Knockout of SITOM1 and SITOM3 results in differential resistance to tobamovirus in tomato

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Abstract
During tobamovirus-host coevolution, tobamoviruses developed numerous interactions with host susceptibility factors and exploited these interactions for replication and movement. The plant-encoded TOBAMOVIRUS MULTIPLICATION (TOM) susceptibility proteins interact with the tobamovirus replicase proteins and allow the formation of the viral replication complex. Here CRISPR/Cas9-mediated mutagenesis allowed the exploration of the roles of SlTOM1a, SlTOM1b, and SlTOM3 in systemic tobamovirus infection of tomato. Knockouts of both SITOM1a and SITOM3 in sltom1a/sltom3 plants resulted in an asymptomatic response to the infection with recently emerged tomato brown rugose fruit virus (ToBRFV). In addition, an accumulation of ToBRFV RNA and coat protein (CP) in sltom1a/sltom3 mutant plants was 516- and 25-fold lower, respectively, than in wild-type (WT) plants at 12 days postinoculation. In marked contrast, sltom1a/sltom3 plants were susceptible to previously known tomato viruses, tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV), indicating that SlTOM1a and SlTOM3 are not essential for systemic infection of TMV and ToMV in tomato plants. Knockout of SITOM1b alone did not contribute to ToBRFV and ToMV resistance. However, in triple mutants sltom1a/sltom3/sltom1b, ToMV accumulation was three-fold lower than in WT plants, with no reduction in symptoms. These results indicate that SITOM1a and SITOM3 are essential for the replication of ToBRFV, but not for ToMV and TMV, which are associated with additional susceptibility proteins. Additionally, we showed that SITOM1a and SITOM3 positively regulate the tobamovirus susceptibility gene SIARL8a3. Moreover, we found that the SITOM family is involved in the regulation of plant development.

KEYWORDS
ARL8, resistance, ToBRFV, TOM1, TOM3, tomato, ToMV
1 | INTRODUCTION

Virus multiplication strongly relies on tight interactions between host susceptibility and virus-encoded proteins (Ishibashi & Ishikawa, 2016; Schie & Takken, 2014). Host susceptibility proteins (encoded by recessive genes) provide essential assistance at different stages of the virus life cycle (Diaz-Pendon et al., 2004; Schie & Takken, 2014; Truniger & Aranda, 2009). Host susceptibility recessive genes serve as targets for the development of crop virus resistance by gene modification through genome editing technologies (Cao et al., 2020; Chandrasekaran et al., 2016; Gal-On et al., 2017).

Tobacco mosaic virus (TMV), a typical member of the genus Tobamovirus and the most studied virus model for more than 120 years (Zaitlin, 1998), interacts with a variety of host susceptibility factors (Nishikiori et al., 2006). During TMV-host coevolution, TMV and the tobamovirus tomato mosaic virus (ToMV, formerly TMV-L) developed interactions with at least 10 proteins involved in virus multiplication (Ishibashi & Ishikawa, 2016). Among these elements, TOBAMOVIRUS MULTIPLICATION proteins TOM1 and TOM3 and the small GTP-binding protein ARL8 play essential roles in the viral replication complex (Ishibashi et al., 2012; Nishikiori et al., 2011). The tobamovirus genome includes two replication proteins, 126 kDa and 183 kDa. The former protein includes methyltransferase (MT) and RNA helicase (Hel) domains, and the latter MT, Hel, and RNA-dependent RNA polymerase (RdRP) domains (Buck, 1999; Goregaoker et al., 2001; Hagiwara et al., 2003). To form a functional TMV replication complex, replication proteins interact with each other and recruit viral genomic RNA, plant transmembrane TOM1, and cytosolic ARL8 proteins through the Hel domain (Ishibashi & Ishikawa, 2016; Liu & Nelson, 2013; Nishikiori et al., 2011). Knockout or silencing of susceptibility genes TOM1, TOM3, or ARL8 in the plant host prevents establishment of a functional viral replication complex. As a result, tobamovirus replication is significantly reduced or abolished.

Previously, TOM1, TOM3, and ARL8 were used as targets for the production of tobamovirus-resistant plants (Nishikiori et al., 2011; Yamanaka et al., 2002). Knockout or silencing of TOM1 and TOM3 resulted in enhanced tobamovirus resistance in Arabidopsis (Ishibashi et al., 2006; Ishikawa et al., 2011). The first tobamovirus-resistant Arabidopsis mutant was successfully isolated from an ethyl methanesulphonate-treated seed library (Ishikawa et al., 1991). Due to a decrease in virus replication, infected mutants accumulated a significantly reduced amount of youcai mosaic virus (YoMV, formerly TMV-cg). The gene involved in tobamovirus replication was identified and named ATTM1 (Ishikawa et al., 1991, 1993). It was shown that ATTM1 encodes a multipass transmembrane protein and serves as an anchor for the tobamovirus replication complex via interaction with the viral helicase domain (Yamanaka et al., 2000). Subsequently, an additional host gene, ATTM3, involved in the tobamovirus multiplication was identified. It was suggested that ATTM3 serves viral replication proteins in the same way as ATTM1 during tobamovirus infection. Complete inhibition of YoMV and ToMV multiplication in Arabidopsis was achieved after a knockout of both genes simultaneously without any effect on plant development (Yamanaka et al., 2002). However, concurrent silencing of NiTOM1 and NiTOM3 in transgenic tobacco plants reduced virus accumulation but did not result in complete inhibition of TMV and ToMV multiplication (Asano et al., 2005).

