ANNUAL REVIEWS

Annual Review of Virology

Breaking Boundaries: The Perpetual Interplay Between Tobamoviruses and Plant Immunity

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Annu. Rev. Virol. 2023. 10:455–76

First published as a Review in Advance on May 30, 2023

The *Annual Review of Virology* is online at virology.annualreviews.org

https://doi.org/10.1146/annurev-virology-111821-122847

Keywords

pattern-triggered immunity, resistance genes, RNA silencing, salicylic acid, ToBRFV, TMV

Abstract

Plant viruses of the genus *Tobamovirus* cause significant economic losses in various crops. The emergence of new tobamoviruses such as the tomato brown rugose fruit virus (ToBRFV) poses a major threat to global agriculture. Upon infection, plants mount a complex immune response to restrict virus replication and spread, involving a multilayered defense system that includes defense hormones, RNA silencing, and immune receptors. To counter these defenses, tobamoviruses have evolved various strategies to evade or suppress the different immune pathways. Understanding the interactions between tobamoviruses and the plant immune pathways is crucial for the development of effective control measures and genetic resistance to these viruses. In this review, we discuss past and current knowledge of the intricate relationship between tobamoviruses and host immunity. We use this knowledge to understand the emergence of ToBRFV and discuss potential approaches for the development of new resistance strategies to cope with emerging tobamoviruses.

INTRODUCTION

Plant viruses of the genus *Tobamovirus* (family *Virgaviridae*) are destructive plant pathogens that cause significant damage to multiple crop species. The *Tobamovirus* genus includes 37 known members that infect plants of different families, including *Solanaceae*, *Brassicaceae*, and *Cucurbitaceae* (1). Tobamoviruses are notoriously infectious pathogens that are transmitted by mechanical contact, including workers' hands, agricultural tools, soil, insects, and seeds (2). Their symptoms include plant stunting, yellow mosaic leaf patterns, leaf blistering and malformations, and fruit blotching and marbling (**Figure 1**). This group contains some of the most economically important plant viruses in the world. In recent years, tobamoviruses such as tomato brown rugose fruit virus (ToBRFV) (3) and cucumber green mottle mosaic virus (4) have caused significant damage to tomato and cucurbit crops, respectively, with massive reductions in fruit quality and yield in various parts of the world.

In marked contrast to their negative impact, tobamoviruses have also played a significant role in the advancement of scientific research and the understanding of fundamental biological principles (5). First and foremost, the initial discovery of tobacco mosaic virus (TMV) by Beijerink in the late nineteenth century as the first "*contagium vivum fluidum*" gave birth to the scientific field of virology (6). In addition, TMV was the first virus to be chemically purified and observed in an electron microscope (7). The use of TMV RNA was one of the first examples to show that nucleic acids contain genetic information and that they are sufficient to promote viral infection (8, 9). The study of TMV movement protein (MP) revealed important insights into how plant cells and organs communicate with each other (10). Importantly, tobamovirus research led to groundbreaking findings in the field of plant immunity that are the focus of this review.

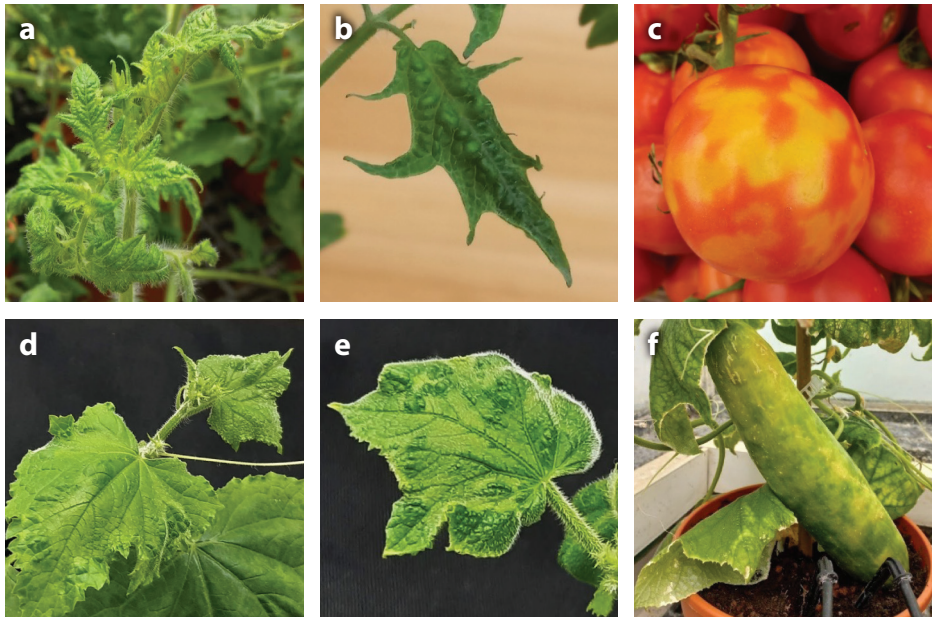


Figure 1

Tobamovirus symptoms in tomato and cucumber plants. (*a–c*) Symptoms of tomato plants infected with tomato brown rugose fruit virus. (*d–f*) Symptoms of cucumber plants infected with cucumber green mottle mosaic virus. Symptoms include leaf curling (*a*,*d*), narrowing and blistering (*b*), mosaic patterns on leaves (*e*), and the development of mottled fruit (*c*,*f*).

To cope with pathogens, plants have evolved a multilayered defense system based on several pathways, including defense hormones, cell surface–localized pattern recognition receptors (PRRs), the intracellular immune receptor family of nucleotide-binding domain leucine-rich repeat-containing proteins (NLRs) encoded by *Resistance* (*R*) genes, and RNA silencing. Remarkably, with only four viral-encoded proteins, tobamoviruses have evolved sophisticated and distinct strategies to effectively evade or counter defense pathways to achieve efficient systemic infection. In this review, we explore the intricate relationship between tobamoviruses and different plant immune pathways. We use this knowledge to gain insights into the emergence and dispersal of the new virus ToBRFV, an agriculturally relevant and devastating pathogen that causes extensive damage in tomato crops. Finally, we discuss potential approaches to developing new resistance strategies against emerging tobamoviruses such as ToBRFV.

