Chapter 12
Research Advances in Negative-Strand Plant RNA Viruses

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Abstract Plant negative-strand RNA viruses cause a number of significant diseases in agriculturally important crops worldwide. As the counterpart of mammalian-infecting viruses, the negative-strand plant viruses share similarities with mammalian viruses in their particle morphology and genome organization. Similar to mammalian-infecting viruses, the genomic RNAs of plant negative-strand viruses are associated with a nucleocapsid protein to form a ribonucleocapsid core which are minimal infectious units and essential for viral replication and transcription. To adapt to the plant host, plant negative-strand RNA viruses have evolved not only movement proteins to aid the viruses moving between plant cells but also RNA silencing suppressors to attack the plant innate immune system. In this article we present an overview of the negative-strand RNA plant viruses classified within the families *Bunyaviridae*, *Ophioviridae*, *Rhabdoviridae* and genera *Tenuivirus*, *Emaravirus* and *Varicosavirus*. We highlight important discoveries over the last decade regarding the replication, transcription, movement, suppression of RNA silencing, and insect transmission of these negative-strand viruses, and antiviral strategies.

12.1 Introduction

The negative-strand RNA viruses infect a wide range of hosts, including mammals, plants and insects. Negative-strand plant RNA viruses have caused a number of significant diseases in important crops worldwide. Genomic RNAs of the

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positive-strand RNA viruses are usually infectious. In contrast to the positive-strand RNA viruses, the genomic RNAs of negative-strand viruses have no infectivity. They are associated with nucleocapsid protein to form a ribonucleocapsid core, which contains with a few copies of RNA dependent RNA polymerase (RdRP). The ribonucleocapsid cores are minimal infectious unit and essential for viral replication and transcription. The negative-strand plant viruses share similarities with mammalian viruses in their particle morphology and genome structure organization. In this article we will present an overview of the negative-strand RNA plant viruses classified within the families *Bunyaviridae*, *Ophioviridae*, *Rhabdoviridae* and genera *Tenuivirus*, *Emaravirus* and *Varicosavirus*. We will mainly focus on the recent advances in understanding the virus life cycle, including replication, transcription, movement, suppression of RNA silencing, insect transmission and antiviral strategies.

12.2 Tospoviruses

The family *Bunyaviridae* includes five genera: *Orthobunyavirus, Phlebovirus, Nairovirus, Hantavirus* and *Tospovirus*. Tospoviruses are the only members in this family that infect plants. Tospoviruses have very large host ranges, infecting more than 1000 plant species, among 80 different families. They rank among the most devastating plant viruses and cause huge economic losses each year, throughout the world (Scholthof et al. 2011).

The genus *Tospovirus* contains more than 30 recognized and tentative species. Those species can be classified into American and European-Asian clade, respectively, according to their nucleocapsid (N) protein and geographical distribution. *Tomato spotted wilt virus* (TSWV) is the type species of *Tospovirus*, and is the best studied virus in this group (Kormelink et al. 2011).

12.2.1 Virus Structure and Genome Organization

Like other members of the *Bunyaviridae*, tospoviruses have spherical, enveloped viral particles with a diameter of 80–120 nm (Fig. 12.1a). The surface of the viral envelope has spiked structures which consist of two glycoproteins: Gn and Gc (n and c refer to the amino- and carboxy-terminal positions, respectively). Within the envelope membrane are contained ribonucleoproteins (RNPs), which consist of genomic single-stranded RNA (ssRNA) tightly associated with N proteins and a few copies of RdRP.

Tospoviruses have three genomic RNAs. According to size, the tripartite genomes are named as large (L), medium (M) and small (S), respectively (Fig. 12.2). The L RNA is 8.9 kb and is negative sense. L RNA encodes a 330 kDa RdRp. The M RNA is 4.8 kb and of amisense polarity. M RNA encodes a 127.4 kDa glycoprotein precursor which processes into two mature glycoproteins, Gn and Gc, from viral complementary (vc) RNA and a non-structural protein (NSm) of 33.6 kDa from viral (v) RNA. The S RNA is 2.9 kb and of amisense

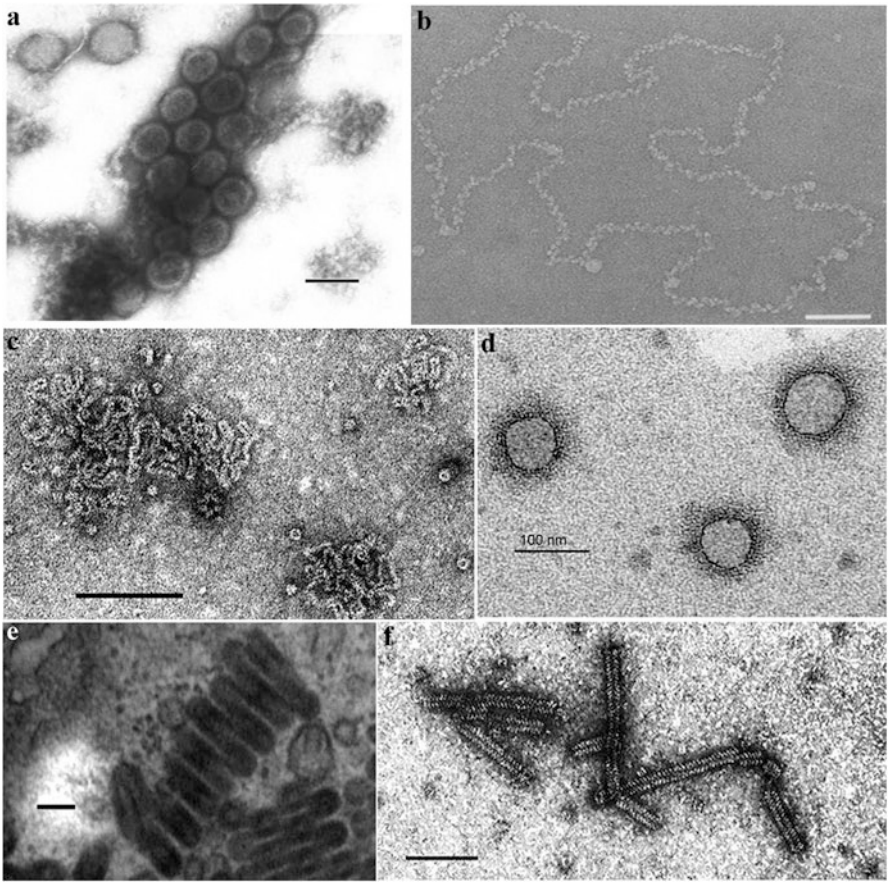

Fig. 12.1 Electron micrographs for particles of TSWV (**a**; provided by Prof. Zhongkai Zhang), RSV [**b**; cited from (Ishikawa et al. 1989)], MiLBVV [**c**; cited from (Vaira et al. 2012)], EMARV [**d**; cited from (Mühlbach and Mielke-Ehret 2012)]; SYNV [**e**; provided by Prof Zhenghe Li]; LBVaV [**f**; cited from (Walsh and Verbeek 2012)]. The *bar* represents 100 nm

coding strategy, encoding a 28.9 kDa nucleocapsid protein from vcRNA and a 52.1 kDa non-structural protein (NSs) from the vRNA.

12.2.2 Replication and Transcription

TSWV not only replicates in plant, but also in insect vectors. A transcription factor (FoTF) from *Frankliniella occidentalis* was found to enhance the efficiency of TSWV replication. Surprisingly, expression of FoTF allows TSWV, a plant-infecting virus, to replicate in mammalian cell lines (de Medeiros et al. 2005). Purified viral particles of TSWV were able to support replication and transcription

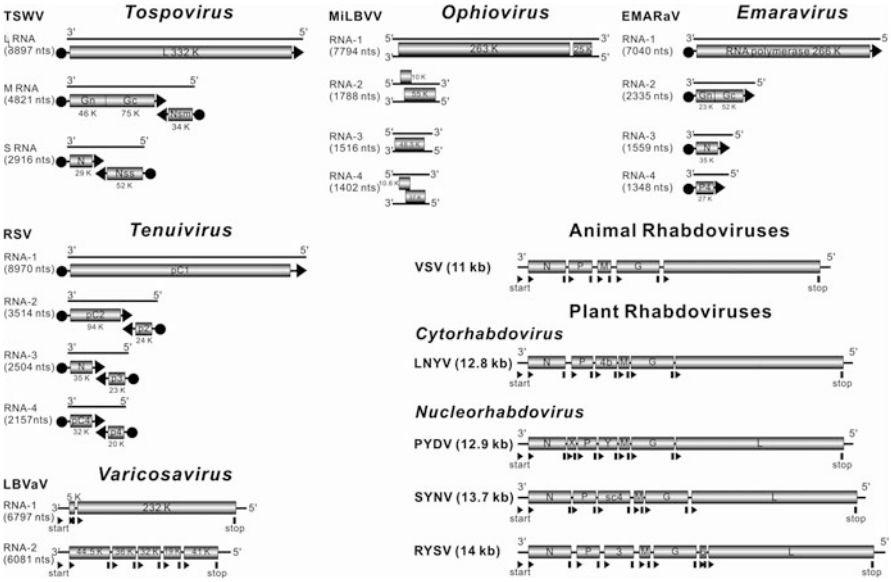

Fig. 12.2 Genome structure of TSWV in *Tospovirus*, RSV in *Teniuvirus*, MiLBVV in *Ophiovirus*, EMARaV in *Emaravirus*, VSV in animal *Rhabdovirus*, LNYV in plant *Cytorhabdovirus*, PYDV, SYNV and RYSV in plant *Nucleorhabdovirus* and LBVaV in *Varicosavirus*

in vitro. Using the *in vitro* system, Komoda et al. 2014 identified a eukaryotic elongation factor (eEF 1A) which could facilitate the transcription and replication of TSWV (Komoda et al. 2014).

TSWV use a cap-snatching mechanism for viral mRNA transcription, during which the viral polymerase cleaves capped RNA leader sequences from host mRNAs, which are then used as RNA primers for transcription initiation (Duijsings et al. 2001; Kormelink et al. 1992; van Poelwijk et al. 1996). Recent investigations on the cap-snatching mechanism of TSWV, showed that cleavage of RNA leader sequences occurred preferentially at multiple-base pairing between donors and the terminal sequence of viral templates (van Knippenberg et al. 2005).

12.2.3 Cell-to-Cell Movement

The NSm protein of TSWV has typical characteristics of plant viral movement proteins (MPs), and is thought to be the result of adaptation of the tospoviruses in plants. NSm associates with, and increases the size exclusion limit (SEL), of plasmodesmata (PD) (Kormelink et al. 1994), induces tubular structures (Storms et al. 1995), and associates with nucleocapsids in both cytoplasm and PD (Kormelink et al. 1994). Feng et al. 2013 have shown that TSWV nucleocapsid

protein is capable of forming highly motile cytoplasmic inclusions that move along the ER and actin networks of cells (Feng et al. 2013). Because nucleocapsid is the main protein for RNP cores, interaction of nucleocapsid with NSm suggests that NSm may facilitate RNP transport from cell to cell. NSm was also able to interact with host factors DnaJ (Soellick et al. 2000) and At4/1 (Paape et al. 2006), respectively. However, their biological roles in these interactions remains unknown.

NSm binds single-stranded (ss) RNA in a sequence-nonspecific manner (Soellick et al. 2000). Consistent with this, NSm was able to complement cell-to-cell movement of a movement-defective *Tobacco mosaic virus* (TMV) (Sin et al. 2005). Using this chimeric system, the domains and motifs of NSm required for movement was extensively characterized (Li et al. 2009). NSm proteins from several tospoviruses were also found to associate with biological membranes. BiFC assays revealed that the NSm protein has topology of N- and C-termini oriented to the cytoplasm when associated with membrane (Leastro et al. 2015). The exposed N- and C-termini of NSm may interact with viral nucleocapsid proteins, and host proteins, for virus cell-to-cell movement.

12.2.4 Particle Morphogenesis

TSWV particle morphogenesis has been extensively characterized in a plant protoplast system (Kikkert et al. 1999). Viral glycoproteins Gn and Gc were found to accumulate in the Golgi, where they wrap the viral nucleocapsids to form double membrane particles. The doubly enveloped particles later fuse to each other and migrate into the endoplasmic reticulum, forming singly enveloped particles that are finally clustered in the ER membranes (Kikkert et al. 1999). Subcellular localization revealed that Gc resided in the ER, whereas Gn was localized in the ER and Golgi. Upon co-expression, Gn rescues Gc from the ER into the Golgi (Ribeiro et al. 2008). Trafficking of glycoproteins from ER to Golgi was dependent on the earlier COP II and COP I secretion pathways (Ribeiro et al. 2009). Truncation analysis of Gn revealed that cytoplasmic tail and transmembrane domains are the key retention signal for Golgi targeting. The cytoplasmic domain of Gn was found to interact with Gc (Snippe et al. 2007a). Gc was able to interact with nucleocapsids (Snippe et al. 2007b), suggesting the possible mechanism for RNP wrapping by glycoproteins.

The nucleocapsid (N) protein binds ssRNA in a sequence independent manner, but does not bind to double-stranded (ds) TSWV RNA (Richmond et al. 1998). The oligomerization of N is formed by interactions of the N terminus with the C terminus of the N protein in a head-to-tail manner (Uhrig et al. 1999). The N protein could form dimer, trimer, tetramer and higher order oligomers (Li et al. 2015a). All these oligomers are able to bind ssRNA. N-RNA homology modeling and mutational analysis demonstrated that the positively charged and polar amino acids in the predicted surface cleft of TSWV N are important for RNA binding. The interaction of TSWV N protein with RNA can partially protect RNA from RNase digestion (Li et al. 2015a).

12.2.5 Suppression of RNA Silencing

RNA silencing is an innate immune system in plants to protect against virus infection. RNA silencing systems recognize the viral double stranded (ds) RNA and cleaves it into siRNA by Dicers. The siRNA is subsequently incorporated into RISC complexes, which target viral RNA sequences, under the guidance of siRNA. As a counter-defense, plant viruses encode specific viral proteins that suppress the RNA silencing. TSWV-specific siRNAs were recently characterized in *Nicotiana benthamiana* and tomato, using deep sequencing (Mitter et al. 2013). Using a GFP-based transient suppression assay, NSs protein from several tospoviruses was shown to suppress RNA silencing by binding to siRNAs and dsRNA (Schnettler et al. 2010). Both the N-terminal and C-terminal domains of NSs are important for RNA silencing suppression activity (de Ronde et al. 2014). In addition to RNA silencing suppression, NSs encoded by groundnut bud necrosis virus (GBNV) was also shown to have ATPase and helicase activity (Bhushan et al. 2015; Lokesh et al. 2010). ATP hydrolysis of GBNV NSs is essential for the helicase activity, whereas the helicase activity is not required for suppressing RNA silencing (Bhushan et al. 2015).

TSWV NSs was also found to enhance the replication of the nucleopolyhedrovirus (AcMNPV), a baculovirus, in Sf9 insect cells, suggesting that the suppressor activity of NSs also functions in insect systems (Oliveira et al. 2011). The critical roles of TSWV NSs in its native insect vector has recently been demonstrated by a mutant virus with a truncated NSs protein (Margaria et al. 2014). The truncated NSs mutant virus can be acquired by *F. occidentalis*, however, it does not demonstrate persistent infection in the insect vector and is no longer capable of transmission by *F. occidentalis*.

12.2.6 Transmission

Tospoviruses are transmitted by a number of thrips species (order *Thripidae*) in a propagative and persistent manner. Characterization of a mutation at site C1375A in the glycoprotein has identified the glycoproteins as the key determinants for thrip transmission (Sin et al. 2005). A soluble form of the glycoprotein Gn (Gn-S), which when expressed and purified in insect cells, was found to specifically bind to the larval thrip midgut. Feeding on the exogenous viral glycoprotein resulted in a significant reduction in virus transmission by thrips (Whitfield et al. 2008). Furthermore, expression of Gn-S protein in transgenic tomato plants interferes with TSWV acquisition and transmission by insect vectors (Montero-Astua et al. 2014).

TSWV is only acquired by larval thrips, and adults derived from such larvae can transmit the virus. Studies of the transmission pathway of TSWV within the body of *F. occidentalis* suggest that the epithelial cells of the midgut are the first organs where virus accumulates. The virus subsequently spreads to muscle tissues of the

midgut during larval stages. In adult thrips, infection is evident in visceral muscle tissues, followed by ligaments, and finally, the salivary glands (Kritzman et al. 2002). Despite the extensive replication of TSWV, there is little to no pathogenic effect on *F. occidentalis*. TSWV infection activates the immune system of *F. occidentalis* and this may protect it from any pathogenic effects caused by virus infection (Medeiros et al. 2004). Although there is little pathogenic effect on *F. occidentalis* by virus infection, TSWV infection may alter the feeding behavior of its insect vector. Male thrips infected with TSWV feed more than uninfected males, thus increasing the probability of virus inoculation (Stafford et al. 2011).

12.2.7 Plant Resistance Strategies

Naturally occurring host resistance genes, *Sw-5* and *Tsw*, respectively, confer resistance to tospoviruses in tomato and pepper. Both *Sw-5* and *Tsw* could be great natural sources for the breeding of resistant plants to viral diseases. However, resistance-breaking TSWV isolates have already emerged for both the *Sw-5* and *Tsw* resistance genes (Lopez et al. 2011; Margaria et al. 2007).

In addition to the use of natural host resistance genes, genetic engineering also provides a promising alternative strategy against tospoviruses. Transgenic plants that expressed fragments from several coding sequences, including the RdRP, Gn/Gc, NSm, N and NSs genes of TSWV, revealed that N or NSm transgenic plants provided resistance to TSWV (Prins et al. 1996). A strategy for combining a conserved region of the RdRP, a 5′ fragment of NSs, and an antisense fragment of N from watermelon silver mottle virus (WSMoV), provided resistance against both the Asian and American types of tospoviruses (Yazhisai et al. 2015).

12.3 Tenuiviruses

Tenuiviruses cause a number of diseases in most important food crops, including rice and maize (Falk and Tsai 1998). *Rice stripe virus* (RSV), *Maize stripe virus* (MSpV), *Rice grassy stunt virus* (RGSV) and *Rice hoja blanca virus* (RHBV), *Echinochloa hoja blanca virus* (EHBV), *Iranian wheat stripe virus* (IWSV) and *Urochloa hoja blanca virus* (UHBV), respectively, are recognized species in genus *Tenuivirus*. *Rice stripe virus* is the type species of this group.

Tenuiviruses share characteristics with the vertebrate-infecting viruses in the genus *Phlebovirus*, belonging to the family of *Bunyaviridae* (Falk and Tsai 1998). Both 5′ and 3′ termini sequences of the genomic RNA between tenuiviruses and phleboviruses are highly conserved. The amino acid sequences of RdRP, glycoproteins and nucleocapsid proteins all show significant similarity between the two groups of viruses, suggesting that they likely evolved from a common ancestor (Fig. 12.3).

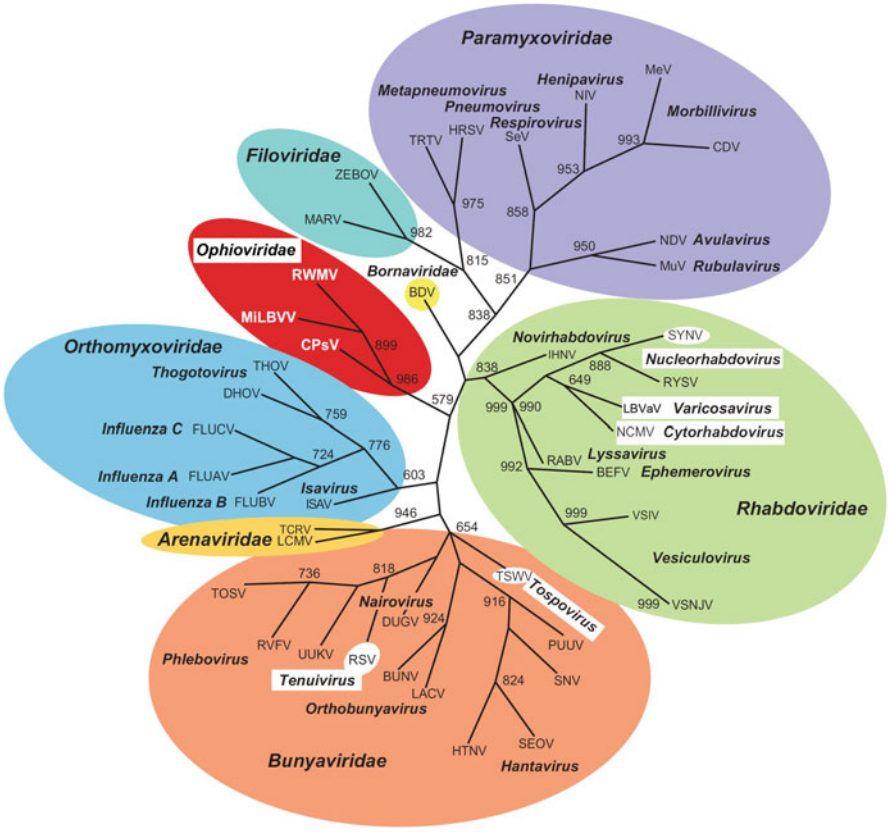

Fig. 12.3 Unrooted phylogenetic tree of the negative stranded RNA viruses based on their conserved RdRp modules. The figure was modified from Ophiovirus genus description in Virus Taxonomy. Oxford: Elsevier, 2011, pp. 743–748. The family and genera described in this article are highlighted. Viruses included in the analysis and the accession numbers used are: BDV [L27077], BEFV [AF234533], BUNV [X14383], CDV [NC_001921], CPsV [AY224663], DHOV [M65866], DUGV [U15018], FLUAV [J02151], FLUBV [M20170], FLUCV [M28060], HRSV [NC_001781], HTNV [X55901], IHNV [L40883], ISAV [AJ002475], LACV [U12396], LBVV [AB075039], LCMV [J04331], MARV [M92834], MEV [K01711], MiLV (MiLBVV) [AF525933], MuV [D10575], NCMV [NC_002251], NDV [X05399], NIV [AF212302], PUUV [M63194], RABV [M13215], RSV [D31879], RVFV [X56464], RWMV [AF335429], RYSV [NC_003746], SEOV [X56492], SeV [M19661], SNV [L37901], SYNV [L32603], TCRV [J04340], THOV [AF004985], TOSV [X68414], TRTV [U65312], TSWV [D10066], UUKV [D10759], VSIV [J02428], VSNJV [M20166], and ZEBOV [AF499101]

12.3.1 Particle Morphology and Genome Organization

Purified ribonucleoprotein particles of tenuiviruses appear as fine filamentous structures (Falk and Tsai 1998). The morphology of the particles resembles the RNPs of members from *Bunyaviridae*, but they are non-enveloped (Fig. 12.1b). The

ribonucleoprotein particles are composed of 4–6 ssRNAs, depending on the virus. RSV and RHBV have 4 segments of genomic RNAs. MSpV and EHBV have 5 segments, whereas RGSV contains 6 segments. RNA-1 is about 9 kb. RNA-2 is 3.3–4.1 kb. RNA-3 is 2.3–3.1 kb. RNA-4 is 2.2–2.9 kb. RNA-5 of EHBV is 1.3 kb. RNA-1, -2, -5 and -6 of RGSV are corresponding to RNA-1, -2, -3 and -4 of other tenuiviruses.

The genomic RNAs for tenuiviruses are either of negative or ambisense polarity (Fig. 12.2). RNA1 is negative sense and encodes an RdRp. RNA2 encodes a 22.8 kDa protein (NSs2) from the viral RNA (vRNA), and a putative glycoprotein (NSvc2) from the viral complementary RNA (vcRNA). RNA3 encodes a nonstructural protein (NS3) from the vRNA and a nucleocapsid protein (NSvc3) from the vcRNA. The NS3 proteins of RSV and of RHBV are suppressors of RNA silencing. RNA4 encodes a NS4 protein from the vRNA and an NSvc4 movement protein from the vcRNA. RNA5 of MSpV encodes an NS5 protein of unknown function from the vcRNA.

12.3.2 Replication–Transcription

A cell line from the small brown planthopper (SBPH; *Laodelphax striatellus*) was recently developed to study the replication of RSV (Ma et al. 2013). Characterization of the mRNAs of several tenuiviruses, including MSpV, RHBV and RSV, has revealed that their 5′ termini contain heterogeneous nucleotide sequences of 10–23 nucleotides (Huiet et al. 1993; Ramirez et al. 1995; Shimizu et al. 1996). The origin of these heterogeneous leader sequences is believed to have been generated by the cap snatching mechanism. Double infection of barley plants with MStV and barley stripe mosaic hordeivirus (BSMV) showed that heterologous BSMV RNAs could serve as primer donors for MStV mRNA transcription. The cleaved RNA leader has size range of 11–14 nt. Cucumber mosaic virus (CMV) RNAs were also found to serve as cap donors for RSV transcription initiation during co-infection of *N. benthamiana* (Yao et al. 2012). Interestingly, RSV can use repetitive prime-and-realignment to convert short capped CMV RNA leaders into longer sizes, which renders a more suitable and stabilized transcription complex for RSV mRNA transcription (Yao et al. 2012).

12.3.3 Cell-to-Cell Movement

NSvc4 encoded by RSV