Resistance to Tospoviruses in Vegetable Crops: Epidemiological and Molecular Aspects

Massimo Turina,[1] Richard Kormelink,[2] and Renato O. Resende[3]

[1]Institute for Sustainable Plant Protection, CNR Torino, 10135 Torino, Italy; email: massimo.turina@ipsp.cnr.it

[2]Laboratory of Virology, Department of Plant Sciences, Wageningen University, 6708PB Wageningen, The Netherlands

[3]Department of Cell Biology, University of Brasília, 70910-900 Brasília, DF, Brazil

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Abstract

During the past three decades, the economic impact of tospoviruses has increased, causing high yield losses in a variety of crops and ornamentals. Owing to the difficulty in combating thrips vectors with insecticides, the best way to limit/prevent tospovirus-induced diseases involves a management strategy that includes virus resistance. This review briefly presents current tospovirus taxonomy, diversity, molecular biology, and cytopathology as an introduction to a more extensive description of the two main resistance genes employed against tospoviruses: the *Sw5* gene in tomato and the *Tsw* in pepper. Natural and experimental resistance-breaking (RB) isolates allowed the identification of the viral avirulence protein triggering each of the two resistance gene products; epidemiology of RB isolates is discussed to reinforce the need for allelic variants and the need to search for new/alternative resistance genes. Ongoing efforts for alternative resistance strategies are described not only for *Tomato spotted wilt virus* (TSWV) in pepper and tomato but also for other vegetable crops heavily impacted by tospoviruses.

INTRODUCTION

Tospoviruses are plant-infecting viruses of the family *Bunyaviridae*, a family that includes mostly arthropod-borne viruses with secondary plant or vertebrate hosts: Only viruses of the *Hantavirus* genus have adapted a vertebrate-to-vertebrate transmission that does not involve arthropod vectors (92). Because tospoviruses most likely have evolved from an insect-infecting bunyavirus ancestor (45), tospovirus-host interactions have been studied from both the insect-host and the plant-host perspectives. A number of reviews have been written on the tospovirus-thrips vector interactions (53, 147, 148), and an exhaustive review about the molecular biology of tospoviruses was published in 2011 (59). Here, we cover the main achievements gained in recent years for the tospovirus–plant host interactions, in particular for the *Tomato spotted wilt virus* (TSWV)-pepper and TSWV-tomato systems.

TOSPOVIRUS DIVERSITY: NEW AND OLD THREATS TO VEGETABLE CROPS FROM TOSPOVIRUSES

An exhaustive review on tospovirus diversity for different crops and different geographical areas was written fairly recently (87); furthermore, two reviews well characterized the tospovirus diversity present in the Mediterranean basin (140) and on the Indian subcontinent (70). Here, we update the information contained therein, with specific attention to new threats to vegetable crops.

At present, at least 29 tospoviruses have been identified as distinct or tentative species (**Table 1**). A phylogenetic tree based on the N-protein coding sequences, one of the ICTV (International Committee on Taxonomy of Viruses) demarcation criteria, is displayed in **Figure 1** and includes all the proposed and established tospovirus species, with the main geographically based origin for each clade. In Asia, a number of new potential threats to vegetable crops have been recently discovered infecting tomato, sweet pepper (50, 110, 150), and ornamental crops (36); particularly worrisome is the recent first report of TSWV in India (97). Meanwhile, *Iris yellow spot virus* (IYSV) belonging to the Euro-Asian clade has been spreading in Europe (5). Similarly, Tomato yellow ring virus (TYRV), which was originally found only in the Middle East on tomato, potato, and some ornamental crops, has recently been reported to be present in Poland and Kenya (9, 151). In the Americas, a number of distinct tospoviruses, which form a new subclade of American tospoviruses (29, 152) and are important for vegetable crops, have been reported, one infecting common bean in Brazil [Bean necrotic mosaic virus (BeNMV)] and another infecting soybeans in the United States [Soybean vein necrosis-associated virus (SVNaV)]. Recently, a new virus infecting *Capsicum* spp. in Peru [Pepper necrotic spot virus (PNSV)] has been characterized (137). Interestingly, *Melon yellow spot virus* (MYSV), formerly reported only in Asia, has for the first time been found outside Asia, in Ecuador (94). Furthermore, in Florida in 2012 (66), the first occurrence of a natural interspecific reassortant *Groundnut ringspot virus* (GRSV)–*Tomato chlorotic spot virus* (TCSV) in tomato fields was reported (144) and its parental TCSV species identified. Another study performed with a number of TSWV isolates showed that reassortment among isolates within a species seems to be a common evolutionary strategy (136), and, in one case, a resistance-breaking (RB) isolate was shown to originate through reassortment (73). Furthermore, in North America a new tospovirus species infecting melon [Melon severe mosaic virus (MSMV)] was found in a number of melon-growing areas in Mexico (24). In addition to the species important for vegetable crops, three new species infecting Lisianthus, Alstroemeria, and mulberry trees have been identified and characterized in Japan, Colombia, and China, respectively (49, 76, 113). For many of these newly characterized tospovirus species, the corresponding thrips vectors still have to be identified as part of a proper risk assessment and an estimate of their economic impact on

Table 1 List of tospoviruses with percentage identities at the amino acid level in the N protein below 90%

Acronym	Virus name[a]	GenBank accession number	Country of first isolation
ANSV	Alstroemeria necrotic streak virus	GQ478668.1	Colombia
BeNMV	Bean necrotic mosaic virus	JN587268.1	Brazil
CaCV	Capsicum chlorosis virus	NC_008301.1	Australia
CCSV	Calla lily chlorotic spot virus	AY867502.1	Taiwan
CSNV	Chrysanthemum stem necrosis virus	KM114548	Brazil
GBNV	*Groundnut bud necrosis virus*	AY871098.2	India
GRSV	*Groundnut ringspot virus*	AF487516.1	Brazil
GYSV	*Groundnut yellow spot virus*	AF013994.1	India
HCRV	Hippeastrum chlorotic ringspot virus	JX833564	China
INSV	*Impatiens necrotic spot virus*	X66972.1	United States
IYSV	*Iris yellow spot virus*	AF001387.1	Israel, United States, Brazil, Netherlands
LNRV	Lisianthus necrotic ringspot virus	AB852525	Japan
MSMV	Melon severe mosaic virus	EU275149.1	Mexico
MVBaV	Mulberry vein banding associated virus	KM819701.1	China
MYSV	Melon yellow spot virus	AB038343.1	Japan
PCFSV	Peanut chlorotic fan-spot virus	AF080526.1	Taiwan
PCSV	Pepper chlorotic spot virus	KF383956	Taiwan
PNSV	Pepper necrotic spot virus	HE584762.1	Peru
PolRSV	*Polygonum ringspot virus*	KJ541744	Italy
SVNaV	Soybean vein necrosis-associated virus	HQ728387.1	United States
TCSV	*Tomato chlorotic spot virus*	AF282982.1	Brazil
TNRV	Tomato necrotic ringspot virus	FJ489600.2	Thailand
TNSV	Tomato necrotic spot virus	KM355773.1	China
TSWV	*Tomato spotted wilt virus*	AF020660.1	Australia
TYRV	Tomato yellow ring virus	AY686718.1	Iran
TZSV	Tomato zonate spot virus	EF552433.1	China
WBNV	Watermelon bud necrosis virus	GU584184.1	India
WSMoV	*Watermelon silver mottle virus*	AB042650.1	Taiwan
ZLCV	*Zucchini lethal chlorosis virus*	AF067069.1	Brazil

[a]Species established by the International Committee on Taxonomy of Viruses (ICTV) are in italics.

vegetable crops and ornamentals must be made. The family *Bunyaviridae* is also under taxonomic revision (see sidebar, A New Taxonomical Framework for the *Bunyaviridae*?).