THE TOBAMOVIRUS GENOME, STRUCTURE, AND FUNCTION

The tobamovirus genome consists of a positive-sense single-stranded RNA molecule of approximately 6.4-kb length that contains four protein-encoding open reading frames (ORFs) (11) (**Figure 2**). ORF1 and ORF2 encode two subunits of the viral replicase—a small one of 122–130 kDa and a larger one of 178–183 kDa—which are distinguished by a stop codon readthrough that extends the ORF1 protein into ORF2. Together, these proteins form a heterodimeric, membrane-anchored RNA-dependent RNA polymerase complex that facilitates viral replication (12). ORF3 encodes the 30-kDa viral MP that enables the cell-to-cell movement of the

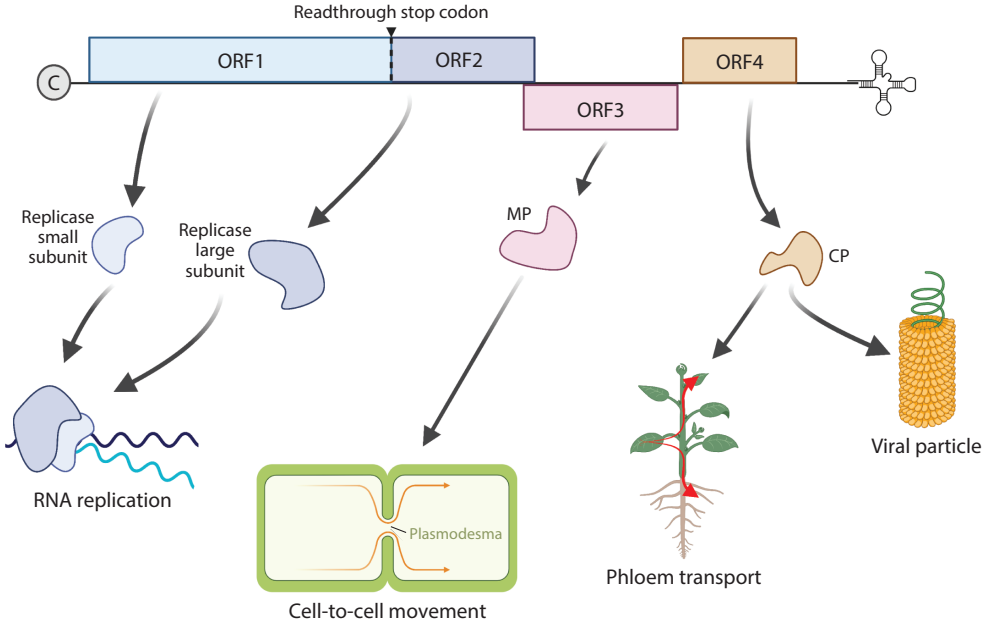


Figure 2

Tobamovirus genome and encoded proteins. This schematic illustration of the tobamovirus 6.4-kb positive-sense single-stranded RNA genome includes the 5′ cap, the 3′ transfer RNA–like structure, and four open reading frames (ORFs) encoding the viral proteins: two subunits of the viral RNA-dependent RNA polymerase (replicase) essential for viral replication; the movement protein (MP), which enables intercellular movement of the virus; and the coat protein (CP), which gives rise to the viral particle and is required for long-distance transport of the virus via the phloem. Figure adapted from images created with BioRender.com.

virus by binding the viral RNA and targeting it to plasmodesmata (PD)—intercellular channels connecting adjacent cells (13, 14). The MP increases the PD's size exclusion limit to facilitate transport of the viral RNA from cell to cell (15). This process involves multiple yet specific interactions with different cellular elements, including the endoplasmic reticulum (ER), microtubules, and proteins (10). ORF4 encodes the viral coat (or capsid) proteins (CPs), which polymerize to give rise to the viral particle that encompasses the viral genome. The CPs form rigid particles of approximately 300 by 18 nm that serve as the conservation and dispersal unit of the virus (16). In addition, the CP is essential for long-distance movement of the virus to distant parts of the plant (17).

The mechanisms of tobamovirus replication and spread have been extensively explored (18). Upon entry into the host cell (**Figure 3*a***), the tobamovirus RNA genome unfolds from its virion particle. Then viral replication complexes (VRCs) are formed at the junctions between ER and microtubules. These VRCs contain the replication proteins, viral RNA, and MP. Particles containing the MP and viral genome then detach from the VRCs to become intracellularly mobile, and they move along the ER to target the MP–RNA complex to PD for intercellular movement. Viruses then move from cell to cell and form infection foci on the infected leaf (**Figure 3*b***). The virus then enters the plant vasculature to infect distant organs (**Figure 3*c***). This movement occurs

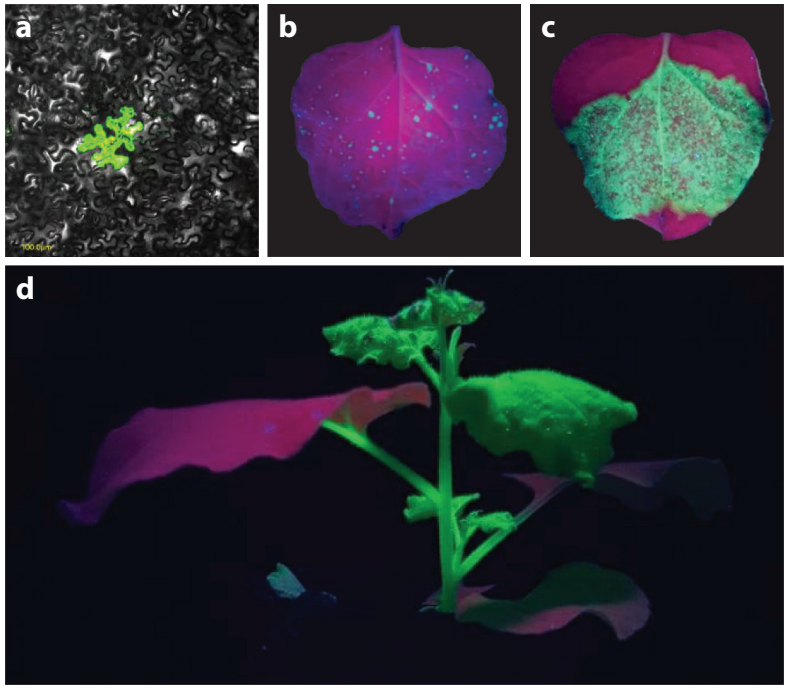


Figure 3

Systemic spread of tobamoviruses. *Nicotiana benthamiana* plants infected with tobacco mosaic virus (TMV) harboring the coding sequence of green fluorescent protein (TMV-GFP) allow monitoring of the spread of the virus. (*a*) Once the virus gains entry into the first cell, viral replication takes place and translation of the viral protein initiates. (*b*) The virus then moves from cell to cell via plasmodesmata—intercellular channels connecting adjacent cells—using its movement protein. The virus then moves into the plant vasculature (*c*), and phloem loading and transport take place. (*d*) The virus is transported to distant parts of the plant with the phloem translocation stream and targets sink tissues such as young leaves, fruit, flowers, and roots.

via the phloem photoassimilate transport system from source, sugar-producing organs, such as mature leaves, to sink, sugar-consuming organs, such as young leaves, flowers, fruit, and roots (**Figure 3*d***). After the virus is well established in infected cells, the VRCs grow into large viral factories for virus transcription, translation, replication, and assembly of newly formed particles that are released to infect new plants. The processes of viral infection are constantly challenged by plant defense mechanisms that act to restrict viral replication, accumulation, movement, and spread. In response, the virus antagonizes or evades the activity of these defenses via the function of specific viral proteins or by the development of specific mutations. In the following sections, we delineate the plants' different antiviral resistance mechanisms and how the tobamovirus counters them to achieve successful infection.