Later, additional players of the tobamovirus replication complex were identified: knockout of two AtARL8 genes completely inhibited tobamovirus multiplication in Arabidopsis without any effect on plant development (Nishikiori et al., 2011). Moreover, it was suggested that AtTOM1 and AtARL8 interact with each other and the helicase domain of ToMV to promote tobamovirus replication complex formation (Nishikiori et al., 2011). As mentioned above, the function of TOM and ARL8 families during plant growth and development remains unknown because mutants in these genes did not show any phenotype (Fujisaki et al., 2006; Ishibashi et al., 2012; Nishikiori et al., 2011).

In the current research, we aimed to study the role of the tobamovirus susceptibility SITOM family in the interaction of tomato with the recently emerged tobamovirus ToBRFV and the well-known tobamoviruses ToMV and TMV. For this purpose, single, double, and triple mutants of the TOM family (SITOM1a, SITOM3, SITOM1b) were generated via CRISPR/Cas9 technology (Feng et al., 2013; Xie et al., 2015). sitolm1a/sitolm3 double-mutant plants exhibited high resistance to ToBRFV infection and were fully susceptible to TMV and ToMV systemic infections. The contribution of each gene of the SITOM family and the SIRL8a3 tobamovirus recessive gene to tobamovirus infection was investigated.

2 | RESULTS

2.1 | Phylogenetic and expression analysis of the SITOM family

A BLASTP query of the tomato genome (SGN ITAG release 2.40 predicted proteins [SL2.40]), with the A. thaliana AtTOM1, AtTOM3, and AtTHH protein sequences allowed identification of the TOM1, TOM3, and THH homologs in tomato. Four open reading frames (ORFs) were identified in the tomato genome. They are encoded by Solyc02g080370, which corresponds to TOM1 (78.5% sequence identity to AtTOM1). Solyc04g008540 and Solyc01g007900 are highly homologous and correspond to TOM3 (81% and 80% sequence identity to AtTOM3, respectively), and both proteins showed high homology to AtTHH (81% and 82.5%, respectively). The fourth ORF, Solyc01g105270, has 67% homology to AtTOM1.

To understand the evolution of the TOM family in Solanaceae, a phylogenetic tree of TOM homologs from seven species was constructed (Figure 1a). Amino acid sequences of a conserved angiosperm TOM1 domain DUF1084 of Arabidopsis, tomato, Solanum tuberosum (potato), Capsicum annuum (pepper), moss Physcomitrella patens, basal angiosperm Amborella trichopoda, and early diverging eudicotyledon Thalictrum thalictroides were used in phylogenetic analysis. TOM proteins are divided into two clades: clade I-TOM1 and clade II-TOM1 homologs. Interestingly, all three moss TOM proteins are related to the TOM3 clade, and the basal angiosperm A. trichopoda has three
TOM genes of both clades. All TOM genes of the early diverging eu-
dicotyledon *T. thalictroides* are related to the TOM1 clade only. The
Arabidopsis THH protein belongs to the TOM3 clade; the sequence
identity between AtTOM3 and AtTHH is 86%. In tomato, and other
Solanaceae potato and pepper, the TOM family is larger than in the
other species (Figure 1a). Despite the high homology of
Solyc01g007900 to AtTOM3, analysis of conserved domains showed that Solyc01g007900 belongs to the
TOM1 clade (Figure 1a). Consequently, we named tomato TOM genes
(Solyc02g080370, Solyc01g105270, Solyc01g007900, Solyc04g008540)
*SitOM1a*, *SitOM1b*, *SitOM1c*, and *SitOM3*, respectively (Figure 1a).

Expression analysis of *SitOM1a*, *SitOM1b*, and *SitOM3* showed
that all three genes are expressed in leaf tissue. *SitOM1a* and *SitOM1b*
genomes were expressed at a similar level and *SitOM3* expression was
twofold higher than the expression of *SitOM1* genes (Figure 1b). *SitOM1c*
expression was undetectable in healthy and virus-infected plants; possibly, it is a pseudogene or tightly regulated at a spatio-
temporal level. Interestingly, during ToBRFV infection, *SitOM1a*
and *SitOM3* were down-regulated, but *SitOM1b* expression level re-
maind similar in infected and healthy tomato plants (Figure 1b).

