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REVIEW

Understanding tobamovirus-plant interactions: implications for breeding resistance to tomato brown rugose fruit virus

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Abstract

The genus *Tobamovirus* comprises a group of single-stranded RNA viruses that affect a wide variety of vegetables of economic importance. Tobamoviruses express a series of proteins that interact with the plant's cellular machinery, allowing viral infection; during incompatible interactions, active defense is mediated by host proteins encoded by resistance genes. The genes conferring viral resistance and tolerance in non-susceptible hosts have been studied for their ability to transfer desired resistance traits to different crops. The *N* gene from *Nicotiana* spp., the repertoire of *Tm* genes in *Solanum* spp., the *L* locus from *Capsicum* spp., and *TOM* genes are the most studied genetic sequences for understanding resistance to tobamoviruses. Through classical plant breeding and genetic engineering techniques, it has been possible to introgress these resistance genes (*R*) into new species. However, new reports highlight the ability of tobamoviruses to overcome *R*-mediated defense. One of the most notorious recent cases is the tomato brown rugose fruit virus (ToBRFV). The main characteristic of ToBRFV is its capacity to overcome the resistance mediated by the *Tm-2*^{2} gene, resulting in a limited repertoire of options to combat the virus. To defeat emerging viruses, it is necessary to apply the knowledge from other tobamoviruses-host relationships and use new technologies such as genome-wide association studies (GWAS) to understand and associate the architecture of resistance genes present in the Solanaceae family for the benefit of plant breeding. Although new genomic tools such as CRISPR systems open the possibility of coping with viral diseases, there are no commercial ToBRFV-resistant tomato varieties. Hence, the world's leading seed suppliers compete to develop and bring these varieties to market.

Keywords Tobamoviruses · ToBRFV · Resistance · Virus-host interactions

Introduction

Tomato (*Solanum lycopersicum*) production has become one of the most profitable agroeconomic activities worldwide. With yields on the rise, according to the Food and Agriculture Organization of the United Nations (FAO), global tomato production exceeded 180 million tons in 2020, with China and India being the principal producers (Guan et al. 2021; FAO 2020). Their nutritional value and antioxidant properties make tomatoes the world's second most important vegetable crop (Viuda-Martos et al. 2014). The increase in product demand has also brought about a paradigm shift at the economic and technological levels. Thus, controlling viral diseases that affect tomato crops is currently one of the main challenges of sustainable farming.

Tobamoviruses have a positive single-stranded RNA genome that codes for four proteins: an RNA-dependent

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RNA polymerase (RdRp) and a small replicase subunit protein (SrSp), both of which are involved in the viral replication process; the coat protein (CP), which is responsible for encapsulating the genetic material; and lastly, a cell-to-cell movement protein (MP) (Oladokun et al. 2019; Panno et al. 2019). In some tobamoviruses, an extra ORF6 has been reported to code for a factor related to pathogenicity (Canto et al. 2004). These proteins allow the development of viral infection through the cells and the plant; therefore, they are critical targets of plant resistance gene products, which in many cases determine the success or failure of the infection (King et al. 2012).

The members of the Brassicaceae, Cucurbitaceae, Malvaceae, and Solanaceae plant families are natural hosts of tobamoviruses (Adkins et al. 2003; Antignus et al. 2001). Tobamoviruses have no natural vectors and are easily transmitted mechanically during cultivation (Candemir et al., 2012). Characteristic lesions caused by tobamovirus infection include yellow necrotic spots on the fruit and a mosaic appearance that leads to the partial discoloration of sick leaves. Some relevant tobamoviruses used as study models are tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), tomato mottle mosaic virus (ToMMV), cucumber green mottle mosaic virus (CGMMV), and tomato brown rugose fruit virus (ToBRFV). All of them are of great importance due to the losses in yield and quality associated with the symptoms they induce in plants or fruits.

ToBRFV causes an emerging disease that was detected for the first time in Jordan in 2015 (Salem et al. 2016). Reports of ToBRFV outbreaks around the world are on the rise (Hamborg and Blystad 2021; Jones 2021), and they translate into significant economic losses. Regarding the dispersal route, ToBRFV, like other tobamoviruses, is transmitted by mechanical contact. Levitzky et al. (2019) showed, through controlled experiments, that ToBRFV can be spread by pollinating insects such as bumblebees (*Bombus terrestris*). Novel experiments suggest that the virus is present in the tomato seed coat and that the infection can be mechanically triggered when in contact with other seeds. This type of transmission is particularly dangerous due to the possible long-distance movement of infected material from one country to another within a short time (Caruso et al. 2022, Salem et al. 2021). A study by Salem et al. (2022) also reported plant species from up to 8 different families (including Amaranthaceae, Asteraceae, Malvaceae, and others) harboring ToBRFV. The studies to date suggest that the ecological niche of ToBRFV is not restricted to tomatoes or peppers alone, but it has a broad range, assuming greater probabilities of infecting essential crops.