INTERACTION OF TOBAMOVIRUSES WITH THE RNA-SILENCING PATHWAY

RNA-silencing-mediated resistance is considered the only immune strategy in plants with adaptive characteristics (19) that is able to confer protection against all types of plant viruses, including RNA and DNA viruses, satellite RNAs, and viroids (20). It is based on the sequence-specific degradation or translation inhibition of the target RNA, in a process termed post-transcriptional gene silencing (PTGS). Throughout their life cycle, RNA viruses form double-stranded RNA (dsRNA) intermediates either as a result of intramolecular RNA interactions or during viral replication. These RNA duplex structures are the hallmarks and initiators of the silencing process, which is based on the formation of small RNA (sRNA) molecules (**Figure 4**). During their evolution, viruses have formed various mechanisms to cope with the plant RNA-silencing machinery. Virus-encoded proteins termed viral suppressors of RNA silencing (VSRs) counter specific components of the silencing pathway to prevent their antiviral activity (21). In the case of tobamoviruses, the replicase small subunit (p122/p130) functions as the VSR (22, 23). This protein sequesters sRNA molecules, thereby inhibiting both their methylation, which is required for their stability, and their incorporation into the RNA-induced silencing complex (RISC) (24, 25). Multiple domains of this protein are independently sufficient to confer VSR activity, suggesting that this function went through tight selection during evolution (26). An additional activity of p122 is the suppression of the expression of an important component of the RISC complex, argonaute 1, by upregulating its regulatory *microRNA 168* (27). These findings suggest that the VSR function of p122 is robust and modular, and that it targets multiple stages of RNA silencing. Interestingly, the MP may counter the VSR activity of p122, as its expression enhances the systemic spread of the silencing signal (28). The contrasting effects of p122 and MP may balance viral propagation with the physiological needs of the plant, which still requires active sRNA-mediated regulation to survive.

Another mechanism regulating RNA levels in the cell is RNA quality control (RQC), also known as the RNA decay pathway. This pathway controls the integrity of endogenous RNA through the degradation of dysfunctional transcripts by a multiprotein complex termed RNA exosome (29). One outcome of RQC is the suppression of PTGS, likely by preventing the entry of dysfunctional RNA molecules into the silencing pathway (30). This tug-of-war between RQC and PTGS is thought to balance the two pathways to ensure appropriate regulation of endogenous RNA levels. A recent study found that expression of TMV CP and MP upregulates the expression of key exosome complex proteins (**Figure 4**), leading to increased rates of RNA decay and a concomitant reduction in antiviral PTGS (31). These findings suggest that in addition to the direct VSR activity of p122, the viral MP and CP have an indirect VSR function by activating RQC at the expense of the PTGS pathway.

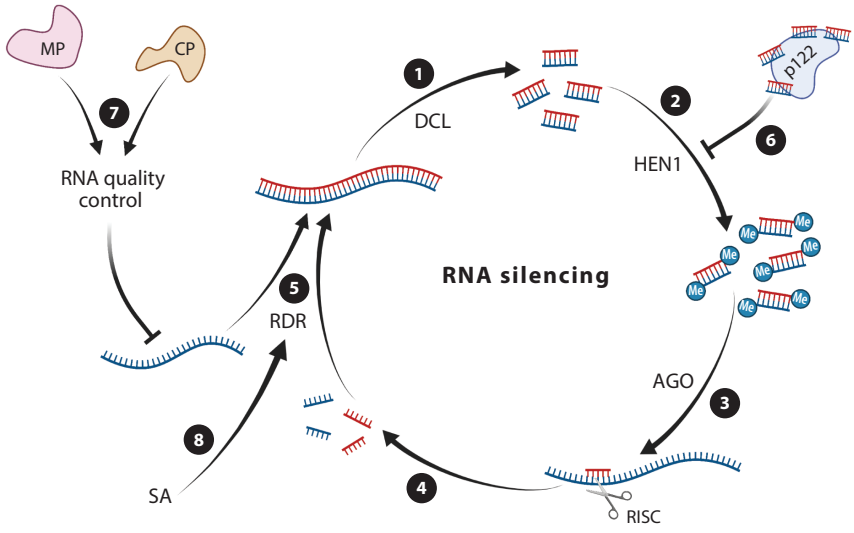


Figure 4

The RNA-silencing pathway and its suppression by tobamovirus proteins. Viral double-stranded RNA (dsRNA) is cleaved by RNase type III enzyme dicer-like (DCL) proteins (①), resulting in short small RNA (sRNA) duplexes of 21–24 nucleotides. The generated sRNAs are further methylated at the 3′ terminal nucleotide by the enzyme HUA enhancer 1 (HEN1) for increased stability and protection against degradation (②). The methylated sRNAs are then loaded onto argonaute (AGO) proteins (③), giving rise to the RNA-induced silencing complex (RISC). The RISC is then guided by the sRNA to the complementary RNA sequence and cleaves it or suppresses its translation (④). In plants, this process is enhanced by RNA-dependent RNA polymerase (RDR) (⑤), which binds to the sRNA as a primer to form additional secondary dsRNA molecules. Tobamoviruses suppress this pathway with their replicase small subunit (p122), which sequesters the sRNA and prevents its methylation by HEN1 (⑥). In addition, expression of the movement protein (MP) and coat protein (CP) upregulates the RNA quality-control pathway (⑦), thereby indirectly suppressing the silencing pathways. The plant hormone salicylic acid (SA) enhances RDR1 activity (⑧) to further enhance the antiviral activity of the silencing pathway. Figure adapted from images created with BioRender.com.

SALICYLIC ACID: A KEY HORMONE IN MEDIATING TOBAMOVIRUS RESISTANCE

Salicylic acid (SA) is a central plant defense hormone that is essential for multiple aspects of plant immunity to biotrophic and semibiotrophic pathogens (32). Numerous studies have established that SA enhances resistance to a variety of plant pathogens, including bacteria, fungi, and viruses (32). This typically occurs via activation of systemic acquired resistance (SAR), which primes the plant against the pathogen attack by upregulating several defense pathways (33). Studies on TMV played a pioneering role in the initial discovery of the role of SA in immunity. The first evidence for SA as a signaling molecule was provided in 1979 by White (34), who found that treatment with SA and its analogs significantly enhances plant resistance to TMV. Later, it was discovered that endogenous SA is upregulated during the systemic immune response to TMV in tobacco (35), suggesting its role as a regulator of plant defense. The essential role of SA in the establishment of SAR was shown by the abolishment of systemic resistance to TMV in plants expressing the SA-degrading enzyme salicylate hydroxylase (36). These groundbreaking studies were crucial for the establishment of SA as a key defense hormone. Further examination of the mechanisms by

which SA induces TMV resistance revealed that it reduces both viral accumulation (37, 38) and cell-to-cell movement (38).