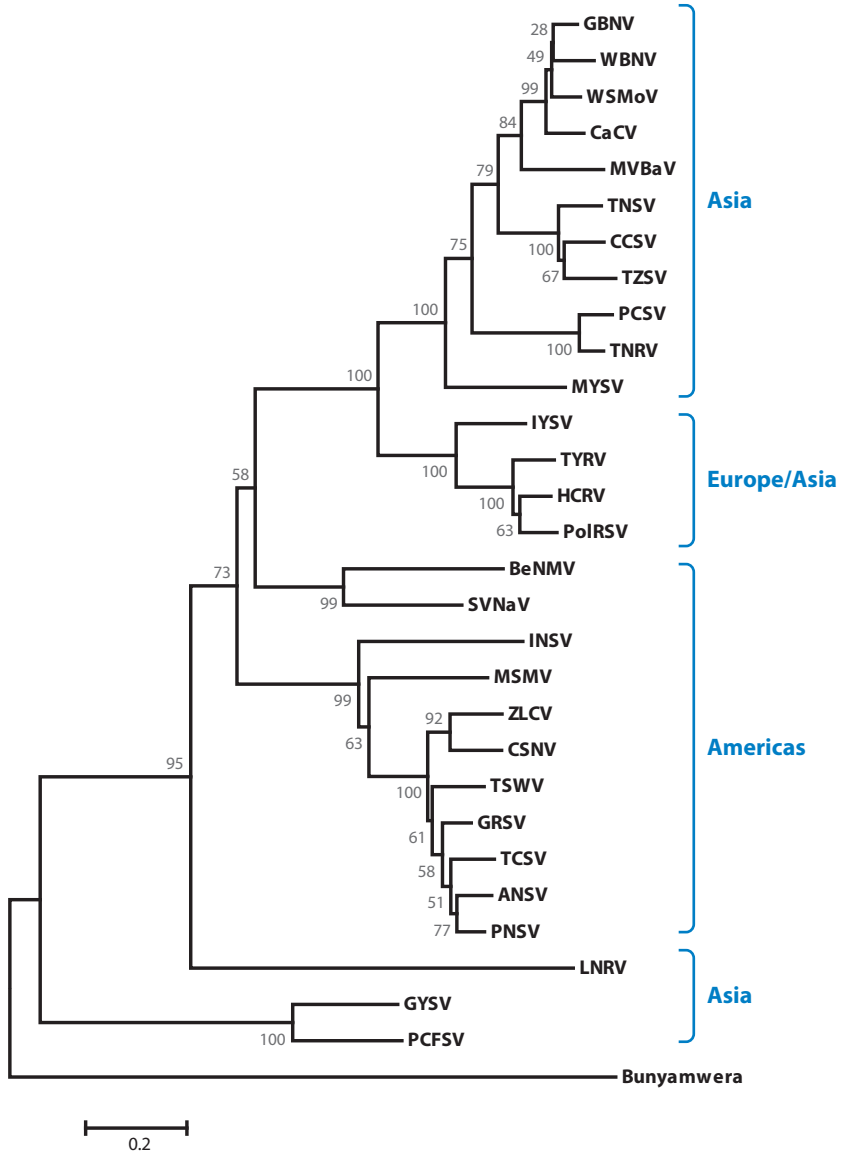
TOMATO SPOT WILT VIRUS MOLECULAR BIOLOGY

Tospovirus Genome Organization, Replication, and Cytopathology

Tospovirus particles are spherical and surrounded by a host-derived membrane with a diameter of 80–120 nm. The membrane contains two glycoproteins (Gn and Gc) that form spikes on the surface and are required for virus acquisition and transmission by thrips vectors. The core of the virus particle contains the ribonucleoproteins (RNPs) that are composed of the three

genomic RNA segments tightly encapsidated by the nucleocapsid protein (N) and molecules of the RNA-dependent RNA polymerase (RdRP; also referred to as L protein). RNPs are generally seen as pseudocircular because of the sequence complementarity of the genomic RNA termini (**Figure 2**).

The genome of tospoviruses is of negative/ambisense polarity and consists of three RNA segments that are denoted large (L, ~8.9 kb), medium (M, ~4.8 kb), and small (S, ~2.9 kb) according to their sizes. The first eight nucleotides (nts) at the termini of all RNA segments are highly conserved and complementary (5′-AGAGCAAU and 3′-UCUGCUUA), which is common for all segmented negative-strand RNA viruses, and play an important role as promoters for transcription and replication.



A NEW TAXONOMICAL FRAMEWORK FOR THE *BUNYAVIRIDAE*?

With the advent of next-generation sequencing (NGS) approaches used in the discovery and de novo assembly of virus genomes from metagenomics studies (79), the number of sequenced genomes is rapidly expanding. A recent paper that used NGS techniques to unveil virus diversity in a collection of 70 arthropod species assembled the genome of 112 novel negative-strand RNA virus species, redesigning some already established taxonomical relationships within this relatively homogenous group of viruses (65). Specifically, for the *Bunyaviridae* family, a Bunya-Arena clade was shown to exist (placing arenaviruses inside the *Bunyaviridae*), and the plant-infecting viruses of the genera *Tenuivirus* and *Emaravirus* were shown also to be inside the *Bunyaviridae* (65), suggesting that they could be treated taxonomically as distinct genera inside this family. The basis of a new genus (*Phasmavirus*) of insect-infecting viruses inside the *Bunyaviridae* was also proposed (6).

The L RNA contains one large open reading frame (ORF) on the viral complementary (vc) strand that encodes the RdRP (~330 kDa). Both the M and S RNAs have an ambisense gene arrangement, i.e., they code for two nonoverlapping ORFs on each of the complementary strands (vRNA and vcRNA). The ORFs do not overlap but are separated by an intergenic region (IR) sequence. The IR contains A- and U-rich stretches and is predicted to fold in a stable stem-loop/hairpin structure. The M RNA codes for the nonstructural, cell-to-cell movement (NS_M) protein on the viral (vRNA) strand, and the protein precursor to the glycoproteins (Gn and Gc, n and c referring to the amino- and carboxy-terminal positions within the precursor protein) on the vcRNA strand. The S RNA codes for the second nonstructural (NS_S) protein, which acts as a suppressor of RNA silencing, on the vRNA strand and for the N protein on the vcRNA strand. The literature related to tospovirus genome organization (**Figure 2**) has been recently thoroughly reviewed (59).

Upon entry into the host-cell cytoplasm, tospoviruses transcribe messenger RNAs (mRNAs), which are near genome-length for the RdRP gene from the L RNA and are significantly shorter than the corresponding genome segments for all genes coded from the ambisense M and S RNA segments (60). Viral mRNAs contain a cap structure but lack the presence of a common eukaryotic

Figure 1

A phylogenetic tree of 29 tospovirus N-protein coding regions with their likely geographic origin. Bunyamwera virus was included as the outgroup. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Abbreviations: ANSV, Alstroemeria necrotic spot virus; BeNMV, Bean necrotic mosaic virus; CaCV, Capsicum chlorosis virus; CCSV, Calla lily chlorotic spot virus; CSNV, *Chrysanthemum stem necrosis virus*; GBNV, *Groundnut bud necrosis virus*; GRSV, *Groundnut ringspot virus*; GYSV, *Groundnut yellow spot virus*; HCRV, Hippeastrum chlorotic ringspot virus; INSV, *Impatiens necrotic spot virus*; IYSV, *Iris yellow spot virus*; LNRV, Lisianthus necrotic ringspot virus; MSMV, Melon severe mosaic virus; MVBaV, Mulberry vein banding associated virus; MYSV, *Melon yellow spot virus*; PCFSV, Peanut chlorotic fan-spot virus, PCSV, Pepper chlorotic spot virus; PolRSV, *Polygonum ringspot virus*; PNSV, Pepper necrotic spot virus; SVNaV, Soybean vein necrosis-associated virus; TCSV, *Tomato chlorotic spot virus*; TSWV, *Tomato spotted wilt virus*; TNRV, Tomato necrotic ringspot virus; TNSV, Tomato necrotic spot virus; TYRV, Tomato yellow ring virus; TZSV, Tomato zonate spot virus; WBNV, Watermelon bud necrosis virus; WSMoV, *Watermelon silver mottle virus*; ZLCV, *Zucchini lethal chlorosis virus*.

