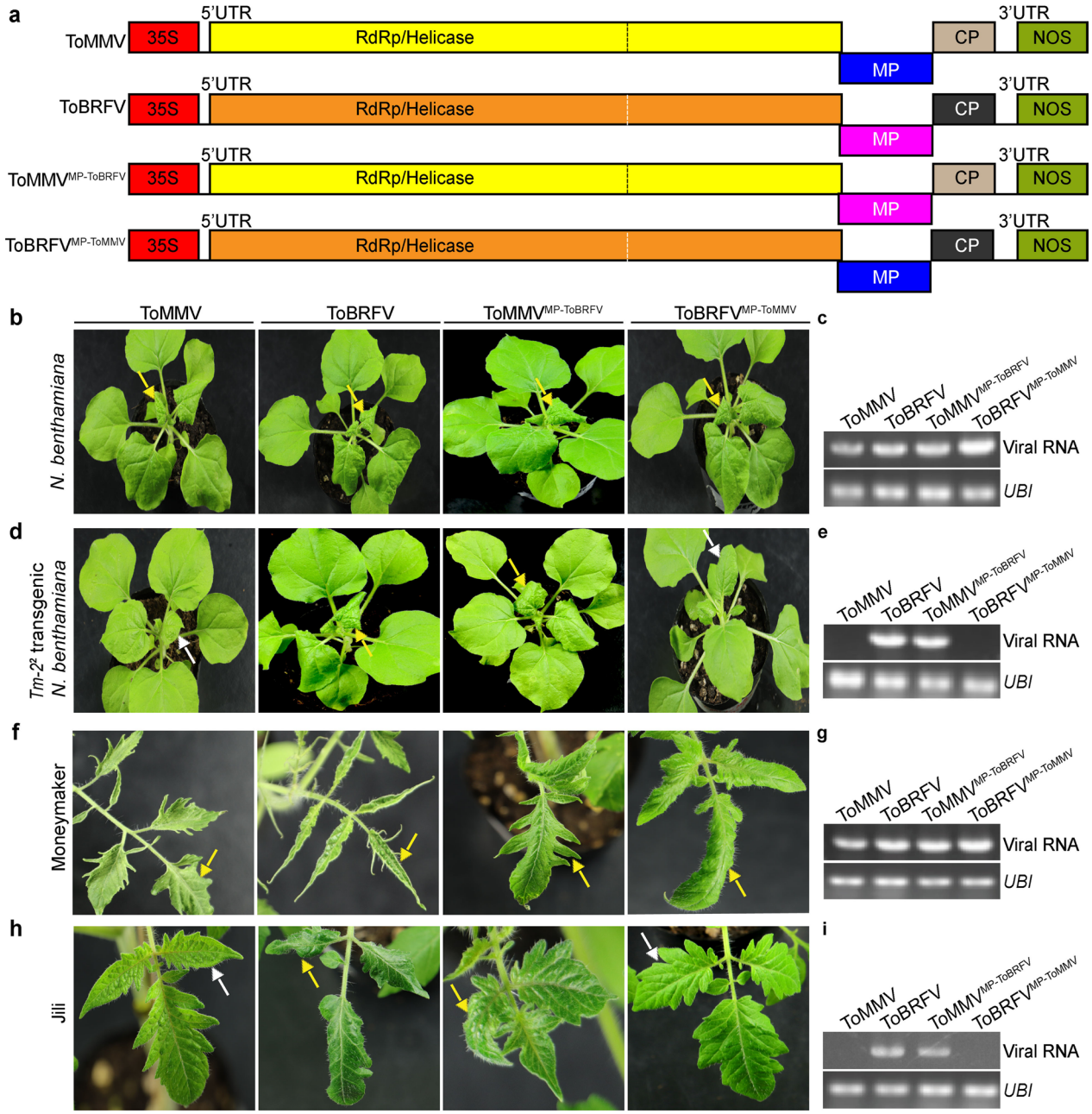

Fig. 2 The MP of ToMMV is the avirulence factor of *Tm-2*^{2}. **a** Diagram of chimeric viruses of ToMMV$^{MP-ToBRFV}$ and ToBRFV$^{MP-ToMMV}$. **b**, **d** Symptom expression in wild-type *Nicotiana benthamiana* (**b**) and *Tm-2*^{2} transgenic *N. benthamiana* plants (**d**) inoculated with ToMMV, ToBRFV, ToMMV$^{MP-ToBRFV}$, and ToBRFV$^{MP-ToMMV}$ at 7 days post-agro-infiltration (dpai). **c**, **e** RT-PCR detection of RNA in the wild-type **(c)** and *Tm-2*^{2} transgenic *N. benthamiana* plants (**e**) inoculated with ToMMV, ToBRFV, ToMMV$^{MP-ToBRFV}$, and ToBRFV$^{MP-ToMMV}$. **f**, **h** Symptom expression in tomato cultivars Moneymaker (**f**) and Jili (**h**) infiltrated with ToMMV, ToBRFV, ToMMV$^{MP-ToBRFV}$, and ToBRFV$^{MP-ToMMV}$ at 14 dpai. **g**, **i** RT-PCR detection of RNA of ToMMV, ToBRFV, ToMMV$^{MP-ToBRFV}$, and ToBRFV$^{MP-ToMMV}$ in tomato cultivar Moneymaker (**g**) and Jili (**i**). Yellow arrows show symptomatic systemic leaves and white arrows show asymptomatic systemic leaves. The *ubiquitin* (*UBI*) gene served as an internal control for RT-PCR detection

the *Tm2*^{2} allelic composition, we obtained the coding sequences of *tm-2*, *Tm-2*, and *Tm-2*^{2} from the NCBI database and aligned them with each other. We observed that the 2135th nucleotides were C, T, and T, and 2136th nucleotides were A, C, and T, respectively, in *tm-2*, *Tm-2*, and *Tm-2*^{2} (Fig. 3d). We amplified and sequenced the region spanning the 2136th nucleotide of the *tm-2*, *Tm-2*, and *Tm-2*^{2} from Jinpeng 1 and Chaobei tomato cultivars.