How SA reduces viral accumulation is not completely understood. However, it has been proposed that it activates RNA silencing. For example, SA induced the expression of RNA-dependent RNA polymerase 1 (RDR1) in *Nicotiana benthamiana* and *Arabidopsis thaliana*, thereby promoting the degradation of viral RNA (39) (**Figure 4**). In addition, SA enhanced the activity of constitutively expressed RDR1 against TMV, leading to recovery from viral infection (40). SA therefore seems to induce both transcriptional and post-transcriptional activation of RNA silencing, and RDR1 in particular.

In recent years, there has been accumulating evidence for the mode by which SA regulates cell-to-cell transport. PD are dynamically regulated in response to various signals through accumulation of the polysaccharide 1-3-β-glucan (callose) near their apertures (41). SA is one of the key positive regulators of plasmodesmal callose biosynthesis, which leads to the closure of PD and restriction of cell-to-cell movement (42) (**Figure 5*a***). This occurs due to SA-mediated transcriptional induction of the callose-biosynthesis enzyme callose synthase 1 (CalS1) and depends on the presence of PD-localized protein 5 (PDLP5), a known regulator of PD function (42–44) (**Figure 5*a***). Blockage of PD, by either SA application or PDLP5 overexpression, indeed suppressed the movement of TMV (44), suggesting that this pathway functions in SA-mediated immunity against tobamoviruses. Therefore, it seems that SA activates multiple pathways, including PTGS and callose biosynthesis, to restrict both viral accumulation and movement.

Because the key role of the MP is to increase the size exclusion limit of PD (15), it can be considered a suppressor of SA-mediated plasmodesmal blockage. It is evident that the presence of MP significantly decreases callose levels near PD (45, 46) (**Figure 5*a***). However, the precise mechanism by which the tobamovirus MP enhances movement in the PD remains unknown. The MP's ability to alter the plasmodesmal size exclusion limit probably involves interaction with multiple PD-localized proteins, such as PDLPs and CalS, and changes to plasmodesmal complex components. However the precise interactions governing these processes remain to be elucidated.

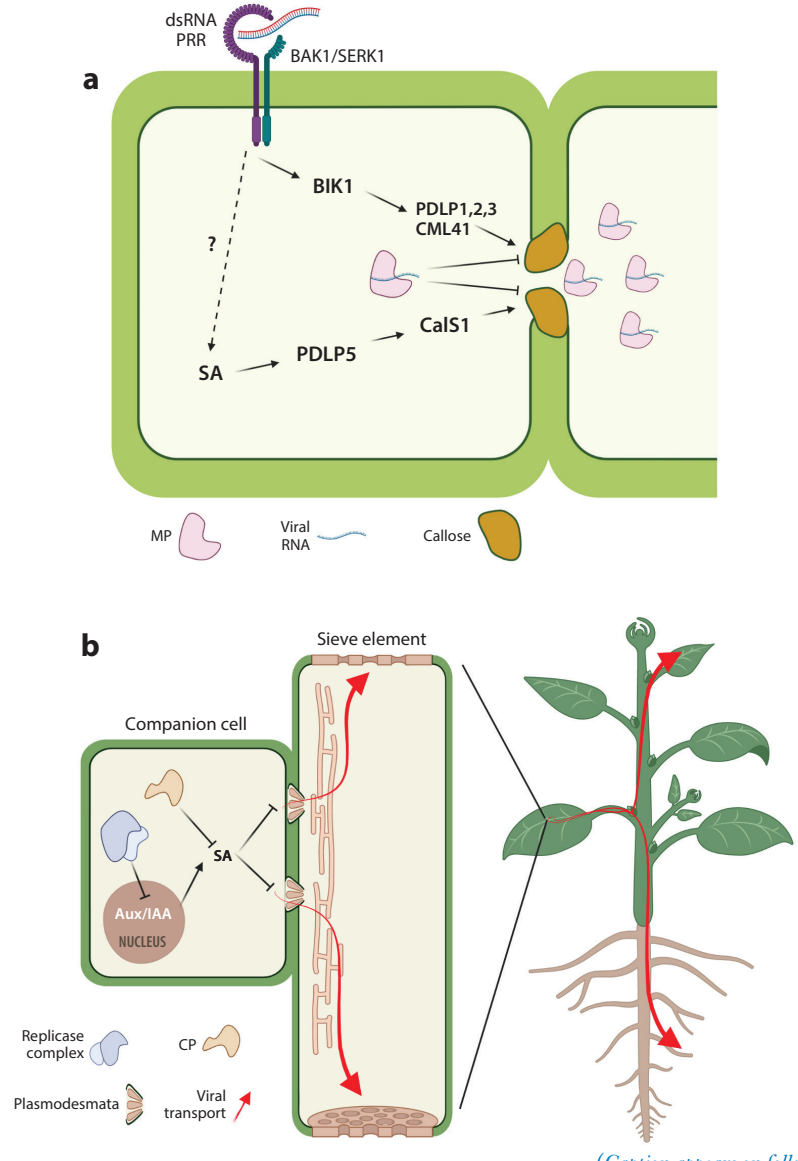
Another tobamovirus protein recently found to be involved in the suppression of SA-mediated responses is the CP. Overexpression of CP in tobacco led to the downregulation of several SA-responsive genes, including *RDR1* (47). Recently it has been shown that this function of the CP allows the long-distance transport of crucifer-infecting TMV (48) (**Figure 5*b***). Downregulation of key regulators of the SA pathway was sufficient to enable the systemic transport of a truncated TMV that lacks the CP.

Various hormonal pathways in plants are intertwined and affect each other through a variety of molecular cross talk involving common regulatory factors. Tobamoviruses exploit these interactions to suppress the SA response. For example, TMV replicase proteins interact with auxin/indole-3-acetic acid (Aux/IAA) proteins—key regulators of the auxin response pathway (49) (**Figure 5*b***). This interaction results in disruption of Aux/IAA protein localization and function, thereby reprogramming the auxin response transcriptional output (50). In turn, this reprogramming enhances the phloem loading of TMV, promoting systemic infection (51) (**Figure 5*b***). Disruption of the auxin response affects the transcription of important regulators of various cellular pathways, including the SA response and plasmodesmal activity, which are crucial for systemic viral infection (51). Collectively, these studies suggest that the movement, coat, and replication proteins jointly suppress the SA pathway to promote cell-to-cell and long-distance movement of the virus.

DOUBLE-STRANDED RNA: A VIRUS-ASSOCIATED MOLECULAR PATTERN

Pattern-triggered immunity (PTI) is a key component of the plant's innate immune system, based on the recognition of conserved microbial determinants termed microbe-associated molecular patterns (MAMPs) by transmembrane PRRs. PRRs include receptor-like kinases and receptor-like proteins, which form heteromeric complexes at the plasma membrane that bind to the MAMP and relay a series of phosphorylation signals that result in a plant immune response (52).