2.2 | Generation of *sitom1a/sitom3* double mutants
using CRISPR/Cas9

Knockout or silencing of AtTOM1 and AtTOM3 resulted in virus re-
stance against YoMV and ToMV in Arabidopsis mutants (Ishikawa
et al., 1993; Yamanaka et al., 2002). Moreover, ToMV replication was
completely suppressed in *attom1/attom3* double-mutant plants

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**FIGURE 1** Characterization of the tomato TOM family. (a) A phylogenetic
analysis of TOM1 and TOM3 homologous genes from seven species based on
amino acid sequences of the conserved angiosperm TOM1 domain DUF1084.
The names of tomato proteins in red, Arabidopsis in black, and other species are indicated near each protein id. A phylogenetic tree was constructed by the neighbour-joining method with 1000 bootstrap replicates (MEGA v. 7 program). (b) Reverse transcription-
quantitative PCR analysis of *SitOM1a*, *SitOM1b*, and *SitOM3* expression levels in healthy tomato and ToBRFV-infected plants 16 days postinoculation. *SitIP41* expression values were used for normalization. Gene expression in healthy and infected plants was calibrated relative to *SitOM1a* expression in healthy plants. Data are means ± SD of three independent biological repeats, each measured in triplicate. The different letters above the bars indicate statistically significant differences in the expression of different genes and asterisks indicate significant differences in gene expression between the treatments (*p* < 0.01). WT-H, wild type healthy; WT-I, wild type inoculated.
In tomato, SITOM1a has a higher similarity to AtTOM1 than SITOM1b (Figure 1a). Additionally, only SITOM1a and SITOM3 expressions were affected during ToBRFV infection (Figure 1b). Consequently, we decided to generate double-mutant sltom1a/sltom3 tomato plants designed to be resistant to tobamoviruses. CRISPR/Cas9 technology was used and two single-guide RNAs (sgRNA) designed to target sequences in the first exon of SITOM1a and SITOM3 genes (Figure 2a). A construct pRCS:Cas9-sgRNA-SITOM1a/ SITOM3, which contains the sgRNAs described above (Figure 2a), was generated and used for tomato agrotransformation. Seven independent T<sub>0</sub> transgenic lines were generated as determined by kanamycin resistance and PCR analysis (File S1). Progenies (T<sub>2</sub>) from each line were screened for homozygous SITOM1a and SITOM3 mutants. Two lines were selected: lines 9 and 11 harbouring mutation in two alleles of SITOM1a and SITOM3 (Figure 2b and File S1). Genotyping of SITOM1a showed in both alleles one-nucleotide deletion and one-nucleotide insertion in lines 9 and 11 upstream of the protospacer adjacent motif (PAM) (Figure 2b). Genotyping of SITOM3 displayed bialleles with one-nucleotide insertion or deletion in line 9, while line 11 had a one-nucleotide insertion in both alleles. Two mutant lines (T<sub>2</sub> generation) designated thereafter as sltom1a/sltom3-9 and sltom1a/sltom3-11, were selected for further experiments. The growth and development of sltom1a/sltom3-9 and sltom1a/sltom3-11 progenies (T<sub>2</sub>) were indistinguishable from the wild type (WT) (File S1).

2.3 | The sltom1a/sltom3 mutant plants exhibited resistance to systemic ToBRFV infection

Progenies of sltom1a/sltom3-9 and sltom1a/sltom3-11 (T<sub>2</sub> generation) were mechanically inoculated with ToBRFV and resistance was evaluated by symptom development and virus accumulation in systemic leaves. In response to ToBRFV infection, sltom1a/sltom3 plants exhibited no symptoms and looked similar to the healthy WT plants, while WT-infected plants developed yellowing leaf mosaic and slight leaf deformation symptoms, and plant development was delayed (Figure 2c). During fruit development, only fruits of ToBRFV-infected WT plants showed yellow patches and the exocarp of sltom1a/sltom3 fruits remained asymptomatic (Figure 2d). The level of viral coat protein (CP) accumulation in sltom1a/sltom3 plants was evaluated by western blot analysis (Figure 3a). ToBRFV-Cp was undetectable in mutant plants at 4 days postinoculation (dpi), except sample 1 from sltom1a/sltom3-11, which accumulated 100-fold virus less than WT-infected plants (Figure 3a). At 12 dpi, virus accumulation was detected in all mutant plants, but the CP accumulation level was 25-fold lower in infected mutant lines sltom1a/sltom3-9 and sltom1a/sltom3-11 lines than in WT-infected plants. In line sltom1a/sltom3-9, the CP accumulation level was slightly lower than in line 11 mutant plants (Figure 3a). In addition to the evaluation of sltom1a/sltom3 resistance by CP accumulation analysis, we tested ToBRFV RNA accumulation at different times after inoculation. At 13 dpi, ToBRFV RNA accumulated about 100-fold higher in WT plants compared to mutant plants, and 30 dpi WT plants accumulated ToBRFV RNA 213- and 55-fold higher than in the infected sltom1a/sltom3-9 and sltom1a/sltom3-11 lines, respectively (Figure 3b). To exclude the possibility that ToBRFV evolved to break resistance in infected mutant plants, the virus from systemic leaves was sequenced twice and no nucleotide changes were observed. These results show that sltom1a/sltom3 mutant plants are highly resistant to ToBRFV.