In this review, we focus on the tobamovirus-host relationships, the spectrum of mechanisms involved in plant defense against tobamoviruses, and efforts to combat them. We emphasize the successful trials in which viral infection was overcome by employing transgenesis, introgression, or RNA interference tools. We also summarize experimental approaches, new technologies, and strategies reported for various study models that can serve as practical examples for developing ToBRFV-resistant tomato germplasm. Lastly, we present the current developmental state of commercial ToBRFV-resistant seeds based on available public information.

Dominant resistance (R) and recessive gene repertoire against tobamoviruses

Successful viral infection is the result of a complex molecular interplay between host plants and invading viruses (Wang 2015). The intricacies of these interactions give a range of options, from the total inability of the virus to replicate to replication of the virus without inducing symptoms. Paudel and Sanfaçon (2018) explain tolerance as an interaction in which viruses accumulate to some degree without causing significant loss of vigor or fitness in their hosts. Although the molecular mechanisms for tolerance are not yet well defined, understanding these mechanisms is a fundamental premise of breeding programs for vegetables that are still susceptible to some tobamoviruses.

According to Ishibashi and Ishikawa (2016), the replication of tobamoviruses is related to the suppression of plant defenses. One of the most studied and characterized plant defense mechanisms is mediated by resistance genes (*R*). Most *R* genes code for a protein with a nucleotide binding site and a leucine-rich repeat domain (NBS-LRR) (Shi et al. 2021). When NBS-LRR-containing proteins recognize avirulence factors encoded by pathogen *Avr* genes, programmed cell death is induced as part of a hypersensitive response (HR) (Jubic et al. 2019; Mur et al. 2008). Genes such as *N*, *L*, *Tm-1*, *Tm-2*, and *Tm-22* encode this type of protein and are fundamental to comprehending the processes of viral infection (Table 1) (Fraile and García-Arenal 2018).

The *N* gene of *Nicotiana glutinosa* was the first characterized tobamovirus resistance gene. Its introgression into commercial tobacco plants (*N. tabacum*) is considered a crucial milestone in combatting viral diseases caused by tobamoviruses, specifically TMV (Scholthof 2017). Although the *N* gene confers partial resistance against TMV, the virus rapidly and systemically moves into the leaves of the plants, developing limited local infections. A new study by Ikeda et al. (2021) showed an increase in the viral resistance of transgenic *N. benthamiana* against TMV by inducing the expression of different introns of the *N* gene. This combination of *N* transgenes revealed that the presence of two specific introns allowed for a more significant accumulation of

Table 1 Tobamovirus proteins and corresponding resistance genes in plants of commercial interest

Virus	Protein	Resistance	Crop	Reference
TMV	CP and p50 helicase domain	*N* gene	Tobacco	Whitham et al. (1994)
ToMV	Helicase domain MP	*Tm*1 gene *Tm*2 gene	Tomato	Ohmori et al. (1998) Hall (1980)
ToMMV	MP	*Tm*2^2 gene	Tomato	Nagai et al. (2019)
CGMMV	Unknown	*cgmmv* 1 and 2	Cucumber	Sugiyama et al. (2007)
PMMoV, PaMMV	Unknown	*L*1-*L*4 genes	Pepper	Antignus et al. (2008) Matsumoto et al. (2008)

premature and mature *N* transcripts and lesser viral spread. Although the mechanisms of intron regulation are still far from being fully understood, the methodology of using introns as transcriptional "enhancers" could be helpful in the development of total resistance against tobamoviruses.

In the *Capsicum* genus, the *L* locus, which is composed of four similar genes (*L*1-*L*4), mediates resistance against tobamoviruses (Kenyon et al. 2014). The type of virus determines the degree of resistance; TMV, ToMV, or TMGMV (tobacco mild green mosaic virus) cannot develop infection; other tobamoviruses with narrow geographic distribution are also incapable of triggering infection in peppers. Similarly, in a study conducted by Vélez-Olmedo et al. (2021), strains of yellow pepper mild mottle virus (YPMMoV) and chili pepper mild mottle virus (CPMMoV) were mechanically inoculated and classified as unable to overcome the resistance mediated by sequences of the *L* locus. However, other tobamoviruses can overcome the resistance mediated by the *L* alleles. A study by Luria et al. (2018) reported a strain of paprika mild mottle virus (PaMMV) capable of overcoming *L*3-mediated resistance in pepper plants. Although PaMMV can infect other plant species, the host range is narrow, and its viral titers are minimal in these plants. In an attempt to determine the behavior of this PaMMV strain in other Solanaceae plants, an increase in infection was found when co-inoculated with other tobamoviruses in tomatoes. Based on the interactions of emerging tobamoviruses, the authors concluded that the breaking of resistance occurred, and there was risk imposed by the possibility of coordinated co-infection by several viruses.