One of the central regulators of PTI is the coreceptor brassinosteroid-associated kinase 1 (BAK1), which complexes with several PRRs to relay the phosphorylation signal (53). BAK1 has



(*Caption appears on following page*)

Figure 5 (*Figure appears on preceding page*)

The plant transport pathways as battlegrounds for tobamoviruses and immunity. (*a*) Cell-to-cell movement is restricted to prevent viral transport. Salicylic acid (SA) activates the transcription of plasmodesmata-localized protein 5 (PDLP5) and callose synthase 1 (CalS1) to synthesize the plasmodesma-blocking polysaccharide callose at the plasmodesmata. Similarly, an unknown pattern recognition receptor (PRR), which recognizes double-stranded RNA (dsRNA), associates with the coreceptors brassinosteroid-associated kinase (BAK1) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1) to activate a cascade of phosphorylation signals, which rely on the serine/threonine protein kinase botrytis–induced kinase 1 (BIK1) to activate callose synthesis via PDLPs 1, 2, and 3 and the calcium-binding protein calmodulin-like protein 41 (CML41). The tobamovirus movement protein (MP) counters the effects of both pathways by reducing callose levels at the plasmodesmata and expanding their size exclusion limit to allow transport of the viral RNA. (*b*) Long-distance transport of viruses involves suppression of key hormonal pathways. The coat protein (CP) suppresses SA responses to potentiate phloem transport of the virus. In addition, the replicase binds to auxin/indole-3-acetic acid (Aux/IAA) proteins to exclude them from the nucleus and alter the expression of key regulators of the SA pathway and plasmodesmal function. Figure adapted from images created with BioRender.com.

been found to contribute to plant immunity to TMV and oilseed rape mosaic virus, as well as several other RNA viruses (54), suggesting the existence of an antiviral PTI pathway; dsRNA was suggested to be the potential PAMP mediating this response (55) (**Figure 5*a***). Application of purified dsRNA from virus-infected plants and the dsRNA analog polyinosinic:polycytidylic acid to *A. thaliana* plants triggered PTI responses resulting in enhanced antiviral defense. These responses were not dependent on a specific dsRNA sequence and did not involve the RNA-silencing machinery. However, they did depend on the PTI coreceptor SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), suggesting dsRNA as a bona fide PAMP.

A recent study showed that dsRNA-mediated PTI leads to the induction of callose near PD, thereby limiting viral cell-to-cell movement (46). This response was dependent on multiple PTI-signaling components, as well as several PD-localized proteins (**Figure 5*a***). In addition, expression of TMV MP suppressed the dsRNA-mediated callose deposition and enabled cell-to-cell movement (**Figure 5*a***). These findings suggested that callose formation at the PD is an important PTI response against tobamoviruses and that the tobamovirus MP counters this response. Because SA is a key regulator of the PTI response, it is likely that dsRNA-induced callose deposition is a result of the activation of SA-signaling pathways as discussed above. However, the role of SA in the dsRNA-mediated PTI response remains to be elucidated.

INTERACTION OF TOBAMOVIRUSES WITH RESISTANCE PROTEINS

Although PRR-mediated activation of PTI provides broad-spectrum resistance, pathogens have evolved to suppress PTI by secreting proteins called effectors into plant cells (56). To counter this, plants have evolved *R* genes that encode NLR immune receptors that recognize pathogen effectors (57, 58). This recognition leads to the activation of effector-triggered immunity, which often culminates in localized cell death at the infection site—termed hypersensitive response (HR)—to limit further spread of the pathogen. The two major classes of NLRs found in plants are the TNLs, which contain a toll-interleukin-1 receptor (TIR) homology domain at the N terminus, and the CNLs, which contain a coiled-coil domain (57, 58).

The Tobacco *N* Gene

The *N* gene (**Table 1**) has long served as a model to study disease resistance in plants. Holmes was the first to report that the *N* gene in *Nicotiana glutinosa* prevents chlorosis symptoms caused by TMV by inducing localized necrotic lesions at the site of infection (59) and the first to introgress

Table 1 Tobamovirus resistance genes, viral targets, and ability to confer ToBRFV resistance

Crop plant	*R* gene	Alleles	Plant of origin	Protein type	Viral target/Avr	ToBRFV resistance
Nicotiana tabacum (tobacco)	*N*	NA	*Nicotiana glutinosa* (59, 60)	TNL (62)	p50 (replicase) (68–70)	Yes (126)
	N′	NA	*Nicotiana sylvestris* (88)	CNL (88)	Coat protein (88)	Yes (126)
Capsicum annuum (bell pepper)	*L*	L^1, L^{1a}, L^2, L^3, and L^4 (85–87)	*Capsicum chinense* (85)	CNL (86)	Coat protein (86)	Yes[a] (124, 125)
Solanum lycopersicum (tomato)	*Tm-1*	Multiple (102)	*Solanum habrochaites* (95)	Unidentified (101, 102)	Replicase (96, 97, 102)	No (117)
	Tm-2	*Tm-2* and $Tm\text{-}2^2$ (104)	*Solanum peruvianum* (95, 105)	CNL (104)	Movement protein (106–108)	No (117)

Abbreviations: NA, not available; ToBRFV, tomato brown rugose fruit virus.

[a]Transient systemic infection was observed and resistance may break in temperatures higher than 30°C.

this gene into cultivated tobacco (60). In 1994, a team led by Barbara Baker used a transposon-tagging approach and exploited the temperature sensitivity of *N* to clone the *N* gene from tobacco (61, 62). The *N* gene encodes a TNL, and it was the first TIR domain identified to contain an immune receptor (62), well before mammalian toll-like receptors were discovered to play a role in immunity (63).

The N protein provides immunity against all strains of tobamoviruses except the resistance-breaking Ob strain of TMV (TMV-Ob), which causes systemic infection in tobacco plants harboring the *N* gene (64). The *N* gene produces two transcripts by alternative splicing, and both transcripts are required to confer full resistance to TMV (65). A detailed structure–function analysis indicated that all domains of N are required for its function in imparting resistance (66). The N protein recognizes the 50-kDa helicase domain (hereinafter referred to as p50) within the 126-kDa replicase subunit of tobamoviruses (67–69) to induce a HR and defense. Although p50 of TMV-Ob (p50-Ob) fails to induce a HR and defense, N associates with p50-Ob, similar to p50 from an eliciting strain of TMV, U1 (p50-U1) (70). Nevertheless, the association between N and p50-Ob is weaker than with p50-U1. Yeast two-hybrid assays revealed that the leucine-rich repeat (LRR) domain of N interacts with p50 (71). However, direct biochemical evidence of this interaction is still lacking. These findings suggest that the activation of N immune signaling relies not only on mere association between N and p50 but also on the strength of association, other protein–protein interactions, and downstream signaling events (70).