2.4 | The sltom1a/sltom3 mutant plants were susceptible to ToMV and TMV infection

As shown above, sltom1a/sltom3 mutant plants exhibited significant resistance to ToBRFV. In view of these results, these mutants were tested for resistance to other tobamoviruses, ToMV and TMV. Unexpectedly, ToMV CP accumulated in sltom1a/sltom3-9 and sltom1a/sltom3-11 mutant plants to a level similar or even higher than in WT plants (Figures 4a and 7a). sltom1a/sltom3 mutant plants exhibited typical ToMV mosaic disease symptoms (Figure 4b). Similar to ToMV infection, TMV induced typical TMV mosaic disease symptoms in double mutants (data not shown), and mutants accumulated variable TMV levels (around 1.4-fold) compared to WT plants (Figure S1).

2.5 | sltom1b mutant plants are susceptible to ToBRFV infection

It has been reported that in tomato-independent RNAi silencing of SITOM1a, SITOM1b or SITOM3 genes resulted in partial resistance to ToMV (Ali et al., 2018). Therefore, to improve plant resistance to ToBRFV infection, we evaluated the contribution of SITOM1b, a third member of the SITOM family, to ToBRFV infection. sltom1b plants were generated by CRISPR/Cas9 technology with sgRNA designed to target the sequences in the fourth exon of the gene at the beginning of the angiosperm TOM1 domain (Figure 5a). A construct pRCS:Cas9-sgRNA-SITOM1b was built and used for tomato transformation. Six independent T<sub>0</sub> transgenic plants were generated. Several mutant plants harboured a four-nucleotide deletion (Figure 5b and File S1) (T<sub>2</sub> generation). Plants designated as sltom1b-6 were chosen for viral resistance analysis. Homozygous progeny of sltom1b-6 mutant line were susceptible to ToBRFV infection and developed disease symptoms, as did the WT-infected plants (Figure 5c). Interestingly, ToBRFV accumulated in sltom1b-6 plants to a higher level (1.3- to 2-fold) than in WT 12 dpi (Figure 5d). Therefore, knockout of SITOM1b alone does not provide ToBRFV resistance and even increases plant susceptibility to ToBRFV, suggesting that SITOM1b is part of the plant defence against ToBRFV.

2.6 | Resistance analysis of sltom1a and sltom3 single mutants to ToBRFV infection

To study the involvement of SITOM1a and SITOM3 in ToBRFV systemic infection, sltom1a and sltom3 mutant lines were isolated from a
FIGURE 2  Knockout of SITOM1a and SITOM3 genes resulted in plant resistance to ToBRFV. (a) Schematic representation of the SITOM1a and SITOM3 genomic maps and the sgRNA target sites. (b) Sequence alignments of DNA fragments from sltom1/asltom3-9 and sltom1a/sltom3-11 (T2 generation) with M82 wild type (WT). DNA deletions or insertions are shown in red. The target sequence is shown in red letters and the protospacer adjacent motif (PAM) is marked in blue letters. (c) Representative images of systemic leaf four at 21 days postinoculation (dpi) from 35-day-old plants (T3 progeny). (d) Fruit representative images at ripening 60 dpi (T3 progeny). WT, wild type; H, healthy plants; I, infected plants.
cross-pollinated T$_2$ population of sltom1a/sltom3-11 and sltom1b-6 (File S1).

Infection of a sltom3 mutant line with ToBRFV showed that ToBRFV accumulated 3-fold less in sltom3 plants at 8 dpi than in WT-infected plants but higher than in sltom1a/sltom3-11 plants (Figure 6). Also, sltom1a plants accumulated ToBRFV to a higher level than the double mutants (Figure 6). Heterozygous triple mutants of SITOM (he*3; sltom1a/sltom1a/sltom3/sltom3/sltom1b/sltom1b) accumulated ToBRFV to a higher level than WT plants, as predicted for recessive genes (Figure 6).

2.7 | Resistance analysis of sltom1a/sltom3/sltom1b triple-mutant plants to tobamoviruses

To generate sltom1a/sltom3/sltom1b tomato plants, the double mutants sltom1a/sltom3-11 were cross-pollinated with sltom1b-6 and sltom1a/sltom3/sltom1b triple-mutant plants were generated (File S1). Triple-mutant plants (sltom1a/sltom3/sltom1b) were infected with ToBRFV at an early developmental stage (first pair of

FIGURE 3 sltom1a/sltom3 plants exhibited resistance to ToBRFV infection. (a) Western blot analysis of ToBRFV coat protein (CP) accumulation in M82 (wild type, WT) and mutant lines 9 and 11 at 4 and 12 days postinoculation (dpi). Accumulation of RbcS showed in Ponceau S-stained blots was used for normalization between samples. (b) Reverse transcription-quantitative PCR analysis of ToBRFV RNA accumulation in WT and mutant lines 9 and 11 at 13, 20, and 30 dpi. SlTIP41 expression values were used for normalization. Data are means ± SD of three or four independent biological repeats (log$_{10}$), each measured in triplicate. Asterisks indicate a significant difference as determined by Student’s t test (∗∗p ≤ 0.01, ∗p ≤ 0.05). WT, wild type; H, healthy plants; I, infected plants.