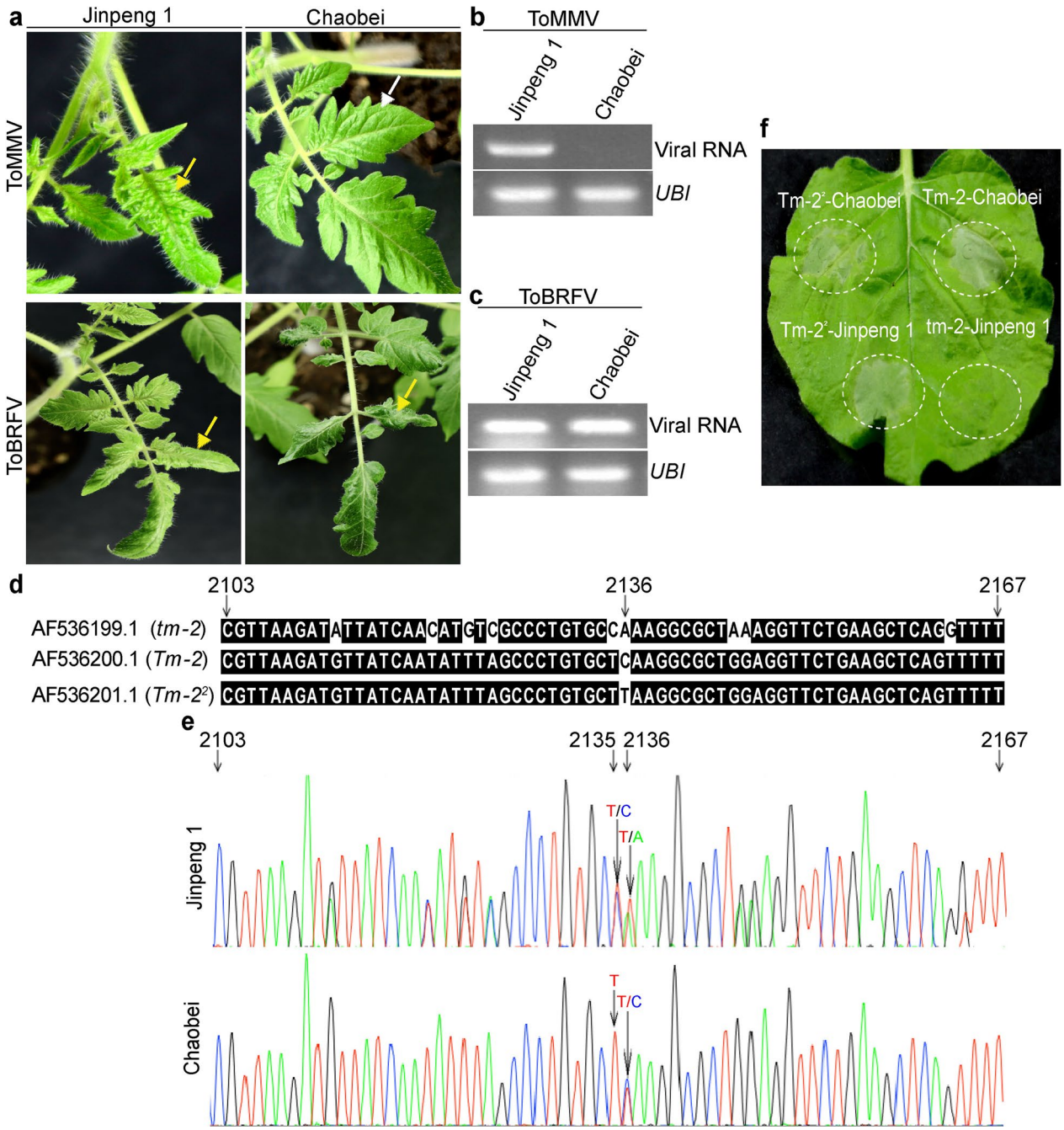

Fig. 3 Effect of *Tm-2²* allele composition of tomato cultivars on the disease resistance against ToMMV. **a** The symptoms of systemic leaves of tomato cultivars Jinpeng 1 (*Tm-2²/tm-2*) and Chaobei (*Tm-2²/Tm-2*) inoculated with ToMMV or ToBRFV at 14 days post-agro-infiltration. **b**, **c** RT-PCR analysis of ToMMV and ToBRFV RNA in the systemic leaves of tomato cultivars Jinpeng 1 and Chaobei. *UBI* gene was used as an internal control. This experiment was repeated three times. **d** Alignment of partial coding sequences of *tm-2* (accession number; AF536199.1), *Tm-2* (accession number; AF536200.1), and *Tm-2²* (accession number; AF536201.1). **e** Sequence chromatogram showing the allele composition of tomato cultivars Jinpeng 1 and Chaobei. **f** Hypersensitive response-associated cell death in wild-type *Nicotiana benthamiana* leaves co-expressed with ToMMV MP and *Tm-2²*, *Tm-2*, or *tm-2*. Tm-2²-Chaobei and Tm-2-Chaobei were derived from Chaobei tomato and Tm-2²-Jinpeng 1 or tm-2-Jinpeng 1 was derived from Jinpeng 1 tomato. The picture was taken at 48 h post-agro-infiltration. Yellow arrows show symptomatic systemic leaves and white arrows show asymptomatic systemic leaves

Based on the sequence chromatograms, we analyzed the allele compositions of the *Tm-2* and *Tm-2²* in tomato cultivars Jinpeng 1 and Chaobei. The tomato cultivar Jinpeng 1 showed an overlap of nucleotides T and C at position 2135 and T and A at position 2136, signifying the presence of both *Tm-2²* and *tm-2* alleles (TC/TA). Also, cultivar Chaobei showed no overlap with nucleotide T at position 2135 and an overlap of nucleotides T and C at position 2136, implying the presence of the *Tm-2²* and *Tm-2* alleles (TT/TC) (Fig. 3e). The full-length sequences of *Tm-2²* and *tm-2* from cultivar Jinpeng 1 or *Tm-2²* and *Tm-2* from cultivar Chaobei were cloned into pCam35S binary vector to produce pCamTm-2²-Jinpeng 1, pCamtm-2-Jinpeng 1, pCamTm-2²-Chaobei, and pCamTm-2-Chaobei. The sequencing results further confirmed the allelic results along with the reference genes (Additional file 1: Figure S2). We co-expressed ToMMV MP with *Tm-2²*-Chaobei, *Tm-2*-Chaobei, *Tm-2²*-Jinpeng 1, and *tm-2*-Jinpeng 1 in wild-type *N. benthamiana* leaves. At 48 hpai, *Tm-2*-Chaobei, *Tm-2²*-Chaobei, and *Tm-2²*-Jinpeng 1 induced HR at the infiltration site, whereas *tm-2*-Jinpeng 1 induced no HR at the infiltration site (Fig. 3f). These results indicated that the *Tm-2²* allele composition might affect the resistance spectrum of tomatoes against ToMMV.