One N-interacting protein required for its activation is the chloroplast-localized N receptor-interacting protein 1 (NRIP1). Upon TMV infection, NRIP1 relocalizes from the chloroplasts to the cytoplasm and nucleus, where it functions as an intermediary interactor between N and p50 (72). These findings and others led to the discovery of chloroplasts' important role in plant immunity (73). During *N*-mediated defense against TMV, chloroplasts induce tubule-like projections called stromules, which are extensions of chloroplast stroma-filled membranous protrusions (74). Stromules make extensive connections with the nucleus during immune response. The formation and extension of stromules are mediated by microtubules, and anchoring of stromules to the nucleus is facilitated by actin filaments (75). The dynamic interaction of stromules with the cytoskeleton promotes perinuclear clustering of chloroplasts during immunity. This clustering

may promote the transfer of chloroplast-produced defense molecules such as hydrogen peroxide or defense proteins such as NRIP1 (75).

Increasing evidence indicates that pathogen recognition by NLRs (sensor NLRs) requires downstream NLRs for immune signaling called helper NLRs (76). In fact, the first helper NLR to be identified was N requirement gene 1, which is required for N to induce TMV resistance (77). Like other TNLs, N also requires a downstream signaling protein termed enhanced disease susceptibility 1 to induce immunity to TMV (78, 79). N also associates with chaperone proteins such as heat-shock protein 90 (HSP90), required for *Mla12* resistance 1, and suppressor of the G2 allele of *skp1* (SGT1), which have been implicated in regulating NLR protein level and NLR folding (79, 80). In plants, N protein level is very low, and TMV infection or introduction of p50 increases it (70, 81). TurboID-based in vivo proximity labeling provided insights into transient protein–protein interactions required for the regulation of N (82). This approach is based on the fusion of a protein of interest with promiscuous biotin ligase, which covalently labels proximal proteins by biotin. This method revealed that N interacts with a putative UBR-box containing E3 ligase called UBR7 that negatively regulates the level of N protein (82). The association between N and UBR7 is disrupted in the presence of p50, leading to increased levels of N and enhanced resistance to TMV. Although UBR7 seems to function in a proteasome-dependent manner (82), how it functions in N stability and defense requires further characterization.

In addition to recognizing p50 in the cytoplasm, N also functions in the nucleus (70, 83), where it interacts with a homolog of squamosa promoter binding protein-like 6 (SPL6) transcription factor from *N. benthamiana* (NbSPL6) (70). Interestingly, N interacts with SPL6 only in the presence of N-activating p50 from the U1 strain, but not in the presence of the non-N-eliciting strain p50-Ob, indicating that the association between N and SPL6 occurs post-recognition of TMV in the cytoplasm. Consequently, silencing of SPL6 in *N*-containing plants leads to loss of TMV resistance, indicating that SPL6 functions as a positive regulator of *N*-mediated immunity (70).

More recently, the transcription factor alfin-like 7 (AL7) has been shown to interact with N to positively regulate *N*-mediated defense against TMV by inhibiting the transcription of genes that encode reactive oxygen species (ROS)-scavenging enzymes (84). In the absence of TMV, AL7 interacts strongly with N and binds weakly to the promoters. Upon TMV infection, SA-inducible protein kinase and wound-inducible protein kinase phosphorylate AL7 at serine 174. This disrupts the interaction between N and AL7, thereby promoting stronger binding of AL7 to the promoters, and reduces the transcription of ROS-scavenging genes, leading to ROS accumulation and HR cell death. These findings suggest that AL7 functions as a transcriptional repressor during *N*-mediated defense against TMV. Therefore, it seems that N recruits both a transcriptional activator (SPL6) and a repressor (AL7) to induce immunity to TMV. Collectively, *N*-mediated defense is a tightly regulated process that involves interaction with multiple plant proteins functioning in various steps of the immune response.

The Pepper (*Capsicum annuum*) *L* Gene

The *L* gene (**Table 1**) is a dominant tobamovirus-resistance gene introgressed from the wild species *Capsicum chinense* into *Capsicum annuum* (85). It encodes a CNL that recognizes the viral CP as its effector to trigger a HR against the virus (86). There are four different alleles of *L*, L^1, L^2, L^3, and L^4, with the number indicating the allele's recognition spectrum. L^1 confers resistance to P_0 viruses such as tomato mosaic virus (ToMV); L^2 confers resistance to P_0 and P_1 viruses, such as paprika mild mottle virus (PMMoV); L^3 protects against P_0, P_1, and $P_{1,2}$ PMMoV pathotypes that overcome L_2; L_4 protects against P_0, P_1, $P_{1,2}$, and $P_{1,2,3}$ PMMoV pathotypes that

overcome L_3 (85, 86). An additional *L* allele is L^{1a}, which is thermosensitive and fails to confer tobamovirus protection under elevated temperatures (87). It should be noted that an ortholog of *L* named *N′* exists in *Nicotiana sylvestris* (**Table 1**) and confers tobamovirus resistance based on CP recognition, albeit with a different resistance spectrum determined by its LRR domain (88).

Distinct mutations in the CP can overcome the various *L* alleles (86). Importantly, a number of PMMoV strains harbor mutations that overcome the durable L^4 allele (89, 90). Because R proteins, such as L, often target protein functions that are essential for pathogen infectivity or survival, resistance-breaking mutations in the CP may have negative effects on the virus. Several studies have investigated the trade-offs between overcoming *L* resistance and overall viral fitness. Long-term field competition assays revealed that L^3 resistance-breaking variants of PMMoV show reduced pathogenicity when competing with nonresistance-breaking isolates (91). These negative effects depended on the specific CP mutation, host genotype, and coinfection with other isolates (92). Resistance-breaking mutations in the CP affected the viral particle's stability by changing the interactions between the CP subunits (93). However, there was no correlation between the intensity of particle instability and the degree to which the various *L* alleles are overcome. A recent study revealed that once an *L* resistance-breaking mutation has stabilized, its reversion may also result in viral particle stability penalties (94). These studies suggested that resistance-breaking mutations in the CP may affect viral fitness. However, because viral infection is affected by multiple factors, it seems that these trade-offs are complex, and depend on genetic and environmental conditions.

The Tomato *Tm-1* Gene

The *Tm-1* gene (**Table 1**) was identified in the 1950s as a single semidominant locus conferring tolerance to ToMV, introgressed into cultivated tomato from the wild tomato species *Solanum habrochaites* (95). However, it could be easily overcome by several ToMV resistance-breaking isolates (96, 97). Several early studies concluded that *Tm-1* does not act as a classical dominant *R* gene. First, inoculation of *Tm-1* protoplasts with ToMV resulted in inhibition of viral replication but not cell death (98). Second, double inoculation of *Tm-1* protoplasts with resistance-breaking and nonresistance-breaking ToMV resulted in infection by the resistance-breaking isolate, suggesting that *Tm-1* does not trigger a general immune response (99). Third, inoculation of *Tm-1*-heterozygous plants with ToMV resulted in incomplete inhibition of viral replication, suggesting that this resistance is semidominant, rather than dominant (100).