FIGURE 4 sltom1a/sltom3 mutant plants were susceptible to ToMV infections. (a) Western blot analysis of ToMV coat protein (CP) accumulation in the sltom1a/sltom3-9 and sltom1a/sltom3-11 and wild-type (WT) plants 8 days postinoculation (dpi). (b) Representative images of systemically infected leaves of ToMV infected plants at 21 dpi. Accumulation of RbcS presented in Ponceau S staining was used for normalization between samples. Each sample represents a single plant. WT, wild type; H, healthy plants; I, infected plants.
true leaves) and viral accumulation was significantly lower than in the WT plants. However, ToBRFV accumulation in sltom1a/sltom3-11 plants (Figure S2), emphasizing that SlTOM1b is not involved in establishing ToBRFV infection in tomato. However, in triple-mutant sltom1a/sltom3/sltom1b plants, ToMV CP accumulation was reduced 3-fold compared to accumulation in WT plants (Figure 7a).

A knockout of three genes from the SlTOM family affected tomato leaf and plant development. The tomato plant cotyledons and leaves became smaller and curly (Figure 7b,c). Also, there was reduced leaf lamina outgrowth (Figure 7b,c) and the plant became bushy (Figure 7d), suggesting the loss of apical dominance.

2.8 | SlTOM1 and SlTOM3 regulate SIARL8a3 expression in tomato

Nishikori et al. (2011) showed the importance of AtARL8a and AtARL8b genes that encode a GTP-binding protein required for tobamovirus replication. A BLASTP query of the tomato genome with the A. thaliana AR8 protein sequences allowed identification of the tomato AR8 homologs. Four ORFs were identified, encoded by Solyc02g064640, Solyc02g092330, and Solyc03g043960, which are very similar and correspond to AtARL8a and AtARL8b from Arabidopsis, and Solyc11g011040, which corresponds to the AtARL8c protein. Consequently, they were named SlARL8a1, SlARL8a2, SlARL8a3, and SlARL8c, respectively (Figure 8a). All SlARL8 genes were expressed at a similar level in WT plants. Moreover, SlARL8a1, SlARL8a2, and SlARL8c expression levels were similar in WT healthy and ToBRFV-infected plants (Figure 8b). However, the expression level of SlARL8a3 was reduced 25-fold in sltom1a/sltom3 double-mutant plants in comparison to WT ToBRFV-infected and healthy plants (Figure 8b). This suggests that SlTOM1a or SlTOM3 positively regulate SlARL8a3.

To explore the contribution of SlTOM1a or SlTOM3 to SlARL8a3 regulation, plants homozygous in sltom1a or sltom1b, but heterozygous in the other two genes were isolated from the T2 self-cross population of sltom1a/sltom3 and sltom1b plants (sltom1a/SITOM3/sltom3/sltom1b [sltom1a*] and SITOM1a/sltom1a/sltom3/SITOM1b/sltom1b [sltom3*], respectively). In sltom1a* plants, the SlARL8a3 expression level was about 1% of the SlARL8a3 expression level in WT plants.
3 | DISCUSSION

During 100 million years of coevolution, tobamoviruses have developed numerous interactions with various plant–host factors and effectively exploit them during infection and host-shifting events (Gibbs et al., 2015; Ishibashi & Ishikawa, 2016; Stobbe et al., 2012). TOM and ARL8 are susceptible host gene families that interact with each other and serve tobamoviruses during the infection (Nishikiori et al., 2011). Both gene families are conserved and even appear in moss (Figures 1a and 8a; Nishikiori et al., 2011).

However, the number of genes and clades within the TOM family has been altered during plant evolution. Selection pressure brought the appearance of a new clade in the TOM family and the enlargement of the new TOM1 clade in angiosperms. Moss has three TOM genes that belong to the TOM3 clade only. However, TOM genes of the early diverging eudicotyledon T. thalictroides are solely related to the TOM1 clade, while tomato has one functional SlTOM3 gene, two functional SlTOM1 genes in leaves, and a third SlTOM1 gene SlTOM1c may express under specific conditions or a specific genetic background. Other Solanaceae members also have an enlarged TOM1 clade. Possibly, during the long divergence, tobamovirus had a strong impact on TOM family evolution in plants and Solanaceae in particular. Possibly, enlargement of the TOM1 clade produced some advantages for Solanaceae during growth and development while also favouring tobamovirus.