Tm-2² accumulation is important for its resistance to ToMMV

To determine whether the expression levels of *Tm-2²* in the transgenic *Tm-2² N. benthamiana* and the tomato cultivar Jili are critical for their resistance to ToMMV, we silenced *Tm-2²* using a TRV-based VIGS vector. At 10 dpai, RT-quantitative PCR (RT-qPCR) results showed that the silencing efficiency of *Tm-2²* in the *Tm-2²* transgenic *N. benthamiana* was about 85% when compared to the control plants that were inoculated with TRV-*GUS* (Fig. 4a). The silenced and the control *Tm-2²* transgenic *N. benthamiana* plants were then inoculated with ToMMV. At 7 dpai, viral symptoms were observed in the systemic leaves of the silenced *Tm-2²* transgenic *N.*

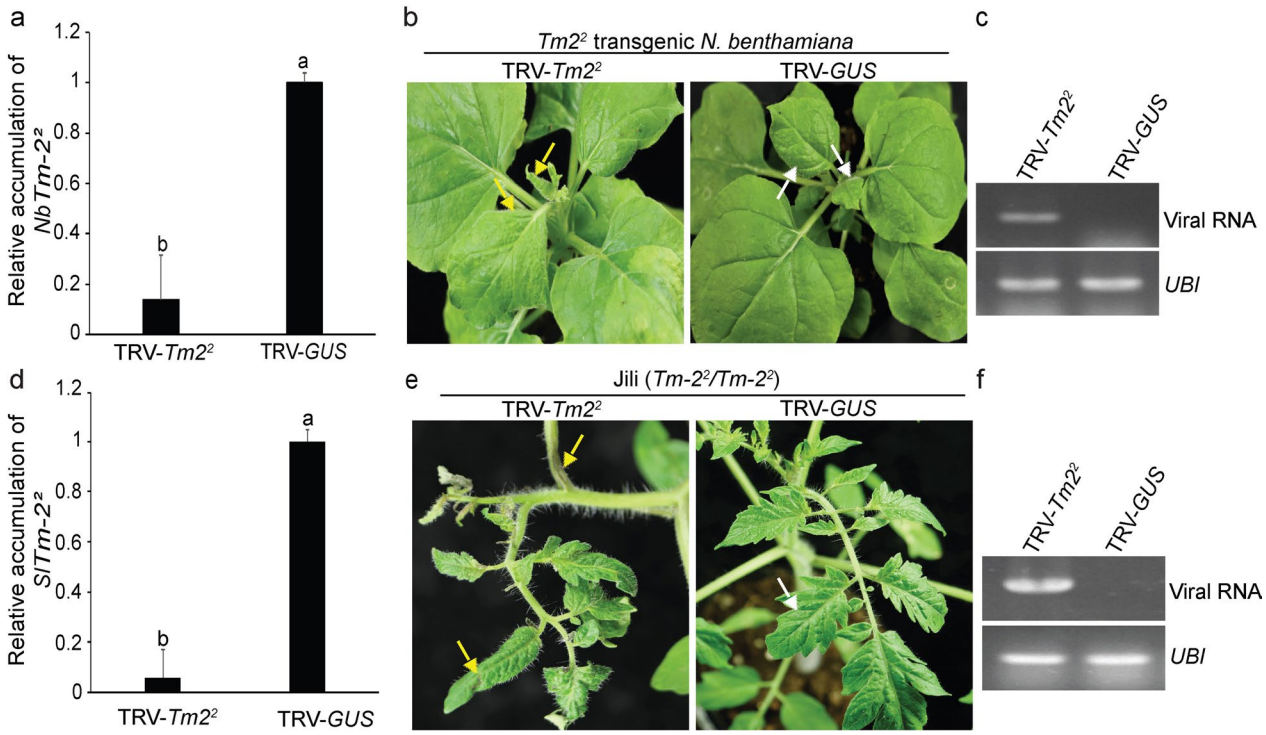

Fig. 4 Effects of *Tm-2²* accumulation on the disease resistance against ToMMV. **a** RT-qPCR analysis of the *Tm-2²* mRNA accumulation levels in the silenced and control *Tm-2²* transgenic *Nicotiana benthamiana* plants inoculated with TRV-derived vector at 10 days post-agro-infiltration (dpai). Yellow arrows show symptomatic systemic leaves and white arrows show asymptomatic systemic leaves. **b** The symptoms of *Tm-2²*-silenced and the control *Tm-2²* transgenic *N. benthamiana* plants inoculated with ToMMV at 7 dpai. **c** RT-PCR detection of ToMMV RNA in the systemic leaves of the silenced and the control *Tm-2²* transgenic *N. benthamiana* plants. **d** RT-qPCR analysis of *Tm-2²* mRNA accumulation levels in the silenced and the control tomato cultivar Jili inoculated TRV-derived vector at 10 dpai. **e** The symptoms of *Tm-2²*-silenced and control Jili tomato plants inoculated with ToMMV at 14 dpai. **f** RT-PCR detection of ToMMV RNA accumulation in systemic leaves of *Tm-2².²*-silenced and control plants of tomato cultivar Jili. The mRNA accumulation of the *UBI* gene served as an internal control. The values indicate the mean + SD of three biological replicates. Letters indicate significant differences in different treatments ($P < 0.05$)

benthamiana plants but not in the control *Tm-2*^{2} transgenic *N. benthamiana* plants (Fig. 4b). RT-PCR results also showed that ToMMV RNA was present in the systemic leaves of the silenced *Tm-2*^{2} transgenic *N. benthamiana* plants but not in the control plants (Fig. 4c). This experiment was repeated with the resistant tomato cultivar Jili. The silencing efficiency of *Tm-2*^{2} in tomato cultivar Jili was about 90% compared to the control plants (Fig. 4d). At 14 dpai, the systemic leaves of silenced tomato cultivar Jili showed leaf necrosis, mottling, and stunting, while no symptom was observed in the control tomato cultivar Jili (Fig. 4e). RT-PCR results confirmed the presence of ToMMV RNA only in the systemic leaves of the *Tm-2*^{2}-silenced tomato cultivar Jili (Fig. 4f). These results indicated that Tm-2^2 accumulation is important to resist ToMMV infection.

