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Paving the Way to Tospovirus Infection: Multilined Interplays with Plant Innate Immunity

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Abstract

Tospoviruses are among the most important plant pathogens and cause serious crop losses worldwide. Tospoviruses have evolved to smartly utilize the host cellular machinery to accomplish their life cycle. Plants mount two layers of defense to combat their invasion. The first one involves the activation of an antiviral RNA interference (RNAi) defense response. However, tospoviruses encode an RNA silencing suppressor that enables them to counteract antiviral RNAi. To further combat viral invasion, plants also employ intracellular innate immune receptors (e.g., Sw-5b and Tsw) to recognize different viral effectors (e.g., NSm and NSs). This leads to the triggering of a much more robust defense against tospoviruses called effector-triggered immunity (ETI). Tospoviruses have further evolved their effectors and can break Sw-5b-/Tsw-mediated resistance. The arms race between tospoviruses and both layers of innate immunity drives the coevolution of host defense and viral genes involved in counter defense. In this review, a state-of-the-art overview is presented on the tospoviral life cycle and the multilined interplays between tospoviruses and the distinct layers of defense.

INTRODUCTION

Tospoviruses belong to the genus *Orthotospovirus*, the family *Tospoviridae*, and the order *Bunyavirales*, of which most members are restricted to animal and human hosts. *Tomato spotted wilt virus* (TSWV), the type species of the tospoviruses (siglum from tomato spotted), ranks within the top 10 most important plant viruses (114), with estimated annual crop losses of more than one billion US dollars. Tospoviruses cause increasing problems in agricultural and ornamental crops worldwide (98, 128) and are transmitted by thrips in a persistent, circulative–propagative manner (111, 139). The western flower thrips (*Frankliniella occidentalis*) is known as one of the most important vectors of tospoviruses, and its worldwide spread starting in the 1980s has contributed to the reemergence and worldwide distribution of TSWV (46).

In the past decade, excellent reviews have appeared on various aspects of the epidemiology, molecular biology, cytopathology, virus–vector interactions, and resistance strategies of tospoviruses (46, 69, 98, 128, 139). In the following sections, an overview of the molecular biology and life cycle of tospoviruses is presented, based on the most important and best-studied member of the tospoviruses, TSWV, with a focus on the achievements obtained in recent years and the multilined interplays with plant innate immunity. Most of this overview is representative for all members of the tospoviruses unless stated otherwise.

MOLECULAR BIOLOGY AND LIFE CYCLE OF TOSPOVIRUSES

The genome of TSWV consists of three negative/ambisense-strand genomic RNA segments (**Figure 1**) that are denoted large (L), medium (M), and small (S) and code for the viral RNA-dependent RNA polymerase (RdRp; 330 kDa), the cell-to-cell movement protein (NSm; 33 kDa), the precursor to the glycoproteins (GP; 127 kDa), the nucleocapsid protein (N; ~28 kDa), and a nonstructural protein (NSs; 52 kDa) (1, 27, 70–72, 131). The GP precursor is processed by host cellular proteases into two mature viral Gn (58 kDa) and Gc (78 kDa) glycoproteins (n and c referring to their amino- and carboxy-terminal positions within the precursor).

Like all animal-infecting members of the former *Bunyaviridae*, tospoviruses are now reclassified as a separate family within the order *Bunyavirales* (86). Tospovirus virions (~80–120 nm in diameter) are spherical and enveloped by a phospholipid membrane (**Figure 1*a***). The two viral glycoproteins (Gn and Gc) are embedded in the lipid membrane and form spikes on the surface. The core of virus particles contains the three genomic RNA elements tightly packaged by the N protein and a few copies of the viral RdRp into ribonucleo(capsid)proteins (RNPs), the minimal infectious unit (70, 98, 128). Crystal structures of TSWV N were recently obtained and point toward N trimerization prior to assembly into RNPs (49, 68), in which critical binding sites for RNA localize in a cleft of the N protein and suggest deep embedding of the genomic RNAs (78).

During a successful infection and dissemination of progeny tospoviruses, three main stages can be recognized in planta (**Figure 2**): (*a*) replication and transcription of the genetic viral elements to produce massive amounts of infectious RNPs; (*b*) their subsequent intra/intercellular trafficking; and (*c*) envelopment by Golgi membranes to support virus acquisition by thrips and dissemination into neighboring healthy crop plants. To accomplish these stages, tospoviruses have evolved to smartly utilize the host cellular machinery, but it is primarily during the first stage that the inducers of antiviral RNAi and *R* gene–based host defense are produced, namely double-stranded RNA (dsRNA) and viral effector proteins, respectively (**Figure 2**).

Tospovirus Genome Replication and Transcription

For tospoviruses, which have a negative/ambisense RNA genome, RNPs present the minimal infectious unit to initiate a multiplication cycle. Replication and transcription of tospoviruses occur