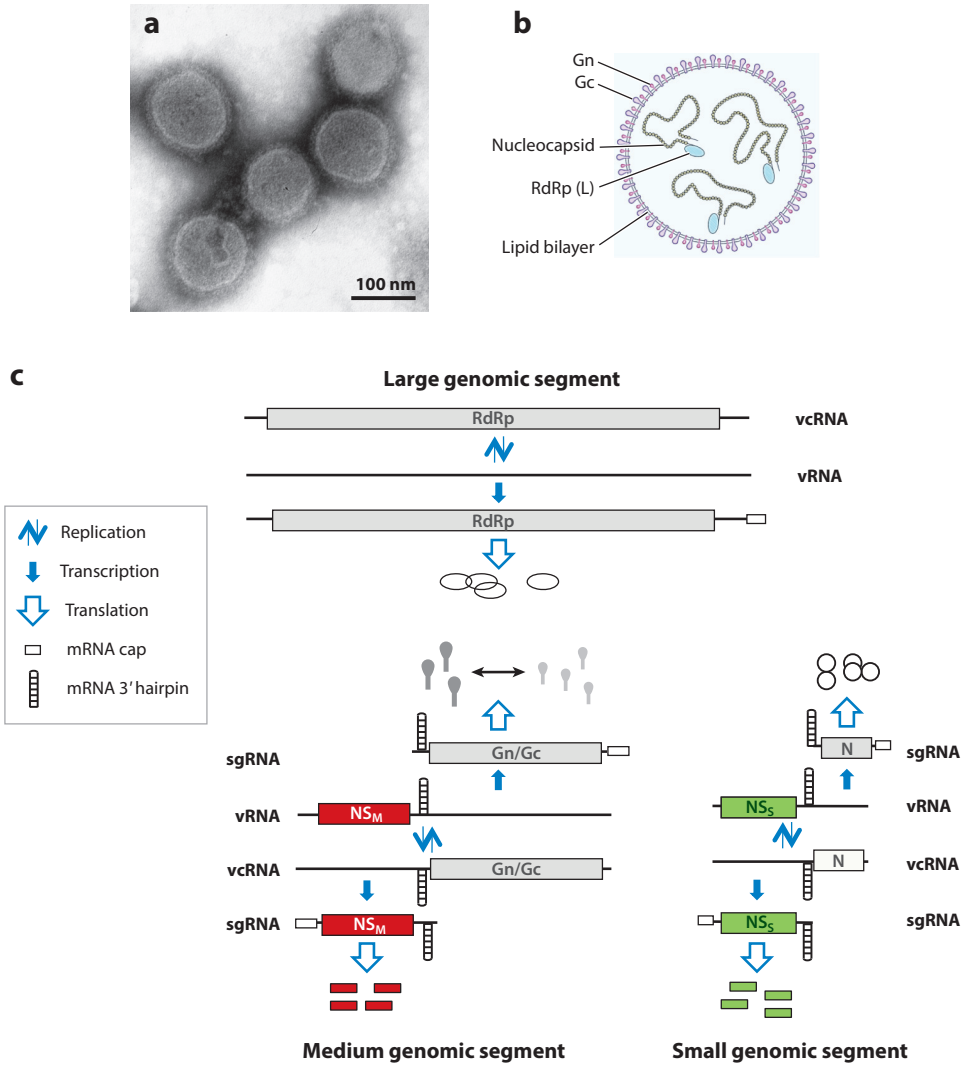


Figure 2

Tospovirus virus particle and organization. (*a*) Electron micrograph of purified virus particles. (*b*) Schematic representation of a tospovirus virus particle. (*c*) Genome organization and replication/expression strategy shown for the tospovirus representative *Tomato spotted wilt virus* (TSWV). The two *AVR* (avirulence) genes involved in the interaction with the resistance genes *Sw5-b* and *Tsw* are in red and green, respectively. Abbreviations: Gc, carboxy terminus of the glycoprotein precursor; Gn, amino terminus of the glycoprotein precursor; N, nucleocapsid; NS_M, nonstructural protein encoded by the M genomic segment; NS_S, nonstructural protein encoded by the S genomic segment; RdRp, RNA-dependent RNA polymerase (also known as L); sgRNA, subgenomic RNA; vcRNA, viral complementary sense RNA; vRNA, viral sense RNA.

poly(A)-tail. Owing to the lack of methyltransferase activity, the viral RdRP is not able to provide viral mRNAs with a cap structure. Instead, cap structures are provided by a mechanism of cap snatching, a process in which the RdRP snatches capped RNA leader sequences from the cytoplasmic pool of host mRNAs (37, 141). The snatched cap leader sequence is then used to prime transcription on the viral RNA genome template (61). During replication/transcription of the

tospovirus RNA genome, the host antiviral RNAi defense system is triggered by (stretches of) dsRNA (double-stranded RNA) that most likely result from secondary folding structures within viral mRNAs (51). The NS_S protein is able to bind long and short dsRNAs and thereby suppresses (local and systemic) RNAi to support the establishment of a successful infection (52, 109).

Although tospoviruses replicate in the cytoplasm, in electron-dense areas generally recognized as viroplasms, not much is known about the host factors involved in transcription and replication or about the cytoplasmic source of capped-RNA leader molecules. During replication, viral genomic RNA molecules concomitantly associate with N and RdRP to give rise to infectious progeny RNPs, which can often be seen in the cytoplasm as electron-dense striated spots in/nearby the viroplasm of virus-infected cells. During the early stages of viral replication, infectious progeny RNPs additionally associate with NS_M to facilitate their transport through modified plasmodesmata in a tubule-guided manner. At later stages, progeny RNPs move toward the endoplasmic reticulum (ER) and Golgi complex in an actin-dependent manner, where they line up and concentrate at foci containing the viral Gn and Gc glycoproteins. This can already take place at the ER where RNP-Gn-Gc complexes move to the Golgi complex and become enwrapped by a Golgi stack, to produce double-enveloped virus particles (DEVs). Specific literature related to RNPs and their association to tubules is detailed in a previous review (59). During maturation, glycoproteins synthesized at the ER leave from ER export sites (ERES) to the Golgi complex by COPII vesicles. To gather at ERES, an interaction between RNPs and the cytoplasmic tail of Gc suffices, but to escape from ERES to the Golgi complex the additional interaction with the Gn protein is required (98–100). Without the interaction with RNPs, Gn and Gc are also able to exit from ER as heterodimers, via ERES, to the Golgi complex. Once RNPs are enwrapped and DEVs are formed, they fuse together and with ER-derived membranes to generate large vesicles containing an accumulation of single-enveloped, mature virus particles (SEVs) (56). Particles remain in these large vesicles until uptake by thrips feeding on virus-infected plants (**Figure 3**). The mechanisms of transmission are persistent, circulative, and propagative, but thrips become viruliferous as adults only if they acquire the virus in the first two larval stages (148). In this respect, preliminary evidence that larval stages have impaired virus defense was recently provided (71). The specifics of virus uptake are not known, but receptor-mediated endocytosis is the most likely mechanism (147, 148). A number of studies have demonstrated the occurrence of homotypic and heterotypic interactions among the N, NS_M, Gn, and Gc proteins encoded by tospoviruses (in plant and animal cells), whereas other studies looked at interspecies interactions among proteins from distinct tospoviruses. For further details, readers are referred to the following references: 34, 63, 98, 100, 114–117, 138, and 139.