The *MIP1* gene is critical for *Tm-2*^{2}-mediated resistance against ToMMV

Silencing of the *NbMIP1* is known to compromise *Tm-2*^{2}-mediated resistance against TMV in *Tm-2*^{2} transgenic *N. benthamiana* (Du et al. 2013). To determine whether *S. lycopersicum MIP1* (*SlMIP1*) or *NbMIP1* is involved in *Tm-2*^{2}-mediated resistance to ToMMV, we silenced *SlMIP1* and *NbMIP1* using TRV-based VIGS vectors. At 10 dpai, the silencing efficiency of *NbMIP1* was found to be about 95% compared to the control plants (Fig. 5a). The silenced and the control *Tm-2*^{2} transgenic *N. benthamiana* plants were then inoculated with ToMMV. At 7 dpai, no clear symptoms were observed for the *NbMIP1*-silenced *Tm-2*^{2} transgenic *N. benthamiana* plants due to distortions of the leaves (Fig. 5b). However, RT-PCR results showed the presence of viral RNA in the systemic leaves of the *NbMIP1*-silenced plants but not in the control plants (Fig. 5c). This result was further confirmed by Western blot analysis. The results showed ToMMV systemic infection in the *NbMIP1*-silenced plants but not in the control plants (Fig. 5d). We further performed the experiments in the resistant Jili tomato plants. The silencing efficiency of *SlMIP1* was about 85% for Jili plants (Fig. 5e). We mechanically inoculated the control and the *SlMIP1*-silenced Jili plant with ToMMV. At 14 dpai, the silenced tomato plants showed epinastic and necrotic leaf symptoms, whereas the control plants showed no

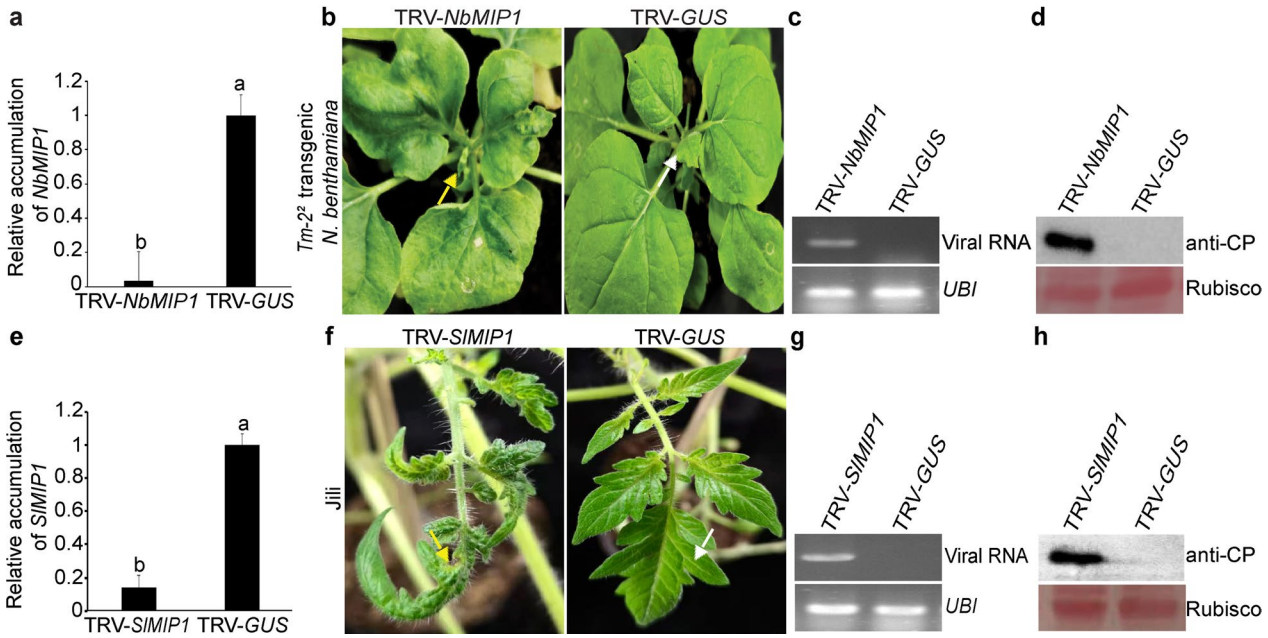

Fig. 5 Effect of *MIP1* on the *Tm-2*^{2}-mediated disease resistance against ToMMV. **a** RT-qPCR analysis of the *NbMIP1* mRNA accumulation levels in the silenced and the control *Tm-2*^{2} transgenic *Nicotiana benthamiana* plants inoculated with TRV-derived vector at 10 days post-agro-infiltration (dpai). **b** The symptoms of the control and *NbMIP1*-silenced *Tm-2*^{2} transgenic *N. benthamiana* plant inoculated with ToMMV at 7 dpai. **c, d** RT-PCR detection of the ToMMV RNA accumulation (**c**) and Western blot analysis of ToMMV CP accumulation (**d**) in the systemic leaves of *NbMIP1*-silenced and the control *Tm-2*^{2} transgenic *N. benthamiana* plant. **e** Silencing efficiency of *SlMIP1* gene in Jili tomato plants analyzed by RT-qPCR at 10 dpai. **f** The symptoms of *SlMIP1*-silenced Jili tomato and the control plants inoculated with ToMMV at 14 dpai. **g** RT-PCR detection of ToMMV RNA in the systemic leaves of *SLMIP1*-silenced and control Jili tomato plants inoculated with ToMMV at 14 dpai. **h** Western blot analysis of ToMMV coat protein (CP) in systemic leaves of the *SlMIP1*-silenced and the control plants at 14 dpai. The *UBI* gene served as an internal control for the RT-PCR and RT-qPCR analysis. The values indicate the mean + SD of three biological replicates. Letters indicate the significant differences in *MIP1* accumulation between the *MIP1*-silenced and the control plants (*t*-test, $P < 0.05$). Yellow arrows show symptomatic systemic leaves and white arrows show asymptomatic systemic leaves

symptoms (Fig. 5f). RT-PCR results showed the presence of ToMMV RNA in the systemic leaves of the silenced tomato plants but not in the control plants (Fig. 5g). Western blot analysis using an anti-CP antibody showed accumulation of ToMMV CP in the systemic leaves of the *SlMIP1*-silenced Jili plant but not in the control plant (Fig. 5h). This result shows that the accumulation of *MIP1* is also critical for *Tm-2*^{2}-mediated resistance.