Resistance-breaking ToMV mutations were located in the helicase domain of the viral replicase, suggesting that *Tm-1* targets the process of viral replication (96, 97). Indeed, *Tm-1* was identified as an 80-kDa protein that binds to the ToMV replicase and inhibits the virus's replication (101). As suspected, Tm-1 was not a NLR-class protein and was not homologous to any other identified protein (101, 102). Instead, it inhibited the assembly of the replication complex that includes several membrane-localized host proteins, among them tobamovirus multiplication 1 and 3 (TOM1/3) and the small GTP-binding protein ARF-like 8, which are essential for viral replication (103). Insights into the function of Tm-1 were provided by the crystal structure of its inhibitory domain and its complex with the viral replicase (102). A study by Ishibashi et al. (102) revealed the precise interface between the two proteins, showing that resistance-breaking mutations in the viral replicase impaired its interaction with Tm-1. Remarkably, a Tm-1 variant with enhanced ToMV-inhibitory function contained a mutation at the same interface. This study provided detailed insights into the coevolutionary arms race between Tm-1 and ToMV. It also provided atomic-level evidence for the red-queen hypothesis, in which evolution is driven by competition between species, selecting for mutations that change their competitive interactions.

The *Tm-2/Tm-2²* Gene in Tomato

Tm-2 and *Tm-2²* are two alleles of the same *R* gene that encodes a CNL; they differ from each other by only four amino acids (104) (**Table 1**). Both *Tm-2* and *Tm-2²* confer resistance to TMV and ToMV, and they were introgressed into cultivated tomato from the wild species *Solanum peruvianum* more than 50 years ago (95). Because resistance-breaking ToMV isolates have emerged for both *Tm-1* and *Tm-2* (97), *Tm-2²* remains the key tobamovirus *R* gene in tomato (105). As a result, *Tm-2²* has been an indispensable part of tomato breeding programs for the last 50 years. The Tm-2² CNL recognizes and associates with the tobamovirus MP as its effector to trigger the defense response against the virus (106–108). Previous studies suggested that Tm-2² recognizes elements in the MP C terminus; however, recently it was shown that other MP elements are also involved in its recognition by Tm-2² (109, 110). Interestingly, while *Tm-2²* lacks a plasma membrane-localization motif, it is located in the plasma membrane, where it interacts with the viral MP (108). Once activated by the MP, Tm-2² self-associates in an ATP-dependent process that is triggered by its nucleotide-binding domain (111). This self-association likely occurs through multiple protein domains of the protein. When activated by the MP, the *Tm-2²* resistance response can manifest as one of three forms, depending on its expression level (112): High expression levels trigger extreme resistance, conferred in the absence of cell death; intermediate expression levels trigger HR-mediated resistance; and low expression levels may result in systemic crawling necrosis that spreads throughout the plant.

Similar to N, Tm-2² is regulated at the protein level by chaperones such as HSP90 and SGT1, which support its stability (113, 114). Another protein required for Tm-2² stability is the J-domain protein MP-Interacting Protein 1 (MIP1) (114). Interestingly, MIP1 also binds to the MP and regulates viral movement (114), suggesting its contribution to both disease and immunity. In addition, the Rubisco small subunit interacts with both Tm-2² and the MP, and it is essential for both proteins' functions (115). These studies suggest that *Tm-2²* resistance is dependent on multiple protein–protein interactions that form a network that allows recognition of the MP and activation of the immune response. In addition, the finding that similar interacting proteins are required for both MP and Tm-2² function suggests that the MP is not recognized by Tm-2² alone but as a complex with other proteins.

TOMATO BROWN RUGOSE FRUIT VIRUS: AN EMERGING TOMATO PATHOGEN THAT OVERCOMES HOST RESISTANCE

The Emergence and Spread of Tomato Brown Rugose Fruit Virus

In 2014–2015, a new tobamovirus emerged in the Middle East, causing significant damage to tomato yield and fruit quality (116, 117). In addition to typical tobamovirus symptoms, which include leaf mosaic, distortions, fruit marbling, and malformations, the new virus occasionally caused brown, rugose, and necrotic lesions on fruit and therefore was named tomato brown rugose fruit virus (ToBRFV) (116). ToBRFV can infect more than 40 plant species from four different families—*Amaranthaceae*, *Apocynaceae*, *Asteraceae*, and *Solanaceae* (3). Importantly, pepper (*C. annuum*) is another major crop threatened by ToBRFV. Similar to other tobamoviruses, ToBRFV is highly infectious due to the stability of its particle and effective mechanical transmissibility (118). In recent years, ToBRFV outbreaks have been documented in different regions worldwide, including Europe, Asia, and North and South America, and currently this virus poses a major threat to the global tomato industry (3, 119). It is still difficult to estimate the economic impact of ToBRFV; however, a potential loss of $262 million a year has been estimated for only the state of Florida (120). Today, ToBRFV is mostly controlled by strict border regulation of imported produce and

seeds, and greenhouse-disinfection protocols. There is concern because the effectiveness of these protocols is limited due to the exceedingly high infectivity of this virus. The precise origin of ToBRFV is unknown; however, sequence analysis suggests that it belongs to a host-shifting clade of tobamoviruses and likely skipped from another host. Phylogenetic analysis localized ToBRFV proximal to two major viral serotypes, which have undergone shifting events from a different plant order, Lamiales (121). One possibility is that ToBRFV originated from weeds proximal to agricultural habitats. Recent studies have shown that ToBRFV can infect 13 different weeds from 8 families, many of which inhabit agricultural surroundings (122, 123). These species could serve as potential reservoirs for ToBRFV in the field and may shed light on the potential origins of this virus.

Interaction of Tomato Brown Rugose Fruit Virus with Host Resistance Genes

The major cause for the ToBRFV epidemic is that this virus overcomes all known tobamovirus resistance genes in tomato (**Table 1**), including *Tm-1*, *Tm-2*, and the durable *Tm-2*^{2} (117). Computational analysis of the ToBRFV genome revealed that this virus contains 9–15% variability (RNA or protein comparison) compared to other members of the *Tobamovirus* genus (121). Among them, 21 amino acids were likely to be involved in the evasion of tomato resistance genes. As previously indicated, prior to the emergence of ToBRFV, Tm-2^2 had conferred exceedingly durable resistance against tobamoviruses (105). Because the tobamovirus MP is recognized by Tm-2^2, it was likely that the unique properties of the ToBRFV MP resulted in Tm-2^2 evasion. Indeed, recombinant clones of TMV and ToMV harboring the ToBRFV MP instead of their original MP were able to overcome Tm-2^2 resistance (109, 110). Mapping of resistance-breaking amino acids in the ToBRFV MP revealed six amino acids that were critical for evading *Tm-2*^{2} (110).