In *Solanum* spp., the *Tm*-1 and *Tm*-2 alleles mediate resistance against ToMV by binding the corresponding R proteins to the viral replication complex (*Tm*-1) or viral MP (*Tm*-2) (Ishibashi and Ishikawa 2013). Although the alleles have shown durability over time, the appearance of new mutant viral variants could easily lead to a hampered plant resistance mechanism (Ishibashi et al. 2012). A study by Hussain et al. (2024) evaluated the variability of 24 tomato lines native to Pakistan and other global reference lines against infection by ToMV and tomato yellow leaf curl virus (TYLCV, genus *Begomovirus*). Twenty-three native lineages were sensitive to ToMV infection but displayed different lesion patterns. Considering parameters such as disease severity and the percentage of infection or symptoms due to ToMV, only one accession (Acc-17,878) was asymptomatic and was considered resistant. The authors highlighted that the available germplasm variability helps achieve genetic improvement to deliver high-yielding resistant tomato varieties.

ToMMV is a tobamovirus that was first reported in tomato greenhouses in Mexico (Li et al. 2013) and has close to 85% sequence identity at the nucleotide level to ToMV and TMV. However, there is special attention to its underrepresented prevalence due to the lack of serological tests that discriminate it from ToMV (Turina et al. 2016). ToMMV can infect pepper plants (Nagai et al. 2019) and overcome resistance to tobamoviruses (Lovelock et al. 2020). Interestingly, some reports show that the tomato *Tm-*2^2 gene could mediate resistance against ToMMV (Nagai et al. 2019). Li et al. (2017) highlighted that two viral proteins, 126 kDa and 54 kDa, were essential to the ToMMV replication process and are therefore important for understanding the mechanism of viral infection. Tu et al. (2021) developed infectious cDNA clones of ToMMV that were able to infect *N. benthamiana* plants. These recombinant ToMMV clones proved to be highly infectious and pathogenic, which introduces the possibility of exploring the pathogen-host relationship through gene silencing or other means.

An alternative to *R*-mediated plant breeding is recessive resistance. Unlike the strategy mediated by a dominant gene, this strategy relies on suppressing recessive genes function. In *Arabidopsis thaliana*, the *tobamovirus multiplication* 1 (*TOM*1) gene family is required for the multiplication of TMV (Ishikawa et al. 1991). Simultaneous loss-of-function mutations of *TOM*1 and its putative paralog *TOM*3 result in near-complete inhibition of tobamovirus multiplication. These and other genes code for proteins in the tobamovirus replication complexes (Yamanaka et al. 2002; Nishikiori et al. 2011). The knockout of genes essential for the virus cycle has shown promising results for ToBRFV control (Ishikawa et al. 2022; Zhang et al. 2022), as described below.

In Cucurbitaceae plants, the wild species *Cucumis africanus* shows high levels of resistance against CGMMV. However, most commercial cucurbits are still susceptible to infection by the virus (Mandal et al. 2008). One of the first approximations to elucidate tobamovirus resistance mechanisms in cucurbits was reported in *Cucumis melo* L. 'Chang Bougi,' a cultivar with partial resistance to CGMMV. The