Temperature modulates *Tm-2*^{2}*-mediated resistance against* ToMMV

To determine whether temperature variations affect *Tm-2*^{2}-mediated resistance, we put ToMMV infected tomato cultivar Jili and *Tm-2*^{2} transgenic *N. benthamiana* plants in bio-chambers at 20, 25, 30, and 35°C. At 14 dpai, no disease symptoms were observed for Jili plants under 20, 25, and 30°C conditions when compared to the control plants, revealing a decreasing disease symptom with the increasing temperatures. However, the systemic leaves (6/18 plants of cultivar Jili with three repeats) showed mild symptoms at 35°C (Fig. 6a upper panel). RT-PCR results showed that ToMMV RNA could only be detected in the systemic leaves of Jili plants exposed to 35°C (Fig. 6b). The tomato cultivar Moneymaker was infected by ToMMV under different temperatures (Fig. 6a lower panel and Fig. 6c). At 7 days post-inoculation (dpi), the systemic leaves (12/18) of *Tm-2*^{2} transgenic *N. benthamiana* showed systemic necrosis at 35°C but not under other temperatures (Fig. 6d upper panel). The control wild-type *N. benthamiana* expressed variable disease symptoms under different temperatures (Fig. 6d lower panel). RT-PCR results showed the viral RNA was only present in the systemic leaves of the *Tm-2*^{2} transgenic *N. benthamiana* plant exposed to 35°C (Fig. 6e), while viral

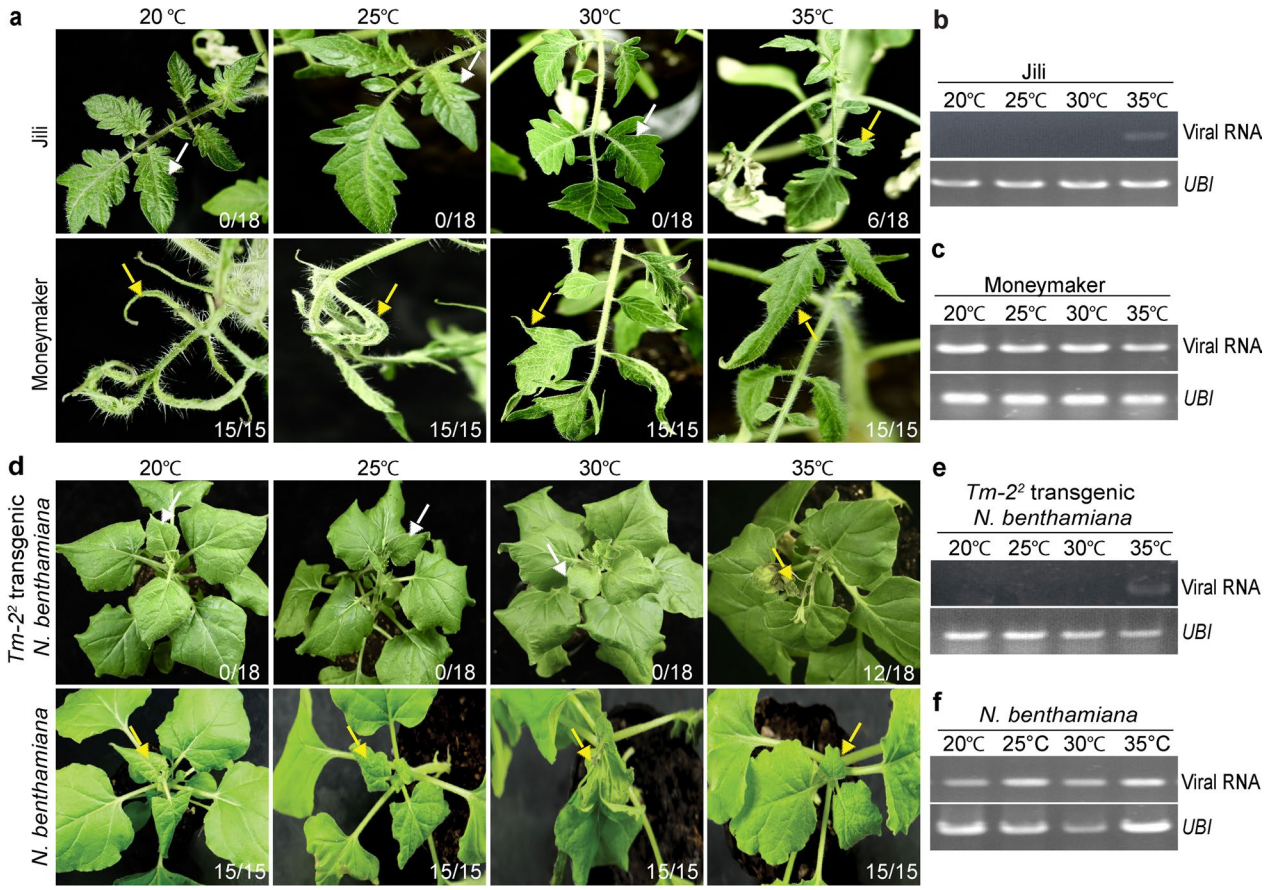

Fig. 6 Effect of temperature on the *Tm-2*^{2}-mediated disease resistance against ToMMV. **a** The symptoms of tomato cultivars Jili (upper panel) and susceptible Moneymaker (lower panel) inoculated with ToMMV and exposed to temperatures of 20, 25, 30, and 35°C at 14 days post-inoculation (dpi). **b**, **c** RT-PCR detection of ToMMV RNA in systemic leaves of tomato cultivars Jili and Moneymaker respectively exposed to different temperatures. **d** The symptoms of *Tm-2*^{2} transgenic (upper panel) and wild-type (lower panel) *Nicotiana benthamiana* plants inoculated with ToMMV and exposed to temperatures of 20, 25, 30, and 35°C at 7 dpi. **e**, **f** RT-PCR detection of ToMMV RNA in the systemic leaves of the *Tm-2*^{2} transgenic and wild-type *N. benthamiana* plants. The numbers in the lower right corner of each picture indicate the number of infected plants out of the total inoculated plants for three experimental repeats. The yellow arrows show symptomatic systemic leaves and white arrows show asymptomatic systemic leaves