Here we studied the role of the tomato TOM gene family on tobamovirus systemic infection caused by TMV, ToMV, and ToBRFV. ToMV and TMV, related to the clade of Solanales tobamoviruses, co-diverged with their hosts for tens of million years, and tomato is considered one of their primary hosts (Gibbs et al., 2015; Stobbe et al., 2012). In contrast to ToMV and TMV, ToBRFV is found in the group of tobamoviruses that have undergone host-shifting events and recently caused a worldwide epidemic disease in tomato (Maayan et al., 2018). It has been shown that elimination of AtTOM1 alone reduced ToMV accumulation by 90% in Arabidopsis, and a double knockout of AtTOM1 and AtTOM3 generated immune Arabidopsis and resistant tobacco (Asano et al., 2005; Yamanaka et al., 2000, 2002). In addition, independent silencing of different TOM genes in tomato plants induced partial ToMV resistance (Ali et al., 2018).

In the current study, simultaneous knockout of SlTOM1a and SlTOM3 genes in tomato resulted in ToBRFV-resistant and ToMV- and TMV-susceptible plants in cv. M82, which does not bear Tm-1, Tm-2 or Tm-2² tobamovirus resistance genes. Very high resistance at the molecular level and lack of symptoms were observed against the recently emerged tomato virus ToBRFV in sltom1a/sltom3 plants (Figures 2.c,d and 3), indicating their importance for ToBRFV systemic infection, as demonstrated in Arabidopsis and tobacco against ToMV (Asano et al., 2005; Yamanaka et al., 2002). Additionally, knockout of SlTOM1a and SlTOM3 separately reduced ToBRFV accumulation, but single-mutant plants accumulated ToBRFV to a higher level than double-mutant plants (Figure 6). This result suggests a considerable role for SlTOM3 during ToBRFV infection compared to Arabidopsis, in which AtTOM1 plays a more important role in tobamovirus infection (Fujisaki et al., 2006; Yamanaka et al., 2002). SlTOM3 has a more profound role in cv. M82 tomato during the infection than AtTOM3, possibly because the SlTOM3 expression level is higher than that of SlTOM1a and SlTOM1b (Figure 1b). These results are different from
those recently observed in single and double tomato mutants of the Craigella GCR26 genotype, which were fully susceptible to ToBRFV (Ishikawa et al., 2022). It is highly probable that the genetic background contributes to this difference: in M82, in contrast to Craigella GCR26, SlTOM3 was expressed to higher level than other genes and SlTOM1c expression was undetectable (Figure 1b). Moreover, in M82 a single mutant, sltom3, was partially resistant to ToBRFV (Figure 6), but this mutant is fully susceptible in Craigella GCR26 (Ishikawa et al., 2022). Moreover, results observed in Craigella GCR26 are different from results observed earlier in Solanum lycopersicum ‘Micro-Tom’ and tobacco plants, where silencing of even one gene contributed to plant resistance (Ali et al., 2018; Asano et al., 2005).

We have demonstrated the negative effect of ToBRFV infection on SITOM1α and SITOM3 expression levels (Figure 1b). As we mentioned above, tobamoviruses and plants have coevolved over a long period. In addition to recruiting susceptibility factors, viruses have developed various strategies to mediate viral levels (Csorba et al., 2015). Reduction in SlTOM1α and SlTOM3 expression levels may point to ToBRFV auto-regulation via fine-tuning of virus replication. The SITOM1b expression level was not affected by ToBRFV infection (Figure 1b), but we could not rule out the involvement of SITOM1b in ToBRFV infection. sltom1b plants were fully susceptible to ToBRFV and accumulated higher virus amounts than WT plants (Figure 5), suggesting that this gene alone is not involved in tobamovirus replication and may belong to the plant

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**FIGURE 8** SITOM1 and SITOM3 regulate SIA RL8a3 expression in tomato.