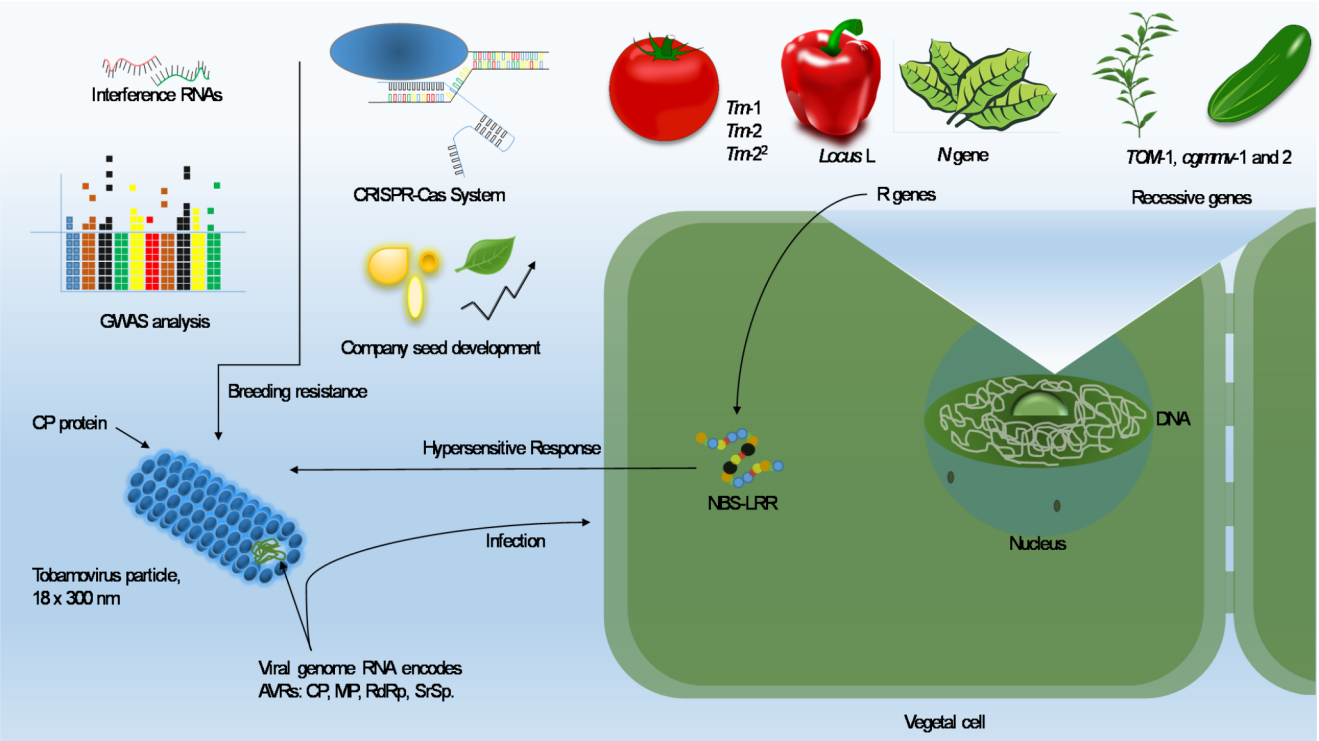

Fig. 1 Plant defense mechanisms against tobamovirus infections and promising methodologies for plant breeding. Tobamovirus infection begins with the internalization of the virion into the plant cytoplasm. The coat that protects the genetic material disintegrates, releasing viral genomic RNA (+). After expressing RdRp, the complementary genomic RNA strand (-) is synthetized and will serve as a template for the synthesis of new genomic RNA strands (+). SrSp plays a fundamental role in viral replication. With the help of MP, the viral replication complex travels from cell to cell through the plasmodesmata to continue the cycle. This process can be hampered by host defenses mediated by resistance genes. *Tm-1*, *Tm-2*, *Tm-2*^{2}, *L*, and *N* are dominant resistance genes from the NBS-LRR class. *TOM*, *cgmmv-1*, and *cgmmv-2* are recessive genes. The CRISPR-Cas system, GWAS, and RNAi are new generation procedures that will shorten the time in the search and/or development of resistance against tobamoviruses.

products of the recessive genes *cucumber green mottle mosaic virus resistance*-1 (*cgmmv*-1) and *cucumber green mottle mosaic virus resistance*-2 (*cgmmv*-2) could be responsible for resistance to the virus and serve as a starting point for developing plant breeding programs in cucurbits (Sugiyama et al. 2007). A study by Ruiz et al. (2021) evaluated 47 different accessions of cantaloupes from Asia and Europe; after the mechanical inoculation of the leaves and the development of the infection, 16 *Cucumis melo* accessions presented partial resistance against CGMMV. Interestingly, the Japanese cultivar Freeman's cucumber and two Spanish accessions (Rochet (BGV004884) and Alficos (BGV004853)) were reported to be resistant to CGMMV.

To date, CGMMV has been positioned as one of the main threats to cucurbits because there are no known commercial cultivars with total resistance to this virus. However, studies such as these introduce the possibility of future breeding programs. A simplified schematic representation of plant-tobamovirus interactions based on *R*-mediated defense and recessive resistance is depicted in Fig. 1, showing promising methodologies for breeding tobamovirus-resistant cultivars of commercial plants.

The search for resistance against ToBRFV

Regarding efforts to obtain ToBRFV resistance, a patent by Hamelink et al. (2019) referred to the invention of a genetically modified tomato with the characteristics of some genotypes of *S. pimpinellifolium* and *S. habrochaites* (which are naturally resistant to ToBRFV). At the time, it was considered a transcendent step in developing resistance against ToBRFV (Ashkenazi et al. 2020; Ykema et al. 2020). In similar attempts, an evaluation of *S. ochrantum* (a close relative of wild tomatoes) exhibited high levels of resistance to ToBRFV and other tobamoviruses. However, transferring these traits to conventional tomatoes is difficult due to sexual incompatibility between *S. ochrantum* and *S. lycopersicum*. A potential alternative to overcome this genetic barrier would be somatic hybridization (Jewehan et al. 2022a; Pertuzé et al. 2002).

In another recent study, Jewehan et al. (2022b) reported the evaluation of wild tomato accessions (*S. habrochaites* and *S. peruvianum*) infected by ToBRFV. From 173 samples, nine accessions of *S. habrochaites* and one of *S. peruvianum* were highly resistant. These plants showed no

symptoms at 24 °C, and no virus could be detected on the inoculated leaves. However, when resistant plants inoculated with ToBRFV were incubated at 33 °C, they expressed mosaic and deformation symptoms, indicating that resistance is broken at high temperatures. These findings demonstrate that some wild tomato species may have unknown ToBRFV resistance genes, opening a new path to discovering sequences involved in resistance against ToBRFV. However, the same research group reported a ToBRFV mutant (Tom2M-Jo) capable of breaking the natural resistance in *S. habrochaites* and *S. peruvianum* (Jewehan et al. 2022c). Tom2M-Jo has two substitutions in the MP gene that result in amino acid changes in the 30 kDa MP (Phe22 → Asn and Tyr82 → Lys). These substitutions have not been reported before in ToBRFV isolates, highlighting the difficulty of finding long-term resistance.