RNA could be detected in the systemic leaves of all the wild-type *N. benthamiana* plants under different temperatures (Fig. 6f). These results suggest that *Tm-2*^{2}-mediated resistance was compromised at 35°C, indicating that temperatures may modulate disease symptoms in susceptible plants.

Discussion

Tm-2^2 recognizes MP to induce resistance against ToMMV

In tomato plants, the *Tm-2* and the more durable resistance gene *Tm-2*^{2} were reported to confer resistance against TMV and ToMV (Lanfermeijer et al. 2003). In this study, we also observed that the *Tm-2*^{2} allele was effective in controlling ToMMV infection in *Tm-2*^{2} transgenic *N. benthamiana* plants and tomato cultivar Jili (*Tm-2*^{2}*/Tm-2*^{2}) (Fig. 1). These results suggest that *Tm-2*^{2} gene confers resistance against ToMMV in tomato plants.

Previous reports showed that *Tm-2*^{2} confers resistance to TMV and ToMV by recognizing MPs (Zhao et al. 2013; Chen et al. 2017), but *Tm-2*^{2} cannot recognize ToBRFV MP to confer resistance against ToBRFV (Hak and Spiegelman 2021; Yan et al. 2021a). Here, we found that ToBRFV and ToMMV$^{MP-ToBRFV}$ but not ToMMV and ToBRFV$^{MP-ToMMV}$ could infect *Tm-2*^{2} transgenic *N. benthamiana* and tomato cultivar Jili harbors *Tm-2*^{2} (Fig. 2). Furthermore, transient expression of ToMMV MP induced HR-associated cell death in the leaves of *Tm-2*^{2} transgenic *N. benthamiana* plants (Additional file 1: Figure S1). These results indicate that ToMMV MP is also the Avr factor of the *Tm-2*^{2} resistance gene as that of TMV and ToMV MPs.

The allele composition and mRNA levels of *Tm-2*^{2} define the strength of *Tm-2*^{2}-mediated resistance to ToMMV

Tomato plants carry alleles of the resistant *Tm-2*, *Tm-2*^{2}, and non-resistant *tm-2* genes (Lanfermeijer et al. 2003). The *Tm-2* and *Tm-2*^{2} genes are both effective in resisting most strains of ToMV by recognizing the virus MP (Weber et al. 2004). However, ToMMV can infect tomato cultivar 'Ailsa Craig', which harbors the *Tm-2*^{2} allele (Nagai et al. 2019). Here, we found that tomato cultivars Jinpeng 1, Chaobei, and Jili contained *Tm-2*^{2}, and ToMMV could infect Jinpeng 1 but not Chaobei and Jili cultivars and induce disease symptoms in the plants (Figs. 1, 3). However, tomato cultivar Jinpeng 1 exhibited resistance to TMV infection (Additional file 1: Figure S3). We found that cultivar Jinpeng 1 consisted of *Tm-2*^{2} and *tm-2* alleles and cultivar Chaobei consisted of *Tm-2*^{2} and *Tm-2* alleles (Fig. 3e and Additional file 1: Figure S2), while cultivar Jili consisted of homozygous *Tm-2*^{2} alleles. Similar to *Tm-2*^{2}, *Tm-2* also recognizes ToMMV MP and induces HR-associated cell death (Fig. 3f). Previous report showed that *Tm-2*^{2}-mediated extreme resistance against TMV depended on the mRNA levels of *Tm-2*^{2} (Zhang et al. 2013). We also found that transgenic *N. benthamiana* and tomato cultivar Jili showed systemic infection of ToMMV when the *Tm-2*^{2} genes were silenced (Fig. 4). The high resistance of cultivars Chaobei and Jili or susceptibility of cultivar Jinpeng 1 to ToMMV may be attributed to the different expression levels of *Tm-2*^{2} and *Tm-2,* and other inherent factors that may also modulate *Tm-2*^{2} function. Therefore, the tomato plants harboring *Tm-2*^{2}*/Tm-2*^{2}, *Tm-2*^{2}*/Tm-2* alleles composition may provide more durable resistance to ToMMV than tomato plants carrying *Tm-2*^{2}*/tm-2*. This knowledge can help tomato resistance breeding programs against ToMMV in future.

The *MIP1* accumulation levels and temperature modulate *Tm-2*^{2}-mediated resistance against ToMMV in the resistant hosts

The *NbMIP1* in *N. benthamiana* has been reported to interact with TMV MP and *Tm-2*^{2} and is required for *Tm-2*^{2}-mediated resistance against TMV (Du et al. 2013). Here, we also found that silencing of the *MIP1* gene in *Tm-2*^{2} transgenic *N. benthamiana* and tomato cultivar Jili plants compromised *Tm-2*^{2} resistance to ToMMV, leading to viral systemic infection (Fig. 5). This note implies that *MIP1* is indispensable for *Tm-2*^{2}-mediated resistance against ToMMV in *N. benthamiana* and tomato plants.