Previously, it was proposed that the high durability of *Tm-2*^{2}-mediated resistance against TMV and ToMV was due to its targeting of processes that are essential for viral infectivity or survival (105). The finding that Tm-2^2 targets the viral MP suggested that *Tm-2*^{2}-resistance-breaking mutations in the MP may also impact viral movement. Indeed, overcoming of Tm-2^2 by the ToBRFV MP was associated with attenuated cell-to-cell and long-distance movement (109). It was therefore logical to hypothesize that the reason for the durability of Tm-2^2 against TMV and ToMV was that it targeted MP elements that are essential for the movement of both viruses; one such element is the amino acid cysteine 68 (C68). The presence of C68 in the tobamovirus MP activates the Tm-2^2 immune response (110, 124). However, replacement of C68 in the TMV or ToMV MP resulted in complete loss of viral movement (124). Interestingly, in ToBRFV, C68 is substituted by a histidine, but viral movement is sustained (124). These findings suggest that prior to the emergence of ToBRFV, *Tm-2*^{2} resistance targeted elements within the tobamovirus MP that are essential for intercellular movement of the virus. This finding may explain the durability of Tm-2^2 against viruses other than ToBRFV.

In pepper, cultivars devoid of the *L* gene are susceptible to ToBRFV, displaying marked tobamovirus disease symptoms. However, in cultivars harboring the *L*^{1}, *L*^{3}, and *L*^{4} alleles, ToBRFV inoculation results in an HR in the infected leaf and plants are largely symptomless (125) (**Table 1**). In these plants, initial systemic infection could be detected; the virus was lost later during infection (125). Another report indicated that *L*^{3}- and *L*^{4}-mediated resistance to ToBRFV is temperature dependent and can break at temperatures higher than 30°C (126). Collectively, these studies suggest that while various *L* alleles are effective against ToBRFV, they likely do not provide complete immunity to the virus. The increasing global dispersal of ToBRFV may therefore lead to the selection of *L*-resistance-breaking isolates, which can adapt to pepper crops. Hence, ToBRFV is a potential threat to pepper crops and may affect them in the same way that it affects tomato.

In tobacco, the existing *R* genes confer efficient resistance to ToBRFV (**Table 1**). The tobacco *N* gene product confers immunity by recognizing the p50 helicase domain in the ToBRFV replicase protein, while the *N′* gene product recognizes the ToBRFV CP for plant resistance (127). Together, these proteins may provide useful models for generating new resistance to ToBRFV in tomatoes.

New Strategies for Tomato Brown Rugose Fruit Virus Resistance

The lack of genetic resistance to ToBRFV in tomatoes calls for the development of new strategies to cope with this destructive virus. The first and clearest strategy for the development of ToBRFV resistance is screening for resistance within collections of tomato varieties and wild species. Indeed, several accessions of the tomato wild species *S. habrochaites* and *S. peruvianum* showed increased ToBRFV resistance, but it was not sustainable at temperatures higher than 33°C and could easily be broken by the virus (128). A more promising approach was based on the screening of 160 tomato genotypes and related wild species for ToBRFV resistance (129). In this screen, 29 lines were found tolerant to ToBRFV. Genetic analysis of the tolerance trait revealed that it is controlled by a single recessive gene. Mapping of the ToBRFV tolerance locus pinpointed a specific location on tomato chromosome 11 that explains 91% of the trait in segregating populations. Strikingly, in the same analysis, a single resistant genotype was identified. Mapping of the resistance trait revealed the involvement of two different quantitative trait loci (QTLs): One was the same tolerance locus on chromosome 11, and the other was on chromosome 2, in the region of *Tm-1*. Together, these loci constituted the first evidence for ToBRFV resistance in natural tomato genotypes. The proximity of the second QTL to *Tm-1* suggests the existence of novel *Tm-1* alleles that may be able to confer resistance to ToBRFV.

Another possible approach to achieving ToBRFV resistance in tomato was based on CRISPR/Cas9-based mutagenesis of tobamovirus *Susceptibility* (*S*) genes termed tobamovirus multiplication (*TOM*). To establish a functional replication complex, the TMV replicase subunits interact with the transmembrane TOM proteins, which anchor it to intracellular membranes (11, 130–132). Consequently, loss or downregulation of *TOM* genes resulted in increased tobamovirus resistance in *A. thaliana* and *Nicotiana tabacum*. Importantly, the function of *TOM* genes is conserved among various plant species, making its mutagenesis a promising approach for tobamovirus resistance in different crops (133–135). In two recent independent studies, mutagenesis of *TOM* genes was performed to confer ToBRFV resistance in tomato (136, 137). In one study, four different homologs of the *Arabidopsis TOM1* gene were identified in tomato. Loss-of-function mutation in all four homologs conferred resistance to several tobamoviruses, including ToBRFV, TMV, ToMV, and youcai mosaic virus (136). In the second study, loss of only two homologs was reported to result in resistance to ToBRFV, but not to ToMV or TMV (137). These studies elegantly demonstrate how CRISPR-based technology can be applied to cope with emerging plant viruses such as ToBRFV.

FUTURE PROSPECTS FOR DEVELOPING PLANT RESISTANCE

Perhaps the most effective strategy for coping with plant viruses in the future originates from past research with transgenes, which express either viral CPs to perturb virion assembly or sRNA to enhance RNA silencing of the virus (138, 139). While the use of this technology was restricted due to negative public opinion and strict regulatory barriers, it still holds great potential for generating efficient resistance against agriculturally relevant viruses, including ToBRFV. Recent revival of transgene technologies for the improvement of crops, including tomato (140), may develop into a new era in which this technology could be reapplied for viral resistance.

Another approach that can be applied for plant protection is the exogenous application of dsRNA. This method was shown to confer protection against TMV (141) and can be modified to cope with other tobamoviruses. However, dsRNA application needs to be significantly improved for agricultural use, including more efficient manufacturing protocols, and stabilization of the dsRNA for prolonged effects. Technical advances in these fields have already been made and may lead to efficient dsRNA-based virocides for agricultural application.

One other blossoming field of research that can be applied for protection against new viruses is the design of NLR receptors that recognize new targets (142). Using several technologies, including protein engineering, random or site-directed mutagenesis, and structure-based predictions, NLRs can be designed for made-to-order pathogen recognition and defense. These approaches may be useful in the development of new tobamovirus-resistance strategies.

SUMMARY

The study of tobamoviruses is essential for learning fundamental processes in viral biology and plant immunity, as well as for the development of new resistance technologies. The evolutionary arms race between plants and tobamoviruses has provided indispensable insights into how plants cope with pathogens and how pathogens counter plant defenses. From an agricultural perspective, this perpetual battle requires the continual development of novel approaches for plant protection. The emergence of novel technologies in the field of plant immunity may boost the development of defense strategies for enhanced antiviral protection and future food security.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by the US-Israel Agricultural Research and Development Fund (BARD) research grant IS-5386-21R and the Israeli Ministry of Agriculture and Rural Development research grant 20-02-0130.

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