(a) A phylogenetic analysis of ARL8 homologs from three species. Tomato proteins in red, Arabidopsis in black, Physcomitrella patens is indicated near each protein ID. A phylogenetic tree was constructed by the neighbour-joining method with 1000 bootstrap replicates (MEGA 7). (b) SIARL8 genes expression in wild-type (WT) and sltom1a/sltom3-9 and sltom1a/sltom3-11 plants healthy (H) and infected (I) by ToBRFV at 12 days postinoculation. (c) Expression levels of SIARL8a3 in sltom1a* (sltom1a/SITOM3/sltom3/SITOM1b/sltom1b) and sltom3* (SITOM1a/sltom1a/sltom3/SITOM1b/sltom1b) mutant plants. SITIP41 expression values were used for normalization. Data are means ± SD of three independent biological repeats, each measured in triplicate. The different letters above the bars indicate statistically significant differences between the investigated plants (p < 0.01).
defence mechanism against ToBRFV. Moreover, ToBRFV accumulation in sltom1a/sltom3/sltom1b triple-mutant plants was similar, as determined in the double mutant sltom1a/sltom3-11 plants (Figure S2). The low ToBRFV replication level in the double mutants suggests an association of ToBRFV with alternative susceptibility proteins. In the absence of SITOM1a and SITOM3, 5’ RNA capping activity of the 126 kDa replication protein does not occur or is severely impaired. As a result, the TMV-encoded 126 kDa protein cannot interact effectively with the host membranes to form a replication complex with the 183 kDa protein, and as a result tobamovirus replication relies solely on the 183 kDa protein (Lewandowski & Dawson, 2000). Possibly, in sltom1a/sltom3, ToBRFV replication was only partially suppressed. This could be due to 183 kDa replication protein activity or inefficient interaction with other susceptibility genes. It was demonstrated that mutant viruses can break recessive resistance (Hashimoto et al., 2016), and therefore the ToBRFV sequence was analysed. However, analysis of the ToBRFV genome from mutant plants identified no changes to the ToBRFV genome sequence.

Despite a high level of ToBRFV resistance observed in sltom1a/sltom3 plants, double mutants were as susceptible to TMV and ToMV as the WT plants (Figures 4 and S1), in contrast to complete ToMV resistance in attom1/attom3 mutant Arabidopsis plants (Yamanaka et al., 2002). Disruption of sltom1b did not contribute to ToBRFV resistance in the triple mutants (sltom1a/sltom3/sltom1b), but improved ToMV resistance by 3-fold in the triple mutant of cv. M82 (Figure 7a). This last contradicts results observed in the Arabidopsis triple mutant attom1/attom3/attth, which was immune to ToMV infection (Fujisaki et al., 2006).

The results observed here underline that the final effects of gene knockouts in the classic model plant Arabidopsis do not always forecast the outcome in other species. Moreover, the genetic background plays a significant role in the effect of mutation on the plant. The divergence between Arabidopsis and tomato occurred around 112 million years ago (Ku et al., 2000). Moreover, the brassica-infesting tobamoviruses originated due to a host-shifting event from asterids to rosids (Gibbs et al., 2015). Consequently, Arabidopsis has been exposed to TMV and ToMV for a shorter period than tomato, and the host-pathogen coevolution in tomato and Arabidopsis has progressed in different paths. As in tomato, the solanaceous tobacco nttom1/nttom3-silenced plants accumulate ToMV during early infection (Asano et al., 2005). Apparently, ToBRFV replication proteins associate with SITOM1a and SITOM3 as their primary membrane docking proteins for ToBRFV replication. In contrast, ToMV and TMV probably developed interactions with additional tomato host factors during their long coevolution. Indeed, it was recently shown that TMV and ToMV multiplication in tomato primarily depends on the interaction with the other susceptibility gene TOM2A because sltom2a plants showed significant resistance to TMV and ToMV (Hu et al., 2021). We might assume that this is why ToMV easily overcomes the knockout of SITOM1a and SITOM3 in tomato. Also, it was demonstrated that the TOM1-TOM2a interaction is well conserved in plants (Hu et al., 2021).

Possibly, the TOM1-TOM2a module is preferable for tobamovirus, but tobamoviruses that have undergone host-shifting events interact primarily with TOM1 and only later develop interactions with TOM2a. Furthermore, during the host-switch event, tobamoviruses do not interact even with all SITOM family members, as in the case of SITOM1b.

Interestingly, we revealed that SITOM1a and SITOM3 genes positively regulate SIARL8a3 expression because knockout of SITOM1a and SITOM3 genes reduced SIARL8a3 expression by 95% (Figure 8b). Moreover, we found that in sltom1a* mutant plants, the SIARL8a3 expression level was lower than in sltom3* mutant plants (Figure 8c). These results indicate for the first time that the SITOM family positively regulates SIARL8a3 expression, while SITOM1a contributes to SIARL8a3 regulation more than SITOM3. slarl8a3 mutant plants were as fully susceptible to ToBRFV as the WT (File S2). Consequently, SIARL8a3 alone does not contribute to ToBRFV resistance, and possibly ToBRFV resistance in cv. M82 is the direct result of SITOM1a and SITOM3 knockout. Apparently, in tomato, as in Arabidopsis and tobacco, several proteins from the SIARL8 and SITOM families provide a platform for effective ToBRFV replication, and the knockout of only one of them is insufficient to produce tobamovirus-resistant tomato plants (Asano et al., 2005; Nishikiori et al., 2011).