Temperature may also affect *R* gene-mediated resistance (Elad and Pertot 2014; Velásquez et al. 2018). The *R-BPMV* gene of *Phaseolus vulgaris* was effective in resisting BPMV infection up to 35°C (Meziadi et al. 2021), whereas others such as the *N* gene of *N. tabacum* lost function against TMV at 28°C (Whitham et al. 1996). TMV also can infect tomato plants harboring heterozygous *Tm-2*^{2} allele under high temperatures of 30–31°C (Pilowsky et al. 1981). In our study, we observed that ToMMV infected tomato cultivar Jili (*Tm-2*^{2}*/Tm*^{2}*-2*) and *Tm-2*^{2} transgenic *N. benthamiana* plants at 35°C but not at 30°C (Fig. 6). However, the mRNA levels of *Tm-2*^{2} in *Tm-2*^{2} transgenic *N. benthamiana* and tomato cultivar Jili at 35°C was higher than those at 30°C (Additional file 1: Figure S4), implying that the infection of plants harboring *Tm-2*^{2} infected by ToMMV at 35°C is not due to a reduction in *Tm-2*^{2} mRNA levels, rather the high temperature (35°C) that affects the basal factors-regulating *Tm-2*^{2}-mediated resistance. Understanding these underlying mechanisms by which temperature modulates *Tm-2*^{2} gene function may help in developing viral disease resistant tomato plants as well as high temperature tolerant.

Conversely, we observed that viral disease symptoms in the susceptible tomato cultivar Moneymaker and wild-type *N. benthamiana* were milder at 35°C than those under lower temperatures (Fig. 6), suggesting that high

temperature can also attenuate symptom expression in susceptible plants. This may be due to the RNA silencing in the plants, which can be enhanced at elevated temperatures as reported earlier (Szittya et al. 2003; Velázquez et al. 2010).

Conclusions

In conclusion, we found that *Tm-2²* confers resistance to ToMMV and the MP of ToMMV was the Avr factor for *Tm-2²* and *Tm-2*. However, the allele composition may affect *Tm-2²*-mediated resistance through its accumulation levels. Also, *MIP1* is required for *Tm-2²*-mediated resistance against ToMMV in *N. benthamiana* and tomato. Moreover, high temperature at 35°C could compromise *Tm-2²*-mediated resistance to ToMMV. This study will benefit the breeding and cultivation strategies to enhance tomato resistance against ToMMV.

Methods

Plants and viruses

The *Tm-2²* transgenic *N. benthamiana,* wild-type *N. benthamiana*, *S. lycopersicum* cultivars Jili (Shandong vegetable industry holding group, Weifang, China) with homozygosis *Tm-2²*, Chaobei (Shandong vegetable industry holding group, Weifang, China) containing *Tm-2²* and *Tm-2* alleles, Jinpeng 1 (Xi'an Jinpeng seed Co., Ltd., Xi'an, China) containing *Tm-2²* and *tm-2* alleles, and Moneymaker with homozygosis *tm-2* were used in this study. All the plants were grown in a greenhouse under temperature conditions of about 23°C in 16 h / 8 h (light / dark) conditions. ToMMV-SD (accession number MW373515) (Tettey et al. 2022), ToBRFV-SD isolate (accession number MK905890) (Yan et al. 2019, 2021b), and TMV-HEB2 isolate (accession number MN186255) were used in this study.

Chimeric infectious clone construction and inoculation

The *MP* gene sequence of ToMMV and ToBRFV was amplified and separately cloned into infectious clones pCBToBRFV and pCBToMMV to generate pCBToMMV$^{MP-ToBRFV}$ and pCBToBRFV$^{MP-ToMMV}$ using Ligation-Free Cloning Kit (Applied Biological Materials, Canada) following the manufacturer's instructions. All the constructs were verified by sequencing and individually transformed into *Agrobacterium tumefaciens* GV3101 competent cells. The agrobacterium cultures containing the binary vectors were incubated overnight at 28°C and were shaken at 200 rpm. The pelleted cell cultures were re-suspended in MMA buffer containing 10 mM MES (pH 5.6), 10 mM $MgCl_2$, and 200 µM acetosyringone at OD_{600} = 1.0. The fully expanded leaves of the test plants were agro-infiltrated with the cells using needleless syringes. The systemic leaves of the inoculated plants were observed for viral disease symptoms. Photographs were taken using a Canon 800D digital camera (Canon, Japan). The experiment was repeated three times using five plants for each treatment. @media print { .ms-editor-squiggler { display:none !important; } } .ms-editor-squiggler { all: initial; display: block !important; height: 0px !important; width: 0px !important; }

Determination of allele composition of the tomato cultivars

DNA was extracted from tomato cultivars using the FastPure Plant DNA Isolation kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. The region spanning the nucleotide position 2,140 was amplified using primer Tm-2²–1720-F/Tm-2²–2586-R (Additional file 2: Table S1). The allele compositions of Jinpeng 1, Chaobei, and Jili were then assessed from the DNA sequence chromatograms. The full *Tm-2²* allele sequences were amplified from DNA extracted from tomato cultivars Jinpeng 1 and Chaobei using primer Tm-2²–1-F and Tm-2²–2568-R (Additional file 2: Table S1) and ligated into a plasmid vector pCam::35S. The vector was transformed into *Escherichia coli* DH5α competent cells and the cells were plated on a selective Luria–Bertani (LB) medium containing 50 µg/ml of kanamycin, and incubated overnight at 37°C. Individual clones were selected and sequenced using the Sanger sequencing platform (Sangon Biotech, China). The sequence chromatograms were analyzed using 4Peaks software version 1.8 (Nucleobytes B.V. Gerberastraat, The Netherlands) and sequences were aligned using BioEdit version 7.2 (Informer Technologies, Inc.). The resulted sequences were aligned against reference sequences from the NCBI database (the accession numbers for *tm-2*, *Tm-2*, and *Tm-2²* were AF536199.1, AF536200.1, and AF536201.1, respectively). The obtained vectors containing *tm-2*, *Tm-2*, and *Tm-2²* were individually transformed into agrobacterium and co-expressed with ToMMV MP into leaves of wild-type *N. benthamiana* plants. The HR-associated cell death on the inoculated leaves was observed at 48 hpai and photographed.