Tospovirus-Host Plant Interactions: Host Factors and Affected Molecular Pathways

Only a limited number of host factors have so far been implicated during various stages of the tospovirus infection cycle in plants. DNA-J protein homologs from *Nicotiana tabacum*, *Arabidopsis thaliana*, and tomato are among the first host plant proteins identified to interact with the tospovirus NS_M cell-to-cell movement protein, which suggests that movement of infectious RNPs possibly involves a mechanism that requires Hsp-70 (118, 143). These very same studies also unveiled interactions with another chaperone protein, At-4/1, which shares homology with α-helical domains of myosin-, ankyrin-, and kinesin-like proteins (143), which are involved in both intra- and extracellular movement (80, 86). In situ localization studies on TSWV proteins in plant cells also revealed an actin-dependent intracellular movement of N-RNPs that requires a myosin XI-K motor protein (39, 100). With relevance to possible breeding approaches, plants treated with

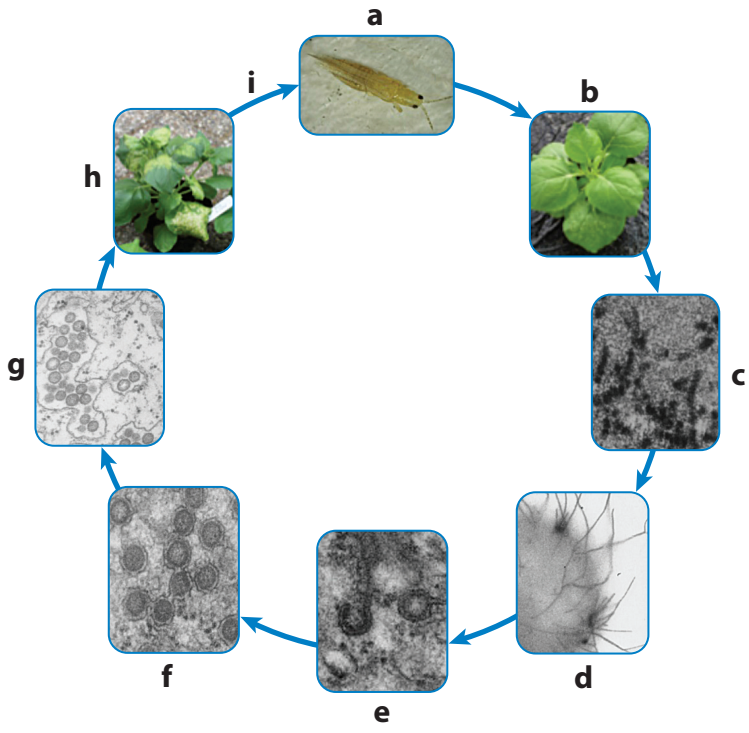


Figure 3

A schematic diagram of the life cycle of tospoviruses. In a clockwise setting, various stages are presented: (*a*) thrips transmission (*b*) onto a healthy plant, (*c*) the earliest stages of viral replication and production of ribonucleoproteins, (*d*) virus spread facilitated by NS_M (nonstructural M protein)-modified plasmodesmata in a tubule-guided manner, (*e*) particle assembly at the Golgi complex, (*f*) formation of double-enveloped viruses (DEVs), (*g*) fusion of DEVs into large vesicles containing an accumulation of mature single-enveloped viruses, (*h*) systemic disease symptoms, and (*i*) acquisition by thrips.

latrunculin-B, an actin-depolymerizing agent, slowed down TSWV infection in both *N. tabacum* and *Nicotiana benthamiana* (39).

During a TSWV replication-transcription study the eukaryotic translation elongation factor 1A (eEF1A) was identified as a host factor from evacuolated tobacco BY-2 protoplast extracts required for viral transcription and replication (58). Application of a specific inhibitor of this factor (didemnin B) inhibited viral genome transcription (58).

Given the paucity of plant host factors identified to interact with the tospovirus RdRP, it is worth mentioning the discovery of a thrips transcription factor required for TSWV replication, which turns human cell lines permissive to TSWV replication (28). A mutant of this factor unable to bind RNA interferes with viral replication in vivo (28).

Recently, healthy and TSWV-infected plants, inoculated by thrips feeding, have been investigated for changes in the JA and SA defense pathways and revealed an upregulation of the SA pathway in only the infected plants. Although thrips feeding normally induces the anti-herbivore (JA) response in plants, virus infection suppresses the JA pathway. These plants not only maintain their attractiveness for thrips but because of viral infection also appear more attractive relative to healthy plants (1, 75), and in this way support dissemination of the virus by thrips.

In another study on the oxylipin biosynthesis pathway, silencing of three genes involved in its biosynthesis (*9-LOX*, *13-LOX*, and *α-DOX-1*) attenuated the PCD associated with TSWV infection, whereas silencing the JA perception gene (*COI1*), in contrast, increased cell death. Furthermore, a double *lox1 α-dox-1* mutant became less susceptible to TSWV infection (42). Regulation of these pathways could contribute to the understanding of a hypersensitive response often associated to the triggering of resistance genes by viruses.

TOSPOVIRUS RESISTANCE IN TOMATO

Natural Genetic Sources Against Tospoviruses for Tomato Breeding

TSWV is the most widely spread tospovirus species worldwide and has been targeted for genetic resistance approaches. The search for natural resistance sources and resistance genes has intensified, and within the past three decades several resistance sources have been reported and incorporated into commercial tomato cultivars (35, 46, 102, 106, 108, 119, 120, 129).

Among these sources (40, 93, 108, 128, 130), recessive (*sw2*, *sw3*, and *sw4*) and dominant genes (*Sw1a* and *Sw1b*) have been reported; however, their resistance was quickly overcome upon challenge with TSWV (40, 93, 108, 128, 130). For the genes denoted *Sw-6* and *Sw-7*, partial resistance to a narrow range of TSWV isolates has been observed (106, 107). Because these reported resistance genes have not been characterized molecularly, it is not clear whether they represent different genes located on distinct chromosomes or represent different alleles from a well-known resistance gene cluster. The exception is the *Sw-5* gene cluster, of which many homologs have been sequenced. This gene cluster originates from *Solanum peruvianum* and has been the most widely deployed resistance source against TSWV because of its durability and the ability to provide stable resistance against tospovirus species and isolates from different geographic locations (14, 15, 47, 106, 127, 129, 130). Although the *Sw-5* gene cluster was cloned and characterized in 2001, only recently have molecular markers that assist in breeding been reported (32, 112).

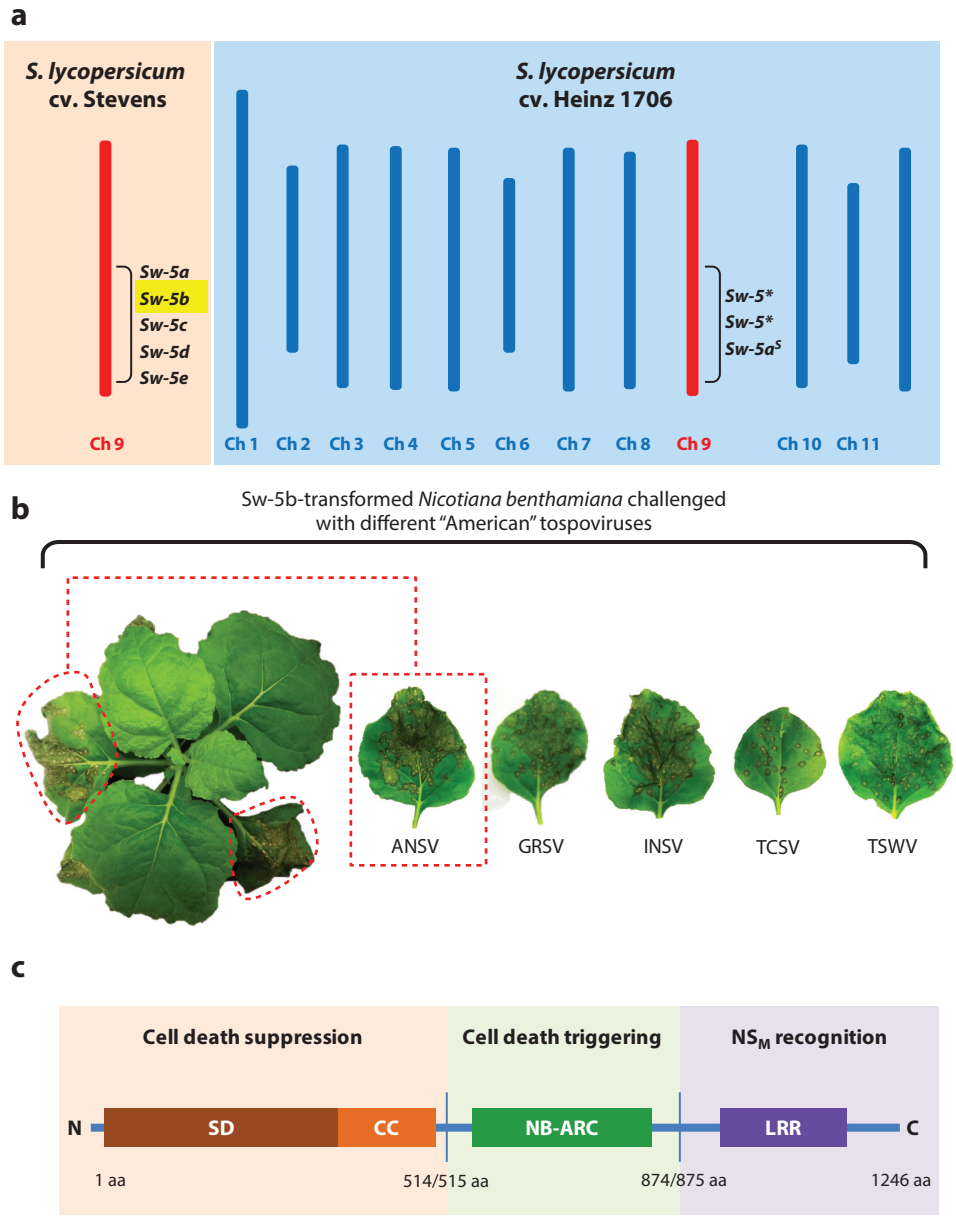
A Still Valuable Resistance Source: The *Sw-5b* Gene Confers Broad Tospovirus Resistance

The *Sw-5* gene cluster is located on the telomeric region of the long arm of chromosome 9. Five paralogs, named *Sw-5a* to *Sw-5e*, have been reported (**Figure 4**), but only the *Sw-5b* copy has been proven to be functional and sufficient for a broad-spectrum resistance against tospoviruses (47, 89, 125). The sequencing and annotation of the tomato genome (cv. Heinz) have helped to map highly conserved *Sw-5* orthologs in susceptible tomatoes, as is the case with *Solanum lycopersicum* cultivars that have not been crossed with *S. peruvianum* (2).

Interestingly and rather uniquely for plant viruses, *Sw-5b* confers resistance not only to TSWV but also to related tospovirus species such as TCSV, GRSV, Alstroemeria necrotic streak virus (ANSV), and *Chrysanthemum stem necrosis virus* (CSNV) (**Figure 1**) and even to the less related *Impatiens necrotic spot virus* (INSV) (33, 47, 125). All these tospovirus species are phylogenetically related and present within a putative American clade (87).

Dominant *R* genes hold an important position in the arms race between pathogen and host, a battle commonly presented as the zigzag model (29b, 55). Often, a single viral protein is the avirulence (Avr) factor responsible for triggering an *R* gene–mediated resistance (30, 47, 134, 146). For *Sw-5b*, the analysis of TSWV reassortants first suggested that the genetic determinant involved in overcoming the resistance conferred by the *Sw-5* gene was associated with the M RNA

(20). Ten years later, by in silico analysis, López et al. (67) proposed that two amino acid substitutions in the NS_M protein of TSWV could be responsible for overcoming the *Sw-5b*–mediated resistance, but only recently was the TSWV cell-to-cell movement protein (NS_M) demonstrated as the Avr determinant for the *Sw-5b* gene. The sole expression of this virus protein from a resistance-inducing (RI) TSWV strain (BR-01) in *Sw-5b*–transformed *N. benthamiana* and in resistant near-isogenic tomato lines (47) resulted in HR triggering, but not with NS_M from a *Sw-5b* RB strain (TSWV "GRAU" strain). This became the first biological demonstration that *Sw-5*–mediated resistance is triggered by the TSWV NS_M cell-to-cell movement protein. Simultaneously,



employing hybrid infectious clones based on *Alfalfa mosaic virus* (AlMV) resulted in the same conclusions (26).

NS_M-*Sw-5b* Interaction: New Molecular Insights into Virus-Host Interactions

All *Sw-5* genes encode putative R proteins of the type CC (coiled coil)-NB (nucleotide binding)-ARC (adaptor shared among APAF-1, R proteins, and CED-4)-LRR (leucine-rich repeat) (77, 125). CC is present at the amino termini; the following NB-ARC domain is the result of the association of an NB domain and the ARC subdomains; and an LRR domain is present at the C terminus (64). Although functions for the three major domains have been explored for some R proteins and mechanistic models proposed (135), many differences are seen that make it difficult to present a single activation model that applies to all *R* genes.

Recently, three Sw-5 R protein homologs, i.e., the known functional resistance protein (Sw-5b), its highest conserved paralog from *S. peruvianum* (Sw-5a), and their highest conserved ortholog from susceptible *S. lycopersicum* (Sw-5aS) were investigated (29a) (**Figure 4**). Analysis of these homologs revealed interesting features on the role of Sw-5 protein subdomains and the genetic basis for their functionality toward tospovirus resistance. The Sw-5b protein is able to trigger HR in an NS_M-dependent and -independent manner (auto-activated), whereas Sw-5a is only able to do this independently from NS_M activation. Their ortholog Sw-$5a^S$ completely fails in both ways. The functions of the CC, NB-ARC, and LRR domains have each been further investigated: For the resistance-effective Sw-5b protein, the NB-ARC domain proved to be sufficient for HR triggering; the LRR domain was instead sufficient for recognition of NS_M as Avr. However, the two other homolog proteins (Sw-5a and Sw-$5a^S$) appear to be hampered in their resistance function because of single-nucleotide polymorphisms (SNPs) in their NB-ARC and/or LRR domains (**Figure 4**). The N-terminal domains CC and SD (Solanaceae domain) were linked

Figure 4

Broad tospovirus-resistance paralogs and topology of the Sw-5b protein subdomains. (*a*) The *Sw-5b* gene is originally from *Solanum peruvianum*, which has other four *Sw-5* paralogs (*Sw-5a*, *Sw-5c*, *Sw-5d*, and *Sw-5e*) located in chromosome 9. With the availability of the tomato genome, three *Sw-5* orthologs have been mapped in *Solanum lycopersicum* cv. Heinz. One of them, *Sw-5a*^{S}, shares high identity with *Sw-5a* and *Sw-5b* and has been functionally studied. The other two (*Sw-5**) share high identity with *Sw-5c*, *Sw-5d*, and *Sw-5e* genes. (*b*) The *Sw-5b* gene is sufficient for broad-spectrum tospovirus resistance against American tospoviruses displaying a clear hypersensitive reaction (HR) (29a, 34). The whole plant shows that only inoculated leaves (dashed in red) induced HR, while the other leaves remain healthy. The presence of HR clearly demonstrates that ANSV (whole plant) and other tospoviruses (individual leaves) activate Sw-5b-mediated resistance. (*c*) The dissection of the Sw-5b protein revealed the function of each domain concerning cell death triggering and NS_M recognition. The overexpression of the NB (nucleotide-binding)-ARC (adaptor shared among APAF-1, R proteins, and CED-4) domain is sufficient for cell death triggering; cell death is suppressed in *cis* and *trans* by coexpression with the SD (Solanaceae domain) and CC (coiled coil) domains. The Sw-5 proteins as well as other Solanaceae NB-ARC proteins present an extended N-terminal domain, and this additional portion corresponds to SD. The leucine-rich repeat (LRR) domain specifies NS_M protein as the avirulence factor (Avr) for *Sw-5b*. A truncated protein containing only the NB-ARC and LRR domains is sufficient for NS_M-dependent HR. The same version of *Sw-5a* is able to trigger HR only when being overexpressed (autoactivation) but not when being expressed in the presence of NS_M under normal conditions, indicating that the LRR of this protein is unable to sense NS_M as Avr. Figures kindly supplied by Athos Silva de Oliveira. Abbreviations: aa, amino acids; ANSV, Alstroemeria necrotic spot virus; Ch, chromosome; GRSV, *Groundnut ringspot virus*; INSV, *Impatiens necrotic spot virus*; TCSV, *Tomato chlorotic spot virus*; TSWV, *Tomato spotted wilt virus*.

to cell death regulation because coexpression of these domains with NB-ARC leads to cell death suppression.

As similarly described for tospoviruses, the role of CC-NB-ARC-LRR domains has been previously reported for other biological systems (8, 68, 69, 95). However, based on all differences detected at HR induction and during regulation, it remains difficult to postulate one single mechanistic model to explain the activation of all R proteins from the NB-LRR type.

Natural Tospovirus Mutants and Synergism During a Mixed Infection with *Tomato chlorosis crinivirus* Result in *Tomato spot wilt virus* Breaking *Sw-5* Resistance

The continuous use of *Sw-5*-resistant cultivars has resulted in the onset of RB isolates worldwide, apart from isolates that can escape the resistance by high inoculum pressure or drastic temperature variation (4, 22, 23, 62, 106). However, these RB TSWV isolates appeared to exhibit lower fitness compared to wild-type TSWV isolates (3, 62, 67).

Efforts have been made to characterize these RB strains and map their molecular changes. López et al. (67) have reported that resistance conferred by *Sw-5b* can be overcome by single–amino acid substitutions in the NS_M protein as a result of positive selection as opposed to recombination. Later, employing infectious clones based on AlMV, Peiró et al. (89) confirmed those predictions showing that the NS_M Y118 or N120 amino acid residues are critical to overcome resistance.

Mixed infections among tomato-infecting viruses are expected in open field conditions. The *Sw-5* resistance was shown to be overcome during mixed infections of TSWV with *Tomato chlorosis virus* (ToCV) (41). A synergistic interaction was described in the commercially grown tomato cultivar Anastasia when challenged with the two viruses. Under normal conditions, this cultivar is susceptible to ToCV, but resistant to TSWV. It was demonstrated that the presence of ToCV in Anastasia plants prior to TSWV inoculation compromised TSWV resistance. These results suggest that ToCV could interfere with the *Sw-5* resistance mechanisms independently of the genetic background of the tomato cultivar tested (18).

A New Allele of the *Sw-5* Gene Cluster and *Sw-5* Analogs in other Solanaceous Species

Although *Sw-5b* provides broad resistance to tospoviruses, sporadic infection by TSWV is observed in *Sw-5*–resistant tomato plants, which has been explained by incomplete *Sw-5* gene penetrance or by gene dosage effects derived from a variable allelic composition within the *Sw-5* gene cluster (15, 125, 130). In addition to RB isolates (4, 22, 23, 62, 106), unique tospovirus isolates have been identified, arisen from genome reassortment during mixed infections (144, 145). The ecological and evolutionary significance of such reassortant events, as well as the risk these resulting new viruses may pose for the durability of genetic resistance genes, remain to be elucidated.

Therefore, an ongoing search for new tospovirus resistance genes remains essential in all breeding programs of tomato (35, 46, 102, 106, 108, 119, 120, 129). More recently, 105 *Solanum* (section *Lycopersicum*: *Solanaceae*) accessions were evaluated for their reaction against TSWV, TCSV, CSNV, and GRSV isolates (33). *S. peruvianum* accessions were found that displayed a broad-spectrum resistance to several tospovirus species, but sources of resistance were also found in accessions of *Solanum pimpinellifolium*, *Solanum chilense*, *Solanum arcanum*, *Solanum habrochaites*, *Solanum corneliomuelleri*, and *S. lycopersicum* "Rey de los Tempranos." Very recently, a patent was launched describing a new tomato *Sw-5b* allele that confers resistance to a common TSWV RB

strain isolated in Southern Italy carrying the typical amino acid substitutions of other RB strains so far characterized (3). Compared to the *Sw-5b* copy, this new *Sw-5b*^{2} allele shows SNPs that result in a polypeptide with differing secondary and tertiary structure and, consequently, a subtle change in its expanded resistance profile.

TOSPOVIRUS RESISTANCE IN PEPPER

Tsw Identification and Deployment in Pepper Crops, and the Limitations of *Tsw*

The second dominant *R* gene deployed in the control of tospoviruses is *Tsw*, identified in *Capsicum chinense* PI accessions and mapping to the distal portion of chromosome 10 (54). This gene has been introgressed into *Capsicum annuum* cultivars (10, 12, 54). Resistance is displayed by an HR, like with *Sw-5b*, that prevents systemic spread of the virus and in the end leads to leaf abscission (13). The resistance only holds to isolates belonging to the species TSWV and not to GRSV, TCSV, or more distantly related tospoviruses (13). As in the case of *Sw-5b*, *Tsw* RB variants of TSWV have been identified (74, 81, 104, 111).

The resistance conferred by *Tsw* is observed to exhibit a reduced performance at prolonged higher temperatures and a lower efficiency when plants are infected during an early growth stage (82, 103, 104, 122). Although RB by TSWV occurs at increasing frequencies (74, 82, 103–105), in some pepper cultivation areas, such as South and Central America, two closely related tospoviruses, TCSV and GRSV, are emerging and seem to be expanding their geographic range and economic impact to Central and North America. For these reasons, the *Tsw* gene is not sufficient for tospovirus control in the longer term, and sweet pepper cultivations are expected to face increasing problems because of infections with TSWV, TCSV, and GRSV. In addition, the effects of (nonrelated) coinfecting viruses on *Tsw* breaking by TSWV, as similarly reported for the tomato *Sw-5* gene upon a coinfection of TSWV and the crinivirus ToCV (41), need investigation. This has become very relevant in light of a recent observation on a mixed infection in pepper containing the *Tsw* gene with a *Tsw*-breaking TSWV and a non-RB isolate that showed severe symptoms likely due to synergism (26). Considering that in many areas where sweet pepper is grown, viruses other than TSWV may occur, a resistance-bypassing event cannot be ruled out.

In contrast to the *Sw-5* gene, the *Tsw* gene has not been cloned yet but because of its single dominant inheritance it is assumed to code for a NBS-LRR protein (10, 12, 54); the recently made available pepper genome sequence should facilitate its identification (57).

Identification of the Avr Determinant

The first step toward the identification of the Avr determinant in relation to the *Tsw* resistance gene in pepper was done through the use of reassortant strains between an RB strain (TSWV A) and a wild-type strain (TSWV D): The RB phenotype in resistant pepper mapped unequivocally to the S segment (54), but the differences among the isolates used in the reassortment experiment did not allow narrowing of the specific mutations within the S segment responsible for the RB phenotype. A few years later, the same results were confirmed using closely related strains that allowed fine mapping of the SNPs that were responsible for overcoming *Tsw* resistance. This study revealed that, contrary to what happens in the *Sw5*-NS$_M$ interaction, a number of different single amino acid substitutions spread along the NS$_S$ sequence are in each case responsible for the RB phenotype present in a collection of field and laboratory RB isolates (72). Later, a TSWV population analysis confirmed that the only ORF displaying positive selection in relation to the pepper RB phenotype is indeed that encoding for NS$_S$ (136). Finally, a technical breakthrough allowed

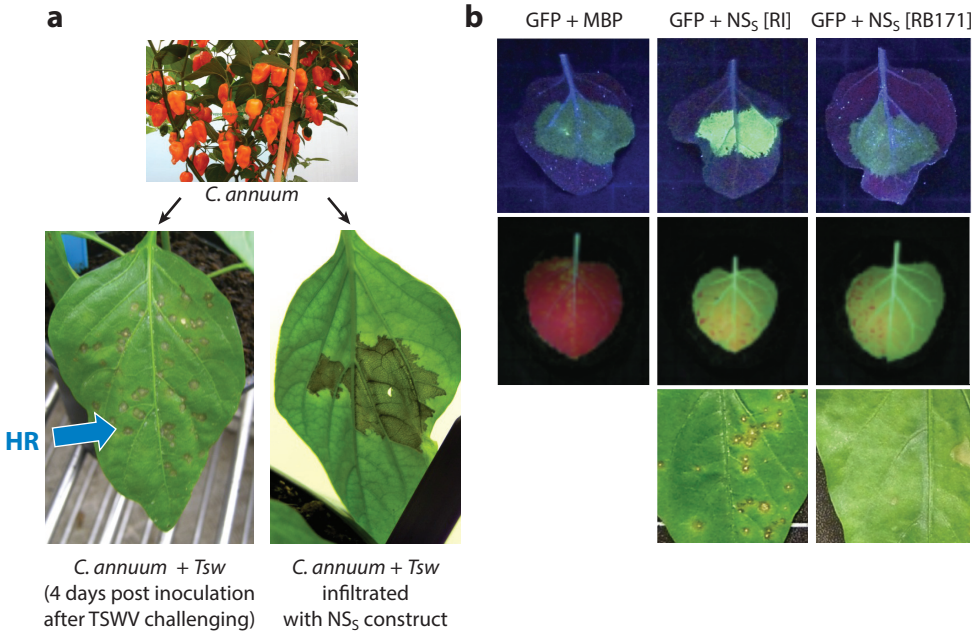


Figure 5

Tsw-mediated resistance against *Tomato spotted wilt virus* (TSWV). (*a*) Triggering of *Tsw*-mediated hypersensitive response upon challenging of *Capsicum annuum* with TSWV (*left*) or after agroinfiltration of a binary vector construct encoding NS$_S$ (*right*). (*b*) Suppression of local (*top row*) and systemic (*middle row*) green fluorescent protein (GFP) silencing in the presence of maltose binding protein (MBP) (negative control), NS$_S$ from a resistance-inducing (RI) strain, and NS$_S$ from a resistance-breaking (RB) strain. Bottom row shows the response on *C. annuum* leaves carrying the *Tsw* gene after local expression of NS$_S$ RI or NS$_S$ RB through agroinfiltration. Abbreviation: HR, hypersensitive response.

the establishment of an expression system in pepper (through agroinfiltration) that reproduced the specific necrotic response associated with NS$_S$ from the wild-type strain but not from RB strain, therefore identifying the silencing suppressor NS$_S$ as the Avr determinant in the interaction with *Tsw* (**Figure 5**) (30). This system, depending on expression of NS$_S$ through agroinfiltration from cDNA, for the first time allows further dissection through mutagenesis of the role of each NS$_S$ domain or amino acid in the Avr phenotype. The observation that the NS$_S$ protein of TSWV-RB isolates had lost the ability to trigger *Tsw*-mediated HR, as well as RSS activity, initially pointed to a link between *Tsw*-mediated resistance and RNA silencing (**Figure 5**). However, a large NS$_S$ mutant screen indicated that RNA silencing suppression and Avr can be functionally separated. Furthermore, during a screen of a larger set of *Tsw*-breaking TSWV isolates, a few isolates were identified that still contained a functional NS$_S$ RNA silencing suppressor (RSS) protein (D. de Ronde, D. Lohuis, R. Kormelink, unpublished data). Interestingly, mutation of a WG/GW motif within the N-terminal domain of NS$_S$, which for a few other RSS proteins was recently shown to mediate binding to AGO1, the core component of RISC (44), also caused a loss of both Avr and RSS activity (31). Although some NS$_S$ proteins from *Tsw*-RB isolates have their ability to suppress local RNA silencing compromised upon transient expression, they still exhibited the ability to suppress systemic silencing to certain levels (52). This would explain why naturally evolved RB isolates maintain fitness and give rise to virus titers in systemic leaves similar to those of a wild-type virus.

Considering the importance of the NS_S protein to defend against the antiviral RNAi machinery in plants and insects, a lowered fitness and/or loss of RSS activity could affect thrips transmission efficiency. Although virus titers of TSWV RB isolates more or less equal those of TSWV RI isolates, some apparently conflicting data have been reported for thrips transmission of TSWV RB isolates. Debreczeni et al. (27) and Roggero et al. (104) reported that RB field isolates (*Tsw* and *Sw-5*) have the same transmission potential as isolates that are unable to infect the resistant tomato and pepper cultivars. A study by Margaria and coauthors (71) limited to RB strains that had deletions in the coding region of the NS_S, resulting in truncated NS_S (obtained via serial mechanical passages) indicated that virus acquisition of NS_S-deficient isolates is not impaired in the early larval stages; in adult thrips, accumulation is much lower compared to wild-type isolates, leading to a loss of thrips transmission of NS_S-deficient isolates. The latter isolates will likely not survive and disseminate under natural field conditions, as their emergence and spread are under tight selection through thrips transmission.

Search for New Resistance Sources in Pepper

Increasing frequencies of TSWV isolates that overcome *Tsw* resistance, some of which result from reassortants (73), together with growing problems of TCSV and GRSV in sweet pepper cultivation, stress the importance of a search for new/additional resistance genes in pepper. A few interesting studies have already appeared on this point. Ngoc Huy and coauthors (85) discovered a new resistance source in *C. chinense* "AC09-207" that is inherited as a single dominant gene and is indicated to present a unique allele at the *Tsw* locus or to be controlled by a different gene tightly linked to *Tsw*. In another recent study from Soler and coauthors (121), a source of resistance was found in *Capsicum baccatum* accession PIM26-1 that conferred high levels of resistance to wild-type TSWV and *Tsw*-RB isolates. Several accessions of *C. chinense* containing *Tsw* have also been discovered to contain resistance to Capsicum chlorosis virus (CaCV). Sublines of these showed a uniform resistance to field isolates of TSWV and CaCV but with the resistance genes segregating independently (90). Although the source of TSWV resistance involves *Tsw*, the resistance against CaCV is thought to involve a yet unidentified single dominant gene.

PERSPECTIVES AND CHALLENGES AHEAD

Sources of Resistance in Different Tospovirus-Crop Systems

More virus-crop combinations are in need of resistance sources to help control the diseases caused by tospoviruses. Given the increasing importance of IYSV, particularly for onion seed production in the United States, some preliminary tests for resistance/tolerance to both onion thrips and IYSV have been carried out and a limited number of selections of potential interest in breeding programs were identified (11, 25, 83).

Another potentially very valuable source of resistance was recently identified against MYSV, a tospovirus of increasing importance in Asia and recently found in Ecuador. A cucumber accession (27028930) was found to be resistant (132), but such resistance is affected somewhat by temperature (133). Mapping of F2 populations resulted in the detection of quantitative trait loci (QTLs) and a resistance gene, and the simple sequence repeats (SSR) marker linked to them, a very useful tool for future breeding programs (131).

A single dominant resistance gene was also found to control resistance in three tomato genotypes (EC 5888, EC 8630, and EC 26512) against *Groundnut bud necrosis virus* (GBNV), the most important tomato-infecting tospovirus in India (96). GBNV also affects mung beans in India, and a screen of mung bean germplasm indeed identified some sources of resistance (126). GBNV also

RESISTANCE GENES AND GLOBAL WARMING

One of the main concerns of global warming is its impact on agriculture. The effects of temperature on plant defense against biotic stress need to be considered in future breeding programs for resistance to pathogens. In general, higher temperatures favor pattern-triggered immunity but have a negative effect on effector-triggered immunity (21). In fact, dominant monogenic resistance genes are often temperature sensitive, i.e., at higher temperature they are not functional and allow systemic infection of the plant, as is the case with the *Tsw* resistance gene in pepper (82, 103). Temperature sensitivity of resistance genes is not an intrinsic quality of a gene, and a mutant screen in *Arabidopsis* showed that it can be improved (153). Furthermore, the same specific substitution that improved temperature sensitivity of the gene in *Arabidopsis* could be engineered in the *N* resistance gene to *Tobacco mosaic virus* (149, 153); the same approach could be extended to other temperature-sensitive resistance genes.

causes stem necrosis on potato in India: A screen of 207 accessions revealed a number of resistant accessions suitable for further resistant potato breeding programs (123).

In some specific situations, tospoviruses have a great impact on the production of vegetables from the *Asteraceae* family, such as lettuce and artichoke, but at present no resistance is available for these crops.

Durability of Resistance

The issue of durability is a key factor in breeding for tospovirus resistance and is one of the main challenges breeders are facing. In fact, *Tsw* and *Sw5* were shown to be different in durability once deployed in pepper and tomato crops, respectively, with *Tsw* being particularly prone to select for epidemics of RB strains even just one year after resistant lines were introduced. A possible explanation could be that many different mutations cause breaking of resistance by the NS_S protein, whereas so far, only specific substitutions cause RB phenotype by NS_M. Two recent, excellent reviews provide a theoretical background needed to understand all the various factors involved in extending resistance durability in the field (17, 84). An issue related to durability of resistance that also needs attention in light of global warming is the effect of elevated temperatures on resistance traits (see sidebar, Resistance Genes and Global Warming).

New Breeding Approaches and Technologies for Resistance to Tospoviruses

Resistance to tospoviruses through grafting (self-grafting or grafting on TSWV-resistant tomato cv. Manduria rootstocks) has recently been shown (124) and has been indicated to involve enhanced RNA silencing. The latter effect was even noticed during self-grafting of a very susceptible variety and provides interesting opportunities for practical applications. Because recovery of infection was the final outcome in those studies, and recovery can also be due to a deficiency in the function of NS_S, the presence of mutations in the TSWV strain is possible (71, 72).

Another resistance approach that so far does not seem to be actively pursued by breeders for tospovirus resistance is the evaluation of quantitative resistance, associated with the introgression of QTLs. Quantitative resistance is characterized by a reduction in disease severity instead of complete resistance or a hypersensitive response; a complex polygenic control is often behind QTLs, and this has generally hampered a wide deployment of this approach.

An alternative approach to breeding for resistance by using single dominant resistance genes is the inactivation of susceptibility genes. Such approaches rely on (*a*) the identification of

plant genes that allow compatible interaction between the host plant and the pathogen and (*b*) the alteration of such susceptibility genes in order to prevent compatibility. The theoretical background and the possible translational breeding aspects have been recently reviewed (88, 142). Although for tospoviruses such a strategy has not yet been deployed, identification of host factors is being pursued (see above), and their possible implications in breeding programs should be taken into consideration.

Once identified, the host factor and its alteration could be pursued through two distinct approaches: the use of TILLING and the use of genome-editing techniques. TILLING is a relatively recent approach that allows the acquisition of specific mutants in a variety of plant species, from model plants to crops. The details of the approach have changed over the years, but what remains constant is a mutagenesis step and high-throughput screening for point mutations in the whole genome (20), which, when combined with knowledge about compatibility genes, could result in new sources of resistance to tospoviruses. A proof of concept of such an approach has allowed the introduction of resistance to two potyvirus species in tomato, a plant species for which efficient TILLING protocols are available (78, 91, 101). EcoTILLING is an approach that allows the detection of natural variability of the allelic variants of a specific gene, an approach that has resulted in the detection of a new *Sw-5b* variant (7).

More recently, alternatives for targeted genome modification (TGM) strategies became available for plants. These techniques use site-specific nucleases (SSNs) such as zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. The interest in such techniques is raised by the possibility that regulation would be different from that applied to conventional transgenic approaches because they act by mutating only a target gene in the genome, without the insertion of (additional) coding and/or noncoding heterologous sequences. Owing to the minimal changes introduced, these strategies are very similar to chemical or physical mutagenesis. The three techniques, their regulatory implications, and their possible usage in breeding programs have been recently reviewed (19). It is noteworthy that tomato was shown to be easily edited in its genome by the use of CRISPR-Cas9 in the first generation (16); therefore, it should be fairly easy to establish a first proof of concept approach for resistance to tospoviruses. Traditional transgenic approaches and these new gene modification techniques allow new experimental approaches, where evolution is mimicked in vitro (see sidebar, Artificial Evolution).

As in many other fields related to agriculture, creative solutions are constantly being envisioned and researched for the new challenges posed by tospoviruses; in our opinion, researchers should always carefully accompany each new solution with a proper communication strategy aimed at the

ARTIFICIAL EVOLUTION

Artificial evolution is another interesting approach for broadening the base of resistance: Work on the R protein encoded by the *Rx* gene for resistance to *Potato virus X* has shown that the NB-LRR gene could be first mutated (through in vitro evolution) to generate a broader spectrum resistance (38). However, in this study the first round of mutants came at a cost because infection with a second potexvirus had a strong necrotic phenotype that killed the plant. Despite this, a follow-up study using the same experimental system has shown that additional mutations in a different protein domain (linked to activation sensitivity) reduced these costs, providing full resistance to the second potexvirus (48). Artificial evolution of resistance genes could be applied to *Tsw*, known to be specific for TSWV only and to be very easily broken, to improve both the activation and recognition phases.

general audience for a deeper understanding of their new technological breakthroughs, preventing the irrational fears that have undermined the use of traditional transgenic approaches in this field.

SUMMARY POINTS

1. Tospoviruses represent a constantly emerging threat to crops worldwide, with new species described every year and the expansion of tospoviruses into new territories.
2. Plant resistance to tospoviruses is still the best option (when available) to limit tospovirus damage in vegetable crops.
3. *Sw-5* is still a valuable resistance gene in tomato, and the corresponding *Avr* gene from TSWV in tomato encodes the NS_M protein.
4. *Tsw* is easily broken by constantly evolving TSWV strains. The resistance gene itself still needs identifying, but the *Avr* gene in TSWV encodes the NS_S protein, the silencing suppressor.
5. The interaction between the gene products for *Sw-5* and NS_M and for *Tsw* and NS_S can be studied in detail through agroexpression of the *Avr* genes, allowing a partial reverse genetics approach for the *avr-R* interaction.
6. It is important to screen for new sources of resistance to tospoviruses other than in the TSWV-pepper and TSWV-tomato crop systems.
7. New approaches to obtain resistant vegetable crop varieties are discussed.

DISCLOSURE STATEMENT

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