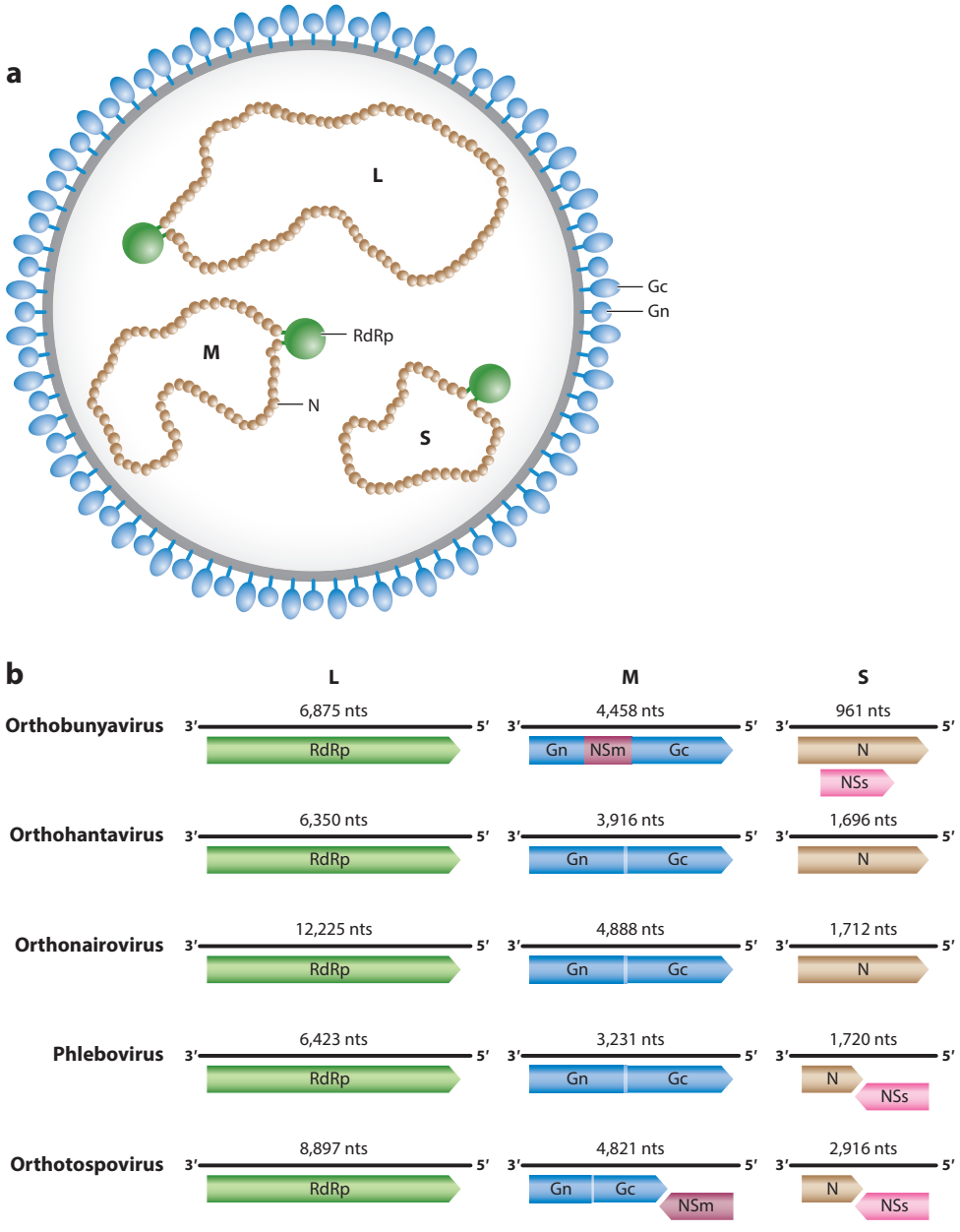

Figure 1

Particle morphology and genome organization of tospoviruses. (*a*) Schematic of an enveloped virus particle of a tospovirus. (*b*) Organization of the tripartite RNA genome of representatives from the animal-infecting bunyaviruses and plant-infecting orthotospoviruses. Abbreviations: Gc, glycoprotein processed from the carboxy-terminus of the glycoprotein precursor; Gn, glycoprotein processed from the amino terminus of the glycoprotein precursor; L, large RNA segment; M, middle RNA segment; N, nucleocapsid protein; NSm, nonstructural protein encoded by the M RNA segment, presents the movement protein; NSs, nonstructural protein encoded by the S RNA segment, presents the RNA silencing suppressor protein; nts, nucleotides; RdRp, RNA-dependent RNA polymerase; S, small RNA segment.

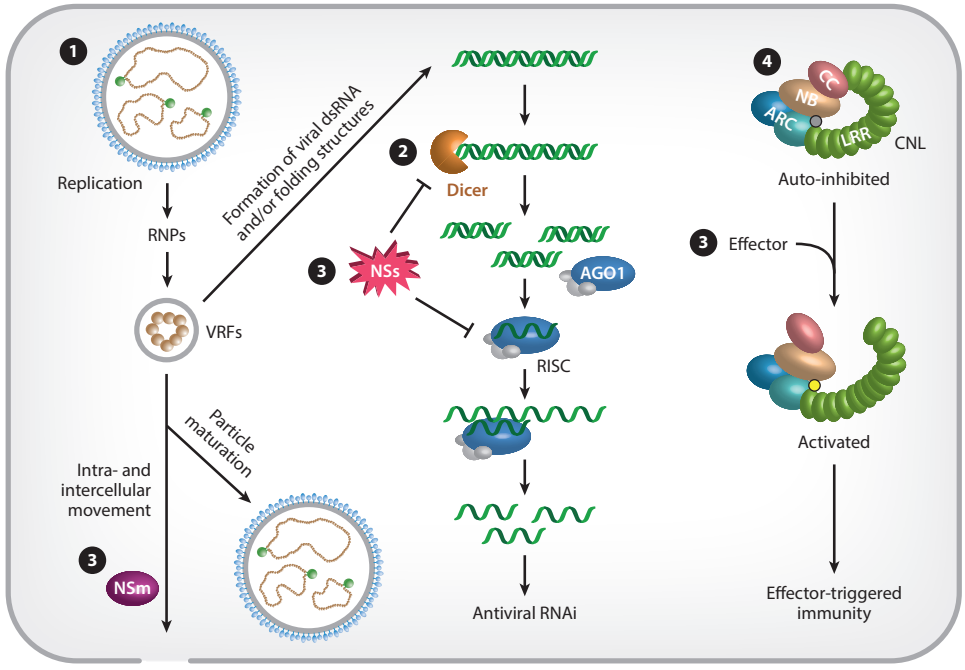

Figure 2

Tospovirus life cycle and interplay with plant innate immunity. ❶ Tospoviruses depend on host cellular machineries to accomplish their life cycle. Ribonucleo(capsid)protein complexes (RNPs) recruit host cellular factors, including eEF1A, to build viral replication factories (VRFs) for genomic RNA replication and transcription. RNPs engaged in transcription snatch capped RNA leader sequences from cytoplasmic mRNAs to prime viral genome transcription. VRFs and/or N/RNPs move along the actin/endoplasmic reticulum (ER) network to meet Gn and/or Gc at endoplasmic reticulum exit sites (ERES) and comigrate to the Golgi complex for assembly of enveloped particles. N/RNPs associating with NSm move intra- and intercellularly via a continuous ER network and through NSm-derived tubule structures to spread systemically. ❷ Viral dsRNA, produced from either viral mRNA by host RNA-dependent RNA polymerases (RdRps) or secondary folding structures of viral mRNA, triggers the first layer of plant innate immunity to defend against tospovirus invasion. DsRNA is cleaved into 21–24-nucleotide viral small interfering RNAs (vsiRNAs) by Dicer-like (DCL) endoribonucleases. Incorporation of siRNAs into Argonaute (AGO) activates the RNA-induced silencing complex (RISC) and enables the sensing and degradation of viral target RNAs. ❸ To counteract antiviral RNAi and successfully establish a viral infection, tospoviruses produce nonstructural proteins NSs and NSm. NSs proteins sequester long dsRNA and siRNAs to prevent the biogenesis of siRNAs and their uploading into RISC, respectively. NSm facilitates movement between plant cells, which allows the systemic spread of the virus. ❹ To further combat tospovirus invasion, tomato and pepper are able to sense the effectors NSm and NSs and mount a second, robust effector-triggered immunity (ETI) mediated by their nucleotide-binding leucine-rich repeat (NLR) receptors, Sw-5b and Tsw, respectively. Abbreviations: ARC, Apaf-1, R-protein, and CED-4 domain; CC, coiled-coil domain; CNL, coiled-coil nucleotide-binding leucine-rich repeat receptor; LRR, leucine-rich repeat domain; NB, nucleotide-binding domain; NSm, nonstructural protein encoded by the M RNA segment, presenting the movement protein; NSs, nonstructural protein encoded by the S RNA segment, presenting the RNA silencing suppressor protein.

in the cytoplasm, but purified RNPs of TSWV, containing the viral RdRp, also support both replication and transcription in vitro (1, 131). In vitro transcription–replication requires the presence of translational machinery, not to support ongoing viral protein synthesis but for ribosome scanning of nascent viral transcripts to prevent premature transcription termination (131, 132). Eukaryotic elongation factor 1A (eEF1A) has been identified to play an important role in the enhancement

of TSWV RNA replication and transcription (67). In *F. occidentalis* thrips, a transcription factor (FoTF) associates with TSWV RdRp, which stimulates replication but not transcription. This factor is also found to turn human cell lines permissive to TSWV replication (27).