Virus-induced gene silencing

About 300 bp fragments of *Tm-2²* (AF536201.1), *NbMIP1.1a* (JX271901.1) and *SlMIP1* (XM_004239689.3) were amplified and individually inserted into the pTRV-2 to create pTRV2-*Tm-2²*, pTRV2-*NbMIP1*, and pTRV2-*SlMIP1*. The pTRV2-*GUS* containing about 300 bp fragment of *GUS* was used as the control. The agrobacterium cultures containing pTRV2 derived vector were mixed with agrobacterium culture harboring pTRV1 (1:1, OD_{600} = 1.0). The mixed cultures were infiltrated into 3-leaf stage Jili and Chaobei tomato plants, and 4-leaf

stage *Tm-2*^{2} transgenic *N. benthamiana* plants using a needleless syringe. The silencing efficiencies of targeted genes were determined by RT-qPCR analyses at 10 dpai. The silenced plants and the controls were mechanically inoculated with ToMMV-SD sap extract. Viral RNA accumulations in the systemic leaves of silenced plants were detected by RT-PCR and CP accumulations were detected by western blot analysis. This experiment was repeated three times using at least four plants in each repeat.

Effect of temperature on *Tm-2*^{2}-mediated resistance

Tomato and *N. benthamiana* were grown at room temperature to the 2 fully expanded leaf stage and 4-leaf stage, respectively. The plants were acclimatized under temperatures of 20, 25, 30, and 35°C respectively, in plant growth chambers for 48 h and then mechanically inoculated with ToMMV sap inoculum (1:10 w/v). Disease symptoms were observed and photos were taken at 7 and 14 dpi for *N. benthamiana* and tomato, respectively. RNA was extracted from inoculated plants to detect the ToMMV accumulation level using RT-PCR. The experiments were repeated three times independently with at least 4 plants.

RT-qPCR analysis

Total RNA was extracted from leaves using TransZol reagent (TransGen Biotech, Beijing, China), according to the manufacturer's protocol. DNA contamination was removed from the RNA by a 4× gDNA wiper mix (Vazyme, Nanjing, China). The complementary DNAs (cDNAs) were synthesized using HiScript II Q RT SuperMix (Vazyme, Nanjing, China) containing random primers. RT-qPCR was done using a 2× Universal SYBR Green Fast qPCR mix (Abclonal) and primers listed in Additional file 2: Table S1, to test for the expression levels of *Tm-2*^{2} and *MIP1* using the LC96 qPCR system (Roche, Basel, Switzerland). The expression level of the *ubiquitin* (*UBI*) gene was used to normalize the expression levels of all the genes studied.

Protein analysis

For protein assays, we extracted total proteins from systemic leaves of the test plants inoculated with ToMMV. The leaves were pulverized in liquid nitrogen using mortar and pestle and transferred into 2 mL centrifuge tubes. Extraction buffer (1:3 w/v) solution containing 100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1% NP40, 5% sucrose, and proteinase inhibitor cocktail (MedChemExpress, NJ, USA) was added. The mixture was vortexed to mix and centrifuged at 13,523 *g* for 10 min at 4°C. The supernatant and equal volumes of 2× sodium dodecyl sulfate (2× SDS) sample buffer were added into 1.5 mL centrifuge tubes and incubated at 95°C for 10 min. The proteins were resolved in 12% SDS-PAGE followed by western blot analysis using antibodies for CP. Chemiluminescence was observed using SuperSignal™ West Dura Luminol Enhancer (Thermo Fisher Scientific, MA, USA), and the image was captured with an SH-Focus 523 Chemiluminescence Imaging System (Shenhua Science Technology, Hangzhou, China).

Statistical analysis

The Student's *t*-test and Duncan's multiple range test were separately used for two treatments and more than two treatments to calculate the statistical difference with a confidence level of 95% ($P < 0.05$). All data were presented as the mean + SD. The graphs were plotted using Microsoft Excel.

Abbreviations

BPMV	Bean pod mottle virus
CC-NB-LRR	Coiled coil-nucleotide-binding site-leucine-rich repeat
CP	Coat protein
dpai	Days post-agro-infiltration
dpi	Days post-inoculation
hpai	Hours post-agro-infiltration
HR	Hypersensitive reaction
MIP1	Movement interacting protein 1
MP	Movement protein
N. benthamiana	*Nicotiana benthamiana*
PBS	Phosphate buffer saline
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
TMV	Tobacco mosaic virus
ToBRFV	Tomato brown rugose fruit virus
ToMMV	Tomato mottle mosaic virus
TRV	Tobacco rattle virus
TSWV	Tomato spotted wilt virus
UBI	*Ubiquitin*
VIGS	Virus-induced gene silencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00162-4.

Additional file 1. Figure S1 Hypersensitive response-associated cell death in *Tm-2*^{2} transgenic *Nicotiana benthamiana* leave expressing MP of ToMMV, TMV, ToMV, and ToBRFV at 48 days post-agro-infiltration. **Figure S2** Alignment of *Tm-2*^{2}, *Tm-2*, and *tm-2* partial nucleotide sequence from position 2132 to 2171 showing nucleotide variation at position 2134 with A, C, and T for *tm-2*, *Tm-2*, and *Tm-2*^{2}, respectively. *Tm-2*-Chaobei and *Tm-2*^{2}-Chaobei indicated that the sequence is obtained from the plant of tomato cultivar Chaobei, while *tm-2*-Jinpeng 1 and *Tm-2*^{2}-JP indicate that the sequence is obtained from tomato cultivar Jinpeng 1. **Figure S3** The symptoms of tomato cultivars Jinpeng 1 and Moneymaker (MM) plants inoculated with ToMMV and TMV at 14 days post-agro-infiltration (**a**), and RT-PCR detection of ToMMV and TMV RNA in the systemic leaves of tomato cultivars Jinpeng 1 and Moneymaker plants (**b**). **Figure S4** RT-qPCR analysis of relative accumulation levels of *Tm-2*^{2} in the systemic leaves of tomato cultivar Jili (**a**) and *Tm-2*^{2} transgenic *Nicotiana benthamiana* plants (**b**) at 14 days of exposure to 20, 25, 30, and 35°C. The expression levels of *Tm-2*^{2} in plants exposed to 20°C were normalized to 1. The *ubiquitin* gene expression level served as an internal control. The values

indicated the mean + SD of three biological replicates. Letters indicate the significant differences among the different treatments (Duncan multiple range tests, $P < 0.05$).

Additional file 2. Table S1 The primers used in this study.

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Authors' contributions
XL and ZY conceived the experimental idea. CKT and ZY designed and conducted the experiments. CKT, YT, and XL wrote the paper. XM, CG, HM, and XC provided technical contributions and helped to revise the paper. All the authors read and approved the final manuscript.

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Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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