The developmental function of the SITOM gene family has been unknown and mutant plants did not show any effect on plant growth and development (Fujisaki et al., 2006; Ishikawa et al., 2022; Schie & Takken, 2014; Yamanaka et al., 2002). In tomato, single and double mutants (sltom1a*, sltom1b, sltom3, sltom3*, sltom1a/sltom3, triple heterozygous) did not exhibit any abnormal morphological or developmental phenotype compared to WT plants as previously observed in tobacco and Arabidopsis TOM mutants (Asano et al., 2005; Fujisaki et al., 2006). However, in the tomato triple mutant sltom1a/sltom3/sltom1b small cotyledons, curly leaves, and bushy plants were observed (Figure 7). Future research will reveal the reasons for observed phenotypes. A reduced cell number or smaller cells could be a reason for smaller cotyledons and leaves. Curly leaves could develop due to abnormal development of the vascular system or irregular development of cells in abaxial or adaxial leaf sides, and a bushy plant phenotype probably emerges as a result of loss of apical dominance. In the future it will be interesting to explore the interactions of SITOM with various developmental factors in tomato.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material, growth conditions, and virus inoculation

Tomato (Solanum lycopersicum) cv. M82 (not bearing Tm-1, Tm-2 or Tm-2* genes) seedlings were grown in a growth chamber with a 16 h photoperiod at a constant temperature of 25°C. Plants were rub-inoculated using carborundum with ToBRFV-IL (KX619418.1), ToMV (similar to isolate 99-1 [KR537870.1]), and TMV (KU321698) mechanically according to (Chandrasekaran et al., 2016) at the two-leaf stage and were grown in a greenhouse at a constant temperature of 25°C or a nethouse with temperatures ranging between 21 and 31°C.
4.2 Generation of SITOM1a, SITOM3, and SIOM1b tomato mutants using CRISPR/Cas9 technology

The knockout of the SITOM1a, SITOM3, SIOM1b, and SIARL8 genes by CRISPR/Cas9 was performed as in Corem et al. (2018). Amplified SITOM1b and SIARL8 sgRNAs were digested and cloned into the pRCS binary vector Sall-HindIII sites. SITOM1a, SITOM3 sgRNAs were synthesized, digested, and cloned into the pRCS binary vector MluI-HindIII sites under the control of the U6 Arabidopsis promoter. Transformation into tomato of binary vectors pRCS:Cas9- sgRNA-SITOM1a/SITOM3, pRCS:Cas9- sgRNA-SITOM1b, and pRCS:Cas9- sgRNA-SIARL8 was performed as in Kravchik et al. (2014). Genomic DNA was extracted by the Dellaporta method (Dellaporta et al., 1983). To detect the transgene in transformed plants sgRNA primers were used (Table S1). Restriction sites of Cfr10I, Sall, Bpil, and Eco130I were used for mutation detection in SITOM1a, SITOM3, SIOM1b, and SIARL8a genes, respectively (amplification primers are designated in Table S1). Additionally, the amplicon used for mutation analysis was cloned into pJET1.2 plasmid (CloneJET PCR Cloning Kit, Thermo Fisher Scientific) and sequenced. To generate sitom1a/ sitom3/sioma-11 tomato plants, the double mutants sitom1a/sitom3-11 were cross-pollinated with sitom1b-6 and triple mutant sitom1a/sitom3/sitom1b plants were generated. From the same population single sitom1a and sitom3 were isolated.

4.3 Total RNA extraction, cDNA synthesis, and reverse transcription-quantitative PCR assay

The upper three or four systemic leaves from three or four independent plants were collected and frozen in liquid nitrogen. Total RNA was extracted with Bio-Tri RNA reagent (Bio-Lab). First-strand cDNA was synthesized from 1 µg of total RNA with a qPCRBBIO cDNA synthesis kit (PCR Biosystems Ltd). The absence of a genomic DNA template in the samples was proved by a negative control (-RT). Real-time PCR was performed using the StepOnePlus Real-Time PCR System with SYBR Green PCR Master Mix (Thermo Fisher Scientific). Three or four independent biological replicates were used for each sample, and quantification was performed in triplicate. Primer sequences are listed in Table S1. SITIP41 was used as a reference gene for the normalization of relative expression levels. The standard curve and 2^ΔΔCT methods were used for relative quantification.

4.4 Protein extraction, SDS-PAGE separation, and western blot analysis

The upper systemic three or four leaflets were collected from independent plants, frozen in liquid nitrogen, and suspended in urea SDS buffer (Levitzky et al., 2019). The proteins were extracted as in Levitzky et al. (2019) and separated on 12% SDS-PAGE. The SDS-PAGE gels were transferred onto nitrocellulose membranes by a semidry transfer blot (Bio-Rad). Ponceau S staining was performed to visualize blotted proteins, and the RuBisCO small subunit (RbcS) band was used as a loading control. Phosphate-buffered saline (PBS)-washed membranes were blocked with PBS containing 4% nonfat milk, then incubated with anti-CP polyclonal antibodies of ToBRFV, TMV, or TMV, labelled with a secondary antirabbit antibody with horseradish peroxidase, and developed with EZ-Link plus activated peroxidase kit (Thermo Fisher Scientific). Signals were captured in an Alliance Q9 Advanced imaging system (UVITEC). Image processing and quantification were performed with ImageJ (National Institutes of Health).

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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

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