The 5′ ends of (subgenomic) TSWV mRNAs contain a nonviral, capped nucleotide (nt) sequence of ~12–20 nt (73, 135). These sequences derive from host cellular mRNAs by a process called cap snatching, as first discovered and described for the influenza virus (11). During this process, the viral transcriptase complex binds to the 5′ cap structure of a host mRNA and performs a cleavage further downstream by an endonuclease activity encompassed in the viral RdRp. The resulting capped RNA leader aligns at the 3′ end of a viral genomic RNA template and is elongated by the viral RdRp to generate messenger transcripts.

During in vitro transcription of TSWV, using either purified virus particles or RNPs, reticulocyte lysate also provides a source of capped RNA (globin mRNAs) (131). During in vivo genome transcription, the virus is able to take any available source of capped RNA. This can also originate from a coinfecting, cytoplasmic-replicating RNA virus like *Alfalfa mosaic virus* (AMV) (35). Extensive in vitro and in vivo transcription studies have shown that TSWV prefers capped RNA leaders with the ability for multiple base pairing to the 3′ end three residues of the viral RNA template, and these outcompete similar leaders with only one base pairing residue (133). Transcription studies with influenza A (43, 44) and tenuiviruses (143), now classified as the plant-infecting members of the *Phenuiviridae* within the *Bunyavirales*, have revealed similar findings and provide support for a paradigm for cap snatching generic to all segmented, negative-strand RNA viruses that employ cap snatching, in which (multiple) base pairing between capped RNA leaders and the viral genomic RNA template promotes their usage during genome transcription initiation.

Although the endonuclease domain has not been functionally mapped within the TSWV RdRp, crystal structure analysis of the influenza virus polymerase PA subunit has pointed toward the identification of the endonuclease domain (146). Similar analysis of the *La Crosse orthobunyavirus* (LACV) RdRp, combined with biochemical assays using RdRp mutants and a structure-based sequence alignment, has revealed that the endonuclease domain of the RdRp is highly conserved among all bunyaviruses, including plant-infecting tospoviruses, emaraviruses, and tenuiviruses, and maps to the N terminus of the RdRp protein (106).

Whereas influenza virus replicates in the nucleus and snatches capped RNA leaders from nascent RNA chains produced by the nuclear RNA polymerase II system (17), TSWV replicates in the cytoplasm and snatches capped RNA leaders from the existing pool of cytoplasmic host cellular messengers. Where and how this takes place is not known, but studies with the related *Sin nombre Hantavirus* (SNV, a former joint member of the *Bunyaviridae*) point toward cytoplasmic RNA processing (P) bodies because the SNV N protein binds to 5′ caps with high affinity and colocalizes with P bodies (92).

Tospovirus Intracellular and Intercellular Movement

Once cytosolic TSWV RNPs are produced, cellular machineries for macromolecular transport are hijacked to take RNPs to the plasmodesmata for spreading into neighboring/systemic plant tissue (via intercellular trafficking) or to the ER and Golgi, where particle maturation occurs (via intracellular trafficking). During intracellular movement, TSWV N/RNPs form motile cytoplasmic protein bodies that depend on the actin cytoskeleton for its movement and are driven by the myosin XI-K (37, 110). Application of latrunculin B, an actin-depolymerizing agent, not only abolishes the intracellular movement of N inclusions but also strongly inhibits the local and systemic movement of TSWV in tobacco (37). In the presence of cytochalasin D, a functionally similar drug, but not colchicine (a microtubule-depolymerizing agent), TSWV N protein bodies are smaller but appear more abundantly, indicating the importance of the actin skeleton in

the formation of N inclusions (110). TSWV N/RNP bodies also move along the ER membrane network (37). The fact that TSWV RNPs play a critical role in viral replication leaves open the possibility for a model in which viral replication factories (VRFs) are not stationary but rather are constantly mobile within the cells. The VRFs moving along the actin/ER network can easily collect cellular host factors required for viral RNA replication–transcription. Trafficking of VRFs along the actin/ER network can also greatly facilitate the envelopment of TSWV RNPs by the ER or Golgi membrane. The observation that the TSWV N protein interacted with ER-resident Gc and concentrated at ER export sites before being rescued by Gn to the Golgi complex (an actin-dependent transport process) nicely fits into this model (110).

During the early stages of infection, RNPs transiently associate with the movement protein NSm. Although these complexes can be widely distributed in the cytoplasm, they ultimately concentrate near and at plasmodesmata (72, 117, 122, 123) to assist in cell-to-cell and long-distance movement of infectious RNPs through plasmodesmata. Owing to the action of the NSm movement protein, NSm forms a tubule structure within the plasmodesmata to guide the transport of RNPs into neighboring healthy cells (80, 122). Plasmodesmata containing NSm also exhibit an enlarged size exclusion limit (123). NSm is also expressed in viruliferous thrips but is not considered to play a role in the infection cycle in thrips (122). Because animal-infecting bunyavirus relatives lack a homolog of NSm, the NSm protein reflects the adaptation of TSWV to infect plant hosts.

Cell-to-cell movement of plant viruses is considered a relatively conserved process. As observed with viral movement proteins from other viruses, the TSWV NSm protein is also able to *trans*-complement movement-deficient viruses that have a plus-strand RNA genome, e.g., *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), and AMV, in their cell-to-cell and long-distance movement (77, 80, 100, 115). The TSWV NSm protein tightly associates with the ER membrane and can move by itself from cell to cell along the ER network (38). In plants, the ER interconnects between neighboring cells via plasmodesmata and forms a continuous ER membrane network throughout the entire plant (120). Mutations within NSm that impair its ER association or pharmacologically disrupt the ER network severely inhibit the cell-to-cell movement of NSm. Likewise, *Arabidopsis thaliana rhd3* mutants containing an impaired ER network show a severe reduction of the intercellular movement of TSWV NSm and, as a result, show reduced levels of systemic viral infection (38). Although NSm facilitates cell-to-cell movement of infectious RNPs in a tubule-guided manner via plasmodesmata, an interplay between NSm and ER clearly plays an important role in the intra- and intercellular movement of viral RNPs.

Tospovirus Particle Maturation

During later stages of infection, TSWV RNPs become surrounded by the lipid membrane of the Golgi apparatus. In contrast to the animal-infecting bunyaviruses, whose RNPs bud into lumenized Golgi stacks, TSWV RNPs are enwrapped by an entire Golgi stack, leading to the formation of double-enveloped virus particles (63). These double-enveloped particles subsequently fuse with each other and ER membranes and generate large vesicles containing accumulating amounts of mature singly enveloped virus particles (63). The viral glycoproteins Gn and Gc are processed from the precursor protein on ER membranes. From there, Gn continues trafficking toward the Golgi complex as a monomer or dimerized with Gc. Upon single transient expression, both glycoproteins are able to induce membrane curving of ER/Golgi membranes (108), a feature typical of reticulons, i.e., proteins that predominantly reside in the ER and play an important role in promoting membrane curvature (136). Whether membrane curving reflects the formation of a spherical enveloped virus particle remains speculative.

Gc expressed on its own is retained in the ER and requires dimerization with Gn to escape and move toward the Golgi complex (108). ER arrest of Gc is not a matter of improper folding but is

due to its (relatively short) transmembrane domain (TMD). The exchange of this domain by the larger TMD of Gn allows Gc to escape from ER arrest and move toward the Golgi (109). This observation fits with the idea that the TMD length of membrane proteins has a major influence on their final destination within the endomembrane system, with shorter TMDs restricting the proteins to the ER (12).

During the early stages of glycoprotein synthesis and processing, the glycoproteins localize at ER export sites (ERESs), distinct foci in the ER, from where concentrated glycoproteins continue in their anterograde trafficking (108, 121). This observation suggests that Gn recruits and subsequently concentrates ER-resident Gc at ERESs, from which anterograde transport to the Golgi complex continues. Inhibition of COPII vesicle formation at ERESs aborts trafficking of Gn and Gn-Gc dimers toward the Golgi complex, which indicates that viral glycoprotein trafficking from ER to Golgi involves COPII-dependent vesicle transport (109).

TSWV N/RNPs rely on ER/Golgi membrane for their envelopment. Intracellular movement of TSWV N/RNPs along the ER membrane network is dependent on actin and myosin (37, 110). The Golgi complex is known to move as a mobile unit along the ER membrane network in an actomyosin-dependent manner as well, meanwhile picking up ER cargo from ERESs in a stop-and-go mode (8, 24, 95). In plants, TSWV N protein colocalizes and interacts with both Gn and Gc at the interface of ER and Golgi (107, 110). The cytosolic N protein of TSWV is able to recruit ER-resident (reticular) Gc proteins to ERESs through interaction with the cytoplasmic tail of Gc (110). From there N/RNP-Gc complexes are rescued by an interaction with Gn and exit the ER. Alternatively, N/RNPs interact with preformed Gn-Gc heterodimers either at ERESs, and traffic to the Golgi, or at the Golgi stacks where particle assembly matures (110).

The actomyosin-dependent movement of RNPs along the ER network ensures RNPs meet ER-associated NSm during the early stages of infection, leading to cell-to-cell movement, or encounter Gn-Gc complexes at either ERESs or Golgi complexes during late stages of infection, triggering membrane envelopment.

RNA INTERFERENCE: THE FIRST LAYER OF PLANT DEFENSE AGAINST TOSPOVIRUS

RNA silencing or RNA interference (RNAi) is known to play a key role in antiviral defense in plant cells. The mechanism is triggered by the formation of dsRNA, which, during a viral infection, arises from viral replication intermediates or secondary folding structures in viral (m)RNA. These are processed by Dicer-like (DCL) endoribonucleases into (primary) 21–24 nt small interfering RNAs (siRNAs). One strand of siRNA loads into an RNA-induced silencing complex (RISC) and guides the RISC to viral RNA target molecules, leading to degradation of the target (50). Aberrant RNAs, resulting from target RNA cleavage, are either further degraded by the cellular decay machinery localized in P bodies or used as a substrate by host-encoded RdRps for further amplification of the antiviral RNAi response (56).

Small Interfering RNAs Derived from Tospovirus

Tospovirus-specific siRNAs [viral siRNAs (vsiRNAs)] from infected *Nicotiana benthamiana* and tomato show the production of 21- and 22-nt vsiRNAs from all three RNA segments (89, 93). The vsiRNA profiles from both hosts are quite similar and show most reads from the M and S RNA and only low amounts from the L RNA. In tomato, vsiRNAs have been found that potentially target host genes involved in many pathways, including those related to plant-pathogen interactions (105). Only a small amount of vsiRNAs is derived from the intergenic region (IGR) sequence

of the ambisense M and S RNA segments. Although synthetic IGR transcripts are recognized and processed into small RNAs by Dicer from *Drosophila melanogaster* embryo extracts, during a natural infection the predicted folding structures within the IGRs are likely not accessible because of masking by other viral/host factors (90).

RNAi-Mediated Resistance Against Tospovirus

In the past, pathogen-derived resistance has been engineered against TSWV. Although transgenic plants expressing the N protein protected against a tospovirus infection (45, 84, 103), resistance was also obtained after expression of untranslatable (partial) *N*, *NSm*, *NSs*, or *RdRp* genes, demonstrating an RNA-mediated defense mechanism (59, 99, 101, 104, 118, 142, 144). Transgenic resistance holds against the homologous virus only and not any other distinct or even relatively closely related tospoviruses (26, 54). Stable tobacco transformants expressing an *NSm* transgene are completely immune at the plant level, but protoplasts collected from these still support replication of TSWV, indicating that antiviral RNAi triggered by a tospovirus infection acts on viral messengers and not (anti)genomic RNA strands (104). Viral (anti)genomic RNAs are not being targeted, likely because these (anti)genomic RNAs are only present in RNPs and tightly encapsidated with N protein and are thereby protected from DCL cleavage.

SUPPRESSION OF ANTIVIRAL RNAi: VIRAL COUNTER-DEFENSE

As a viral counter-defensive strategy, almost all plant viruses encode RNA silencing suppressor (RSS) proteins to inhibit key steps of host antiviral RNAi. Viral RSSs suppress RNA silencing pathways through sequestering dsRNA to inhibit biogenesis of siRNA, sequestering siRNA duplexes to prevent the loading of small RNA into the AGOs, or inhibiting antiviral activity of the host RISC machinery (50).

Tospovirus Encodes an RNA Silencing Suppressor to Counteract Host RNAi Antiviral Defense

Using a GFP-based transient silencing suppression assay, the NSs protein of TSWV and other tospoviruses such as *Impatiens necrotic spot virus* (INSV), *Groundnut ringspot virus* (GRSV), and *Tomato yellow ring virus* (TYRV) has been identified as the RSS (14, 54, 113, 124). Electrophoretic mobility shift assays with NSs from different tospoviruses, either using NSs-containing crude plant/insect cell extracts or *Escherichia coli*–expressed purified NSs, have shown that tospoviral NSs proteins are able to bind dsRNA independent of size. siRNAs and microRNA (miRNA)/miRNA* duplexes as well as long dsRNA are bound (55, 113). In agreement with the binding of long dsRNA, TSWV NSs protein inhibits the cleavage of long dsRNA by Dicer in vitro and suppresses gene silencing induced by an inverted repeat GFP (55, 113). Together, these findings suggest that tospovirus NSs exerts RNAi suppressor activity by sequestering long and small dsRNA to prevent the biogenesis of small RNAs, their uploading, and the activation of antiviral RISCs. Using alanine substitution analysis of the NSs protein, both the N- and C-terminal domain of NSs have been shown to be required for RNA silencing suppression, but mutations introduced in the N terminus almost always rendered NSs dysfunctional in the suppression of local GFP silencing assays (33). TSWV NSs also contains a GW/WG motif, a dipeptide sequence that has been reported to be involved in the interaction of several RSS proteins with AGO1 (47). Mutation of this motif within NSs also impaired its (local) RNA silencing suppression activity (33) and implies a possible interference at AGO1-RISC. Mutations introduced into NSs affect the ability to suppress local RNAi

and do not always render this protein completely dysfunctional. Several dysfunctional NSs mutants still are able to suppress GFP silencing in systemic leaves, most likely because of their ability to sequester siRNAs from local leaves and to prevent their movement and subsequent activation of antiviral RISC in systemic tissue. This also applies to the NSs GW/WG mutant and supports the idea that this mutant is functionally hampered from suppressing local GFP silencing further downstream from the biogenesis (and binding) of siRNAs (57).

The ability of NSs to sequester siRNAs independent of sequence also implies that it might interfere in the antiviral RNAi response toward a coinfecting virus. This is nicely exemplified with stable transformants of *N. benthamiana* containing a partial *N*-gene sequence of TYRV-t (tomato strain) that are resistant to this virus but not to TYRV-s (soybean strain). When these transformants are co-challenged with TYRV-t and TYRV-s, a systemic infection of both viruses is observed. Delivery of TSWV-s NSs protein from a PVX replicon prior to TYRV-t infection also allows the latter to break the resistance. This shows that even engineered resistance that provides total immunity can be directly broken when the virus target strain is *trans*-complemented by a viral RNAi suppressor from a coinfecting related strain, or possibly from an unrelated virus as well (54). NSs has been observed to *trans*-complement (heterologous) RNAi-suppressor-deficient viruses as well. A nice example of this is observed in *Turnip mosaic virus* (TuMV). When the NSs gene was inserted into a HC-Pro-defective TuMV-GFP, NSs compensated for the functional loss of HC-Pro and supported a systemic TuMV infection of the plant (41, 42).

Tospovirus NSs Suppresses RNAi in Insects

Because RNAi also acts as an antiviral defense mechanism in arthropods, NSs may also suppress RNAi in insects and even ticks. Evidence for this has come from studies in which TSWV NSs recombinantly expressed from a baculovirus enhanced the replication of this virus in Sf9 insect cells (97) and increased its virulence in caterpillars (30). In tick and mosquito cells, expression of TSWV NSs from a semliki forest virus replicon impaired RNAi induced by a semliki forest virus replicon expressing luciferase (7, 56).

During infection of arthropods with arboviruses, the activity of RNAi in the midgut epithelium has been postulated to play an important role in the midgut barrier and vector competence (74). In thrips, antiviral RNAi has not yet been investigated, but it is generally assumed that the virus encounters an antiviral RNAi response and requires NSs to counter-defend during propagative transmission. Recent studies by Margaria et al. (87) pointed toward an important role for TSWV NSs during acquisition/transmission by *F. occidentalis*. A TSWV isolate containing a truncated NSs protein and compromised in its ability to suppress RNAi could still be acquired by *F. occidentalis*. However, this virus was not able to establish a persistent infection and was not transmitted by *F. occidentalis* (87), suggesting that the observed reductions in vector competence and virus titers are likely due to the lack of RNAi suppression by these NSs-defective isolates.

EFFECTOR-TRIGGERED IMMUNITY: A ROBUST SECOND LAYER OF PLANT DEFENSE AGAINST TOSPOVIRUS INVASION

Plants employ diverse intracellular innate immune receptors to recognize invading pathogens and to induce host defense (61). The largest class of intracellular receptors is presented by the nucleotide-binding leucine-rich repeat receptors (NLRs). During pathogen invasion, plant NLRs recognize specific pathogen effectors to trigger so-called effector-triggered immunity (ETI) (15, 61), which often comes along with a programmed cell death (PCD)–based hypersensitive response (HR) at the initial infection site. Effectors recognized by NLRs are referred to as elicitors or

avirulent (Avr) determinants. On the basis of the difference at the N terminus, plant NLRs are further classified into Toll/interleukin-1 (TIR)-NLRs (TNLs) and coiled-coil (CC)-NLRs (CNLs).

Tomato Immune Receptor Sw-5b-Mediated Resistance Against Tospoviruses

The tomato *Sw-5* gene is the most commonly used resistance gene in tomato resistance breeding to control tospoviruses. Two groups have independently cloned this single dominant resistance gene (13, 119). Sw-5b belongs to the CC-NLR-type immune receptors, which contain a CC domain, a central nucleotide-binding adaptor shared by ApaF-1, resistance proteins, a CED-4 (NB-ARC) domain, and a C-terminal leucine-rich repeat (LRR) domain (13, 18, 29, 119).

Sw-5b confers broad-spectrum resistance to American-type tospoviruses through recognition of a conserved 21-amino acid epitope in NSm. Currently, almost thirty recognized and tentative tospovirus species are known (128), and these classify into American and Euro-Asian clades based on their geographic origin and the amino acid sequence of the N protein. Analyses of *Sw-5b* stable transgenic *N. benthamiana* plants and (near-isogenic) tomato lines carrying *Sw-5b* showed that *Sw-5b* confers broad-spectrum resistance to various American-type tospoviruses but not to Euro-Asian-type tospoviruses (**Figure 3**) (76, 149).

Sw-5b resistance is triggered by the cell-to-cell movement protein NSm (51, 76, 82, 100, 149). The activation does not rely on the presence of motifs required for plasmodesmata targeting, tubule formation, or cell-to-cell movement (76, 148). Instead, the central domain, specifically a 21-aa (amino acid) peptide region positioned at amino acid 115–135 in TSWV NSm (NSm^{21}),

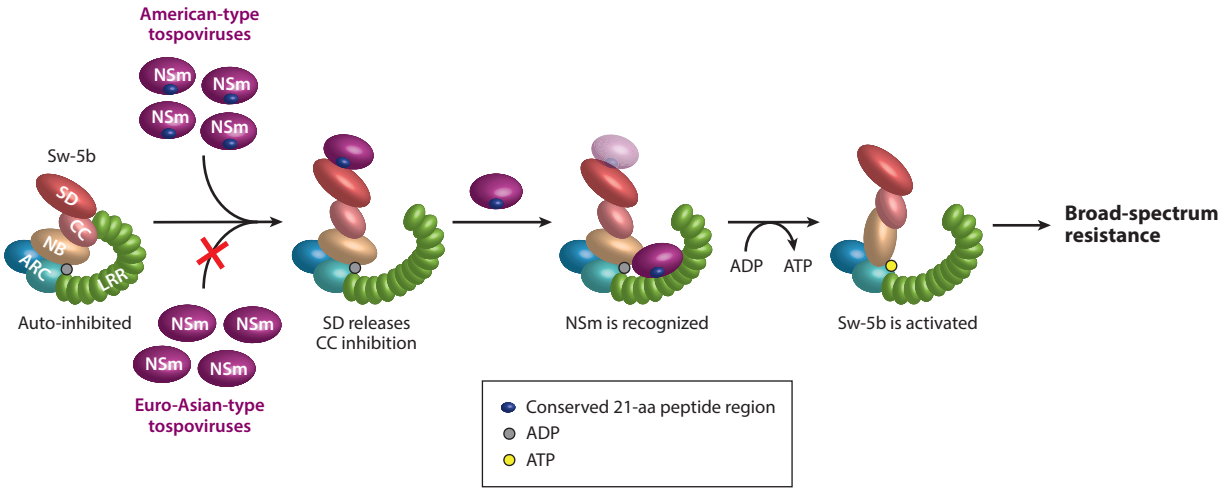

Figure 3

Model for Sw-5b-mediated resistance against tospoviruses. Sw-5b confers broad-spectrum resistance to various American-type tospoviruses, but not to Euro-Asian-type tospoviruses, through the recognition of a conserved 21-aa (amino acid) peptide region of the nonstructural protein NSm. NSm binds to the extended SD of Sw-5b, which releases the inhibition of the CC domain on the central nucleotide-binding leucine-rich repeat domain (NB-ARC-LRR). The NB-ARC-LRR further recognizes NSm, exchanges ADP (*gray dot*) for ATP (*yellow dot*), and switch activates the receptor, leading to a robust defense response against tospoviruses. Abbreviations: ARC, Apaf-1, R-protein, and CED-4 domain; CC, coiled-coil; CNL, coiled-coil nucleotide-binding leucine-rich repeat receptor; LRR, leucine-rich repeat domain; NB, nucleotide-binding domain; NSm, nonstructural protein encoded by the M RNA segment, presenting the movement protein; NSs, nonstructural protein encoded by the S RNA segment, presenting the RNA silencing suppressor protein; SD, *Solanaceae* domain.

is sufficient to trigger Sw-5b-mediated HR. This 21-aa region is highly conserved among the American-type tospoviruses only. Importantly, the Euro-Asian-type tospovirus NSm protein can be converted into an inducer of Sw-5b-mediated HR by introducing the NSm^{21} peptide to their sequence (149). Mutations C118Y and T120N, which have been reported in two natural TSWV-NSm resistance-breaking (RB) isolates, map within this NSm^{21} region. Introduction of these mutations into either the full-length NSm of a resistance-inducing (RI) strain or the NSm^{21} peptide only impairs its ability to elicit Sw-5b-mediated HR (76, 149). The mutations in this conserved peptide region have been shown to strongly affect intercellular movement of TSWV NSm (38). This suggests that the conserved NSm^{21} region plays a critical role in viral cell-to-cell movement. Introduction of the T120N mutation into NSm compromises the avirulence and leads to a fitness cost during viral infection (100).

Plant pattern-recognition receptors (PRRs) typically recognize conserved pathogen-associated molecular patterns (PAMPs) to provide broad-spectrum pathogen resistance, whereas plant NLRs generally recognize strain-specific pathogen effectors and confer race-specific resistance. The findings showing that Sw-5b NLR recognizes a conserved NSm^{21} peptide region to confer broad-spectrum resistance to American-type tospoviruses strongly support the notion that this conserved NSm^{21} peptide acts as a PAMP (149), similar to the plant PRRs FLS2 and EFR, which (extracellularly) recognize a conserved 22-aa peptide of flagellin (flg22) (19) and an 18-aa peptide (elf18) of elongation factor Tu (EF-Tu) (150), respectively, and provide broad-spectrum resistance to bacterial invasions. The intracellular triggering of Sw-5b NLR by NSm^{21} provides the first case of a plant NLR conferring broad-spectrum resistance by means of a PAMP-like structure–triggered immune response. The Mi-1.2 NLR, a close Sw-5b homolog from tomato, also provides broad-spectrum resistance to four completely different pests: nematodes, aphids, whiteflies, and psyllids (16, 91, 96, 137). Although speculative, it is not unlikely that triggering the broad-spectrum Mi-1.2-mediated resistance, as with Sw-5b, might also involve a conserved PAMP-like structure in the cognate pest effectors.

Multilayered auto-inhibition and activation of Sw-5b. Plant NLR immune receptors typically function as molecular switches to initiate robust defense responses against pathogen invasion. The central NB-ARC domain of NLRs binds to ADP or ATP to switch from an auto-inhibited "off" state to an activated "on" state (85, 125, 134, 141). The CC domain and the LRR domain suppress NB-ARC nucleotide exchange through intracellular domain interaction and maintain the NLR protein in an auto-inhibited state. Only once the presence of the pathogen is perceived does the status change to an activated one. Although Sw-5b belongs to the CNLs, like other *R* genes from *Solanaceae*, it contains an extended N-terminal *Solanaceae* domain (SD) (94). With four domains, Sw-5b has evolved a more complex multilayered mechanism to regulate and switch between its auto-inhibited, or "off," and activated, or "on," state (18). In the absence of NSm, Sw-5b LRR suppresses the NB-ARC domain and keeps the NB-ARC-LRR in an auto-inhibited state. The N-terminal SD and the CC domain actively suppress the NB-ARC-LRR to prevent activation of the receptor and concomitant onset of PCD (18, 29). In the presence of NSm, the N-terminal SD, CC, and LRR-mediated inhibition of NB-ARC are sequentially relieved and switch Sw-5b to an activated form, leading to a (visual HR) defense response (**Figure 3**) (18, 29).

Domain swaps between Sw-5b and Sw-5 paralogs from *Solanum peruvianum* or orthologs from susceptible *Solanum lycopersicon* (domesticated Heinz Tomato) have shown that LRR domains can be exchanged and revert susceptible/nonfunctional homologs into functional resistance-conferring copies (29, 149). Identification and characterization of the NSm binding site in the LRR domain by means of site-directed mutagenesis and domain swaps discerned four

polymorphic sites in the Sw-5b LRR domain that are critical for NSm or NSm^{21} recognition. Structure modeling of the Sw-5b NB-ARC-LRR revealed that these four polymorphic sites are all clustered and surface exposed (149). Furthermore, the R927 contact site between the NB-ARC and LRR domain was also identified; it is required to maintain Sw-5b NB-ARC-LRR in an autoinhibited state and is located adjacent to the four polymorphic sites in the LRR domain of the homology model. Considering that the R927 contact site and the NSm^{21} binding sites are located next to each other on the LRR domain surface, the recognition of NSm^{21} by Sw-5b LRR likely disrupts the R927 contact site and weakens the intramolecular interaction between the NB-ARC and LRR domain, thus translating NSm^{21} ligand recognition into activation of the receptor (149).

Although many plant NLRs recognize pathogen effectors via their N-terminal domain (22, 34, 61) and switch to an activated state in the presence of the effector, the situation with Sw-5b seems more complex. Recently, Li et al. (79) demonstrated that, in addition to the LRR domain, the extended N-terminal SD of Sw-5b also plays a critical role in sensing NSm and enhances the ability of NB-ARC-LRR to detect a low amount of NSm. These findings suggest that Sw-5b NLR has evolved an extra pathogen sensor (SD) next to the main switch activator (NB-ARC-LRR) in one single immune receptor and uses two distinct domains to detect the viral movement protein NSm. In this way, Sw-5b has adopted a two-step recognition mechanism to significantly enhance its sensitivity to tospovirus infections.

Evolutionary selection of Sw-5b homologs from wild tomato species in South America. The resistance allele *Sw-5b* originates from a wild species of *S. peruvianum* from South America. American-type tospoviruses (5, 25, 102, 140) and tomato species (81, 126) both originate from South America. The observation that *Sw-5b* confers resistance to the American-type tospoviruses only supports the idea that *Sw-5b* and these tospoviruses coevolved. The importance of the four polymorphic sites (M3–6) and R927 within Sw-5b LRR for NSm recognition and activation of NB-ARC-LRR is emphasized by the observed natural variation among Sw-5b homologs analyzed from ~90% of all wild tomato species from South America (149). The polymorphic site 8 (M8) in Sw-5 SD (79) has recently been identified and has also shown to be critical for NSm recognition (79). Natural covariation analysis showed that only those Sw-5 homologs containing conserved M8 sites in the SD and conserved M3–6 sites in the LRR domain were able to induce HR in the presence of NSm or NSm^{21} and confer resistance to TSWV infection (79, 149). These findings support the idea that co-selection of the critical NSm recognition sites in both the SD and LRR domain is necessary for conferring the resistance against tospoviruses.

Pepper Immune Receptor Tsw-Mediated Resistance Against Tospovirus

In contrast to the broad-spectrum resistance mediated by *Sw-5b* to various American-type tospoviruses, *Tsw*, originating from *Capsicum chinense*, only confers resistance to TSWV isolates (9, 10). Similar to *Sw-5b*, HR is triggered in *Tsw* cultivars upon TSWV infection to prevent local and systemic viral spread (10).

Tsw recognizes tospovirus RNA silencing suppressor to elicit a hypersensitive response. In the past, the identity of the effector of Tsw has been debated. Margaria et al. (88) obtained indirect evidence indicating that NSs triggered Tsw-mediated resistance. Lovato et al. (83), however, found that the N protein triggered PCD in *Capsicum chinense* plants carrying the *Tsw* gene after transient expression from a *Potato virus X* (PVX) vector. In 2013, de Ronde et al. (31) indisputably identified NSs as the effector of Tsw-mediated resistance. Using *Agrobacterium tumefaciens*

transient expression assays (ATTAs), de Ronde and colleagues found that NSs, but not N, triggers HR in *Tsw*+ pepper plants (31).

Considering that NSs from TSWV acts as an Avr factor as well as an RSS suggests that there was an arms race during coevolution between the virus and plant innate immunity. Analysis of a series of NSs mutants indicated the importance of the N terminus of the protein for both functions, although some mutations do uncouple the RSS activity and HR triggering of NSs (33). Chimera of TSWV NSs and GRSV NSs, two closely related tospoviruses, do not trigger *Tsw*-mediated resistance, indicating that Avr functionality cannot simply be transferred. Furthermore, the NSs GW/WG motif mutant that was previously shown to be impaired in its local but not systemic RNA silencing suppression activity also lost the ability to trigger Tsw-mediated resistance (33, 57).

Tsw is a CC-NLR immune receptor. Screening of *Capsicum* spp. lines identified three *C. chinense* accessions ('PI152225,' 'PI159236,' and 'Panca') resistant to TSWV (6, 9). Allelic relationship analysis of these lines showed that resistance was provided by the same gene (9). Subsequent genetic mapping of the *Tsw* locus linked the gene to the marker SCAC568 c. on chromosome 10 (58). Meanwhile, the *Tsw* gene has been cloned using a comparative genome-guided fine-mapping approach. Within a 295-kb region of chromosome 10, eight intact CNLs are found, of which only one candidate induced HR upon co-expression with NSs in *N. benthamiana*. Transient and stable expression of this candidate in *N. benthamiana* plants conferred resistance to TSWV and was therefore confirmed as *Tsw* (64).

The 6,351-bp-long *Tsw* gene has nine exons and encodes an NLR protein of 2,116 amino acids with a CC domain, a nucleotide-binding domain, and eight LRRs (64). Of these eight LRRs, seven are highly similar and differ by only a few amino acids. The susceptible allele *tsw* lacks four of these similar LRRs and does not induce an HR response. *tsw* also encodes a different C-terminal end than *Tsw*. As the LRR domain of NLRs is generally associated with the recognition of effectors (22, 34, 61), the absent LRRs, as well as the different C-terminal domain in *tsw*, are likely involved in the recognition of NSs. The role of the LRR as effector recognizer is further illustrated upon comparison of Tsw and Pvr4, an NLR receptor from *Capsicum annuum* with high structural homology to Tsw that confers resistance against potyviruses upon recognition of the potyviral RdRp (64).

Strains of *Tomato spotted wilt virus* that break Tsw-mediated resistance. Various Tsw RB strains of TSWV have been reported (2, 31, 32, 39, 60, 88). Almási et al. (3) discovered that a single-point mutation at amino acid position 104 of wild-type NSs was sufficient to break Tsw-resistance. When position 104 of wild-type and RB NSs was changed, their phenotypes reversed, which suggests that this point mutation likely affects the (in)direct interaction with Tsw. In addition to this single point mutation, other mutations associated with RB strains were found that localized further toward the C terminus of NSs.

At temperatures above 30–32°C, the Tsw NLR is no longer functional (20, 32) and any TSWV isolate, including a wild-type (RI) TSWV, is able to establish a systemic infection. Besides the common RB strains, de Ronde et al. (32) discovered another class of strains that break Tsw-mediated resistance at 28°C, a temperature at which Tsw is still functional. At standard greenhouse conditions (21–22°C), these temperature-dependent RB strains induce Tsw-mediated resistance. Temperature-shift assays indicated that de novo synthesis of this RB NSs protein at lower temperatures was required to induce resistance.

PERSPECTIVES

Employment of Recessive or Dominant Resistance Genes to Prevent Tospovirus Infection

Plant viruses are obligate pathogens whose life cycles are dependent on host-plant machineries. Disruption or mutation of cellular host factors essential for virus replication, transcription, or movement can result in a recessive resistance against virus infection in plants. Many recessive resistance genes against virus infection have been identified in diverse plant species, including *Arabidopsis*, pea, pepper, lettuce, barley, melon, white lupin, and wild tomato (53). One of the best examples is eIF4E: Its isoform, eIF(iso)4E, carries a mutation in the recessive allele and fails to interact with viral VPg, thereby conferring resistance to potyviruses (53). Later, this gene was found to confer resistance to a broad spectrum of other plant viruses as well (112, 126). Several plant proteins have now been characterized as important factors needed for plant virus life cycles (53) and are potential resources of recessive resistance. For TSWV, RHD3 has been shown to play a critical role in movement (38), whereas TSWV N/RNPs move intracellularly in an actomyosin-dependent manner. Viral replication and transcription are enhanced by eEF1A (67). Whether these host factors present interesting targets for the development of recessive resistance against TSWV/tospoviruses remains to be further investigated.

Although several new sources of tospovirus resistance are being described (128), e.g., *Sw-2*, *Sw-3*, *Sw-4*, and *Sw-7*, a continuing search for new sources of resistance is needed. Several wild species of tomato do not possess the Sw-5b resistance allele but could confer resistance to TSWV infection and present interesting targets for further analysis (149). Further resistance screening can be done to combat not only TSWV in tomato but also Euro-Asian-type tospoviruses in a wide range of other host plants/crops.

Breeding for Resistant Cultivars Against Tospoviruses Through Targeted Genome Editing

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology has emerged as a powerful tool for precise targeted genome editing, including gene knockout and knockin (145, 147). For tomato breeding, TSWV-susceptible cultivars are known to contain an Sw-5b homolog with only one or a few SNPs relative to the functional Sw-5b. Because tomato is suitable for genome editing by CRISPR-Cas9 technology (23, 116), susceptible/dysfunctional Sw-5 alleles can now be (ex)changed to reintroduce Sw-5b-mediated TSWV resistance. The same applies to the susceptible *tsw* allele from pepper, which lacks four LRR domains compared with the functional *Tsw*. Their reintroduction could rescue Sw-5b- or Tsw-mediated resistance in susceptible tomato or pepper and simultaneously allow these cultivars to enter introgression breeding programs.

Molecular Engineering of NLR Receptors to Expand the Spectrum of Pathogen Recognition

Artificial evolution has been successfully used to expand the spectrum of pathogen recognition by plant NLRs, which is nicely exemplified with Rx. This TIR-NLR receptor confers resistance to several strains of PVX. A natural mutation at amino acid residue 121 in the PVX coat protein (CP) sequence allows the virus to overcome Rx-mediated resistance in potato (48, 62, 66). Using an in vitro artificial evolution approach, a newly generated (mutant) Rx not only recognized the noneliciting PVX strain but also, along with other strains of PVX, the distantly related *Poplar mosaic virus* (PoMV) (36, 52). Applying this approach to *Sw-5b* and *Tsw* is tempting, considering

that RB isolates have been documented for both genes (4, 21, 60, 75, 82, 128) and the narrow resistance spectrum has been observed for *Tsw*.

Although many plant NLRs share high amino acid sequence identity, they mostly recognize very different pathogens. Several good examples exist: Rpi-blb2 NLR shares 82% amino acid sequence identity with Mi-1.2 but confers resistance to *Phytophthora*, whereas Mi-1.2 confers resistance to a nematode and several insects (16, 91, 96, 129, 137). Rpi-vnt1.1 and Tm-2^2 share 72% identity but confer resistance to potato late blight and *Tomato mosaic virus*, respectively (40). Rx and Gpa (88% amino acid sequence identity) respectively confer resistance to PVX and the nematode *Globodera pallida* (130). R8 shares 89% amino acid sequence identity with Sw-5b but confers resistance to potato late blight disease (138). Pvr4 shares 66% amino acid sequence identity with Tsw, but both genes recognize different viruses (64). It is likely that these homologs share a common ancestor but have evolved to recognize different pathogens/pests through natural selection. Recently, the *Arabidopsis* immune receptor RPS5 has been engineered to switch from recognizing bacteria to viruses (65). In light of this achievement, and with the availability of new and fast gene editing tools, there is now the possibility to molecularly engineer plant NLRs with a changed or expanded pathogen recognition spectrum. Additional NLRs also increase the number of genetic resources that can be used for crop resistance (introgression) breeding programs to defend against different tospoviruses or plant pathogens/insect pests.

SUMMARY POINTS

1. Tospoviruses are important plant pathogens and pose a serious threat to agricultural and ornamental industries worldwide.
2. Tospoviruses depend on host cellular machineries, including virus replication, transcription, movement, and particle maturation, to accomplish their life cycles.
3. As the first layer of defense, the plant immune system utilizes an RNA silencing mechanism to prevent tospovirus invasion.
4. Tospoviruses are known to encode RSSs to counteract the host's first defense layer and facilitate virus infection in plants.
5. To further combat tospoviruses, the plant immune system has evolved NLR immune receptors (e.g., Sw-5b and Tsw) to recognize different viral effectors (e.g., NSm and NSs) and induce robust ETI as the second layer of defense against tospovirus infection.
6. Sw-5b- and Tsw-mediated resistance has been broken by naturally evolved new TSWV strains. The arms race between tospoviruses and plant innate immunities is endless and drives the coevolution of host defense mechanisms and virally encoded proteins. New strategies, including targeted gene editing and artificial evolution of NLRs, can be adopted to reprogram plant innate immunity to control tospovirus diseases.

DISCLOSURE STATEMENT

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

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