A critical study to develop ToBRFV-resistant tomato varieties by Zinger et al. (2021) reported the identification of loci related to resistance and tolerance against ToBRFV. For this purpose, tomato varieties susceptible (VC532) and tolerant (VC554) to ToBRFV were crossed and self-pollinated to obtain the F2 population ($n = 160$). By characterizing the ToBRFV response in parental, F1, and F2 plants and high-throughput sequencing, it was found that parental plants share common genome regions (on chromosome 11) that control tolerance to ToBRFV. The results indicate that the resistance trait is partially dominant and that *Tm*-1 and *Tm*-2 are ineffective at controlling ToBRFV infection in tomato plants. The new sequences found in the locus of chromosome 11 will allow for a better understanding of how some tomato varieties' natural resistance to ToBRFV is governed. Although the evaluated alleles are strongly associated with tolerance control, the authors concluded that other loci participate in the resistance process. Studies on the interactions between loci as a way to search for resistance against ToBRFV open up new possibilities for developing phenotypes resistant to the virus. The introgression of these resistance alleles can be assisted by plant breeding with gene-editing technologies.

The suppression of functional genes and viral proteins is also emerging as a promising alternative to confer resistance against tobamoviruses. Examples worthy of note are the works of Ishikawa et al. (2022) and Kravchik et al. (2022), in which sequences homologous to genes indispensable for tobamovirus replication from *Arabidopsis* were edited in tomato using CRISPR/Cas9 (see below). Notably, several genetic sequences with unknown functions have been proposed as potential candidates for inhibiting ToBRFV components (e.g., *NBS*, *RLP*, and *RLK* genes reported by Andolfo et al., 2013). However, the characteristics of the new ToBRFV mutants (Tom2 M-Jo) raise the possibility of a change in the current paradigm, in which the main resistance trait relies on the presence of *R* genes. New approaches to addressing ToBRFV infection are needed, and the previously mentioned recessive resistance examples seem to support this idea. In either case, the functional analysis of ToBRFV proteins is essential to advance the understanding of their role in the viral infection and resistance processes, as described below.

Characterizing ToBRFV proteins, a fundamental step for breeding resistance

Currently, the breeding of plants of commercial interest is performed by gene introgression and the specific suppression of gene expression (Nishiguchi et al. 2019). In tomato plants, some *R* genes have been introduced from wild tomatoes, such as *S. pimpinellifolium* or *S. habrochiates*, to commercial lines of *S. lycopersicum* to observe and preserve the desired phenotype. During the breeding of plants resistant to ToBRFV, it is imperative to know which genes and proteins interact in the infection process and to determine which sequences are more likely to develop the desired resistance.

Weber et al. (2004) described one of the first approaches to understanding the function and characteristics of the MP avirulence factor of ToMV (MP^{ToMV}). By using Craigella tomato cultivars GCR 26 (without any resistance gene against ToMV), GCR 236 (*Tm*-2/*Tm*-2) and GCR 267 ($Tm\text{-}2^2/Tm\text{-}2^2$), and different MP^{ToMV} transgenes, the authors elucidated some similarities and differences between resistance genes *Tm*-2 and $Tm\text{-}2^2$ against ToMV. Both genes can induce HR after recognizing MP^{ToMV}, but *Tm*-2 recognizes a domain at the N-terminus of the MP, while $Tm\text{-}2^2$ most likely interacts with more than one region of the MP.

Upon studying the complete genome sequencing of ToBRFV (Salem et al. 2016, Luria et al. 2017, Chanda et al. 2020), it was clear that ToBRFV has a high identity and a short evolutionary history with other tobamoviruses. However, one main characteristic that differentiates ToBRFV from other tobamoviruses is its ability to avoid the polypeptide encoded by the $Tm\text{-}2^2$ gene (Luria et al. 2017). Any tobamovirus infectious process is partly mediated by its MP, which has been described as a crucial viral element that triggers intracellular invasion by increasing virus permeability and movement within the plasmodesmata (Sheshukova et al. 2020).

To understand the function of MP^{ToBRFV}, which breaks the resistance mediated by the $Tm\text{-}2^2$ gene product, Hak and Spiegelman (2021) reported the essential characteristics of this viral protein. First, they demonstrated that expressing MP^{ToBRFV} alone is not enough to activate the function of $Tm\text{-}2^2$, as opposed to MP^{TMV}. Then, using hybrid infective clones with other tobamoviruses, the authors demonstrated

that the amino acid sequence of MP^{ToBRFV} from residues 1 to 216 plays a determining role in the development of the infection, because the C-terminal fraction of MP alone is not sufficient to activate *Tm*-2^2 gene-mediated plant resistance. Lastly, ToBRFV infection spreads slower than TMV, as evidenced by the detection of a higher signal in *N. benthamiana* leaves inoculated with a TMV-GFP (green fluorescent protein) hybrid compared to plants inoculated with a TMV-$GFP^{MP-ToBRFV}$ hybrid.

Yan et al. (2021) evaluated the role that MP^{ToBRFV} plays in triggering (or not triggering) HR. Using MP constructs (GFP-MP^{ToBRFV} and GFP-MP^{TMV}) and chimeras, they established that the central amino acid region of MP^{ToBRFV} is involved in overcoming *Tm*-2^2-mediated resistance. By interchanging regions of MP^{ToBRFV} and MP^{TMV}, the authors determined that residues 60–186 of the virulence factor MP^{ToBRFV} are responsible for overcoming resistance by ToBRFV; specifically, residues H67, N125, K129, A134, I147, and I168. Since MP^{ToBRFV} is essential to overcoming *Tm*-2^2-mediated resistance, evaluating the spread of viral infection by monitoring HR-related markers could accurately determine the behavior of new hybrids and help better understand the interrelation between the MP protein and the *Tm*-2^2 product. Recently, Rivera-Márquez et al. (2022) used an *in silico* approach to find new and potential mutations for *Tm*-2^2 that would increase the binding affinity to MP^{ToBRFV}, H384W, and K385L. Studies such as this one show the relevance of bioinformatics to help design strategies that can be confirmed by in vitro methods later on.

Silencing, editing, and new approaches for the development of ToBRFV-resistant tomato cultivars

RNA interference (RNAi) is a cellular mechanism that regulates gene expression via small RNAs (Hung and Slotkin 2021). The study of the RNAi pathway in tobamovirus-plant models allows us to use this information to design control strategies, for example, designing small interfering RNAs (siRNAs) specific to critical sequences of the genome or viral transcripts (hot spots). Virus-derived small interfering RNAs (vsiRNAs) and artificial microRNAs (amiRNAs) are some of the most commonly used methodologies for gene silencing against viruses.

Jiao et al. (2022) characterized small RNAs against pepper mild mottle virus (PMMoV). Leaves of peppers (*Capsicum annuum* L. cv. Zunla-1) were inoculated with PMMoV, and viral small interfering RNAs (vsiRNAs) were identified. These vsiRNAs were characterized *in silico* using the miRanda algorithm, and their expression was evaluated. PMMoV infection in peppers generates a wide variety of vsiRNAs, and those from (+) RNA are the most abundant. Additionally, PMMoV infection significantly increased the proteins that play crucial roles in generating vsiRNAs, such as CaDCL2 and CaRDR1. In light of the abundance of 21–22 nt vsiRNAs found in their study, the authors suggested that they were produced during the development of a successful infection.

One of the most effective attempts to combat tobamovirus in cucurbits was reported by Liang et al. (2019), who developed and evaluated three amiRNAs to target viral CP, MP, and replicase gene sequences through a gene silencing methodology. After *N. benthamiana* infiltration, a positive correlation was observed between amiRNA expression and tolerance to CGMMV, as evidenced by a reduced viral load. The most promising results were observed after CP silencing. Subsequently, in a follow-up to Liang's work, Miao et al. (2021) reported on transient expression assays with polycistronic amiRNA and synthetic trans-acting small RNAi in *N. benthamiana* and cucumber protoplasts. The study demonstrated that the polycistronic amiRNA construct conferred long-lasting resistance to CGMMV in cucumbers.

In a study by Li et al. (2016), vsiRNAs were characterized in cucumber seedlings infected with CGMMV 14 days post-infection. RNAs measuring 21–22 nt in length were predominant in leaves infected with CGMMV, suggesting that 21-nt vsiRNAs represent the main antiviral silencing component in the plant, which are produced by the DCL4 protein. Although the effects of 21-nt vsiRNAs are known in plants (Mitter et al. 2013; Zhang et al. 2015), the authors suggested that vsiRNAs could also have a role during the CGMMV infection cycle since several genes involved in cellular processes, regulation, and structure were predicted to be targeted by these vsiRNAs.

Using gene silencing in plants has benefited the development of varieties with better responses to stress. Under this premise, an *in silico* study by Gaafar and Ziebell (2020) reported possible therapeutic targets of microRNAs against ToBRFV. On the assumption that *S. lycopersicum* encodes mature miRNAs that may have a protective effect against ToBRFV infection, a total of 147 tomato miRNA sequences were analyzed by five different RNA target prediction tools (miRanda, RNAhybrid, RNA22, Tapirhybrid, and psRNATarget). Up to 11 miRNAs were found to share some regions of the ToBRFV genome as a common target. The authors concluded that the sequences they found may effectively increase *S. lycopersicum* immunity against ToBRFV; however, it is also necessary to validate their results using amiRNAs (Song et al. 2014). Considering the scope of the study and if those results could be extrapolated to *in vitro* technology, the strategy would undoubtedly serve as a model for developing ToBRFV-resistant tomato plants.

Gene silencing in *S. lycopersicum* provides an obvious opportunity to understand the genetic architecture of the RNAi machinery and the sRNAs (length or sequence) and

how they are used against ToBRFV. The following steps toward understanding the ToBRFV-*S. lycopersicum* interactions should be aimed, among other goals, at unveiling the genetic similarity among specific sRNAs synthesized under infections in *S. lycopersicum* by close tobamoviruses such as ToBRFV vs. ToMMV. To date, no studies have addressed this question, which could clarify how the viral defense system of *S. lycopersicum* against tobamovirus has evolved.

In the quest for ToBRFV control, genetic editing is another strategy that has regained particular interest over the past decade since the development of the clustered regularly interspaced short palindromic repeats (CRISPR) editing system and its applications in plants.

One of the cases in which genetic editing was successfully used in plants to confer resistance against a virus was the work described by Aman et al. (2018). They achieved interference to turnip mosaic virus (TuMV, genus *Potyvirus*) in recombinant *N. benthamiana* lines using the CRISPR-Cas 13a system. First, transactivating CRISPR RNAs (crRNAs) were designed to target four different regions of the TuMV-GFP hybrid genome. After infiltrating leaves with crRNAs targeting *HC-Pro* (coding for a helper component-proteinase silencing suppressor) and *GFP*2 sequences, an ~ 50% reduction in the GFP signal level was observed. The authors concluded that Cas 13a is an RNA-guided ribonuclease that can be programmed to target and degrade viral RNA genomes.

Although drawbacks in using CRISPR-Cas 13 have been described due to RNA degradation (Ali et al. 2018), other systems have been used to obtain plant phenotypes with desired characteristics. For instance, Ghorbani et al. (2020) reported a significant reduction in viral DNA compared to control plants when *S. lycopersicum* (cv. Moneymaker) seeds were inoculated with TYLCV. Single guide RNAs (sgRNAs) were designed from the intergenic region (IntR) and CP sequences of TYLCV. Then, sgRNAs were separately infiltrated into tomato plants; these plants presented a lower viral load than the control plants when inoculated with the virus. The authors demonstrated the effectiveness of using the CRISP-Cas 9 system in targeting TYLCV. Although off-target effects are one of the main limitations of the CRISPR-Cas 9 system, this problem was overcome in this study due to modifications in the activating promoter of endonuclease Cas 9.

Another interesting report regarding resistance to ToBRFV infection in tomatoes is the work of Ishikawa et al. (2022). Based on the nucleotide sequence of *TOM1* (gene essential for tobamovirus multiplication) from *A. thaliana*, similar sequences were identified in *S. lycopersicum*; subsequently, using CRISPR-Cas 9, a quadruple knockout of the homologous *TOM1* genes (*SlTOM1a–e*) was performed in tomato. A single mutation in the *TOM1* homolog did not affect ToBRFV accumulation in the inoculated plants. However, in *Sltom1* triple mutants, CP^{ToBRFV} accumulation was reduced compared to that in wild-type plants. It is also suggested that the contribution of *SlTOM1* genes to ToBRFV multiplication was in the order *SlTOM1a* > *SlTOM1c* > *SlTOM1d* > *SlTOM1b*. The knockout of homologous *TOM1* genes generated long-lasting resistance in tomatoes, suggesting that genome editing using CRISPR-Cas 9 could aid in the development of ToBRFV-resistant tomato plants.

Using the same editing tool, Kravchik et al. (2022) reported resistance to ToBRFV in *S. lycopersicum* cv. M82. After both *SlTOM1a* and *SlTOM3* were knocked out, the resulting plants were asymptomatic in response to ToBRFV infection, and their *SlARL8a3* susceptibility gene expression was reduced. Although *SlARL8a3* alone did not contribute to ToBRFV resistance, it was observed that the double mutants were susceptible to TMV and ToMV, establishing that sometimes the effects observed in some study models cannot be extrapolated to other viral species.

As noted in these reports, the study of recessive resistance in *S. lycopersicum* (*TOM* genes) has made it possible to establish different action methods in the struggle to develop tomato plants resistant to ToBRFV infection. The CRISPR-Cas system has overcome limitations (such as low target recognition and stability) present in alternative methodologies (e.g., site-directed mutagenesis, the Cre-Lox system (Cre recombinase and Lox sequences), or the TALEN system (transcription activator-like effector nucleases)). In this way, CRISPR-Cas allows gene editing with greater precision and control over the study model.

To achieve resistance to ToBRFV and other viruses, researchers need to develop new strategies to search for host sequences capable of constraining ToBRFV infection alongside new strategies to look for them. After completing the reannotation of the NB-LRR genes of *S. lycopersicum* (cv. Heinz 1706) using the resistance gene enrichment and sequencing (RenSeq) approach (Andolfo et al. 2014), Andolfo et al. (2021) determined the impact of the vast repertoire of NB-LRR genes on plant breeding strategies. In general, the NB-LRR genes showed genetic expansion and divergence with respect to other Solanaceae family members, such as potatoes. Interestingly, approximately 80% of the annotated genes have a single catalytic domain whose function is still unknown. In the tomato genome, chromosome nine harbors signaling-related sequences such as *Sw*5 (resistance against tospovirus) and *Tm*-2^2 genes that lead to plant cell immunity. The sequences of the NB-LRR gene repertoire open the door to gene editing techniques to improve the immune defense machinery of plants of agroeconomic interest, such as tomatoes.

Complementary to these efforts, genome-wide association studies (GWAS) could serve as a starting point for

Table 2 ToBRFV-resistant seeds are expected to be launched on the market in the coming years

Company	Product launch date	Product (seed)	Resistance against ToBRFV	Reference
Bayer (Germany)	2024	NA	Intermediate	Bayer News (2021)
BASF (Germany)	2020*	Teenon F1	Intermediate	de Domènech (2020)
Syngenta (Switzerland)	2021*	Barosor Lansor	High Intermediate	Syngenta Group News Service (2021)
Enza Zaden (Netherlands)	2022	NA	High	Enza Zaden Group (2020)

*Original estimated closing. Due to the COVID-19 pandemic, new launching closings and market availability in regions or countries are not available. NA: Not available.

searching sequences of interest. In tomato, GWAS have been aimed at seeking the usefulness of genetic sequences associated with the organoleptic traits of the product, such as flavor (Tieman et al. 2017), weight, and ripening time (Wang et al. 2019). However, with the fast-rising -omics sciences, the utility of this tool diversified. Following a GWAS, Bauchet et al. (2017) reported more than 11,000 single nucleotide polymorphisms (SNPs) in accessions of different tomato varieties and classified the sequences into six genetic groups associated with agroeconomic interest traits, such as fruit weight and resistance to diseases. For the disease resistance trait, up to seven genes were identified on three different chromosomes. The diversity of genetic sequences associated with resistance is due to a strong impact of genetic introgression developed partly by breeding different tomato species.

Patents and companies involved in ToBRFV-resistant seed development

To date, reports on the development of commercial tomato varieties resistant to ToBRFV are lacking (Zhang et al. 2022). A considerable number of patents are registered (Supplementary material 1) in the World Intellectual Property Organization (WIPO). Most of these inventions describe phenotypes in which ToBRFV replication is delayed, reduced, or inhibited in the plants of interest. With a focus on a commercial perspective, transnational companies engage in research efforts to develop varieties of tomato seeds with total resistance to ToBRFV. The varieties intended for introduction to the agricultural market are defined by the degree of resistance to ToBRFV. The seeds with a new genotype supporting intermediate resistance (IR) are among the first to be developed. Although infected (PCR tests may be positive for the presence of ToBRFV), IR plants are asymptomatic or develop only mild symptoms in their leaves and fruits. Although this finding represents a genuine advance, studies on the agricultural and economic impact of IR tomato varieties are missing. Additionally, introducing highly resistant phenotypes stands out as one of the short- and medium-term leading prospects.

The development of tomato seeds with high resistance to ToBRFV is still in the experimental phase, with the idea of formulating seeds that limit the infection and spread of the disease (Table 2). Undoubtedly, this viral disease represents a challenge for companies dedicated to developing seeds, not because of their access to monetary funds or technology but because of each country's agricultural regulations for using and commercializing seeds. Disagreements between developer agents and farmers can be expected due to the use of intellectual property and the benefit that the seed represents as innovation.

Conclusion and future trends

The *Tobamovirus* genus represents a threat to commercially important species of Brassicaceae, Cucurbitaceae, Malvaceae, and Solanaceae. Although members of these groups of plants naturally present a resistance response against viruses, sometimes the defense is overcome. Like other tobamoviruses, CGMMV and ToBRFV can be combated by avoiding mechanical transmission; however, no commercial cultivars resistant to these viruses are available to help prevent outbreaks.

Since MP^{ToBRFV} can overcome host resistance mediated by the product of *Tm*-2^2, an *R* gene present in commercial tomato varieties, new unknown sequences from wild Solanaceae plants are becoming relevant to look for means to inhibit this emergent virus. Nevertheless, the search should not be limited to dominant resistance genes; the study of recessive resistance in *S. lycopersicum* has attracted renewed interest and opens a new promising picture for developing tomato lines resistant to ToBRFV.

We consider that the new trends in the short term should be aimed at understanding the genomic architecture and the functioning of a wide range of resistance mechanisms that still remain unknown in a more robust way. We may face a new scenario in plant breeding research, in which obtaining ToBRFV-resistant tomato varieties could rely on next-generation sequencing and gene editing tools such as CRISPR-Cas and gene silencing. These strategies will shorten research times and have very high confidence thresholds. Additionally, prior to testing in experimental crops, it is

necessary to construct effective in vitro models to inhibit virus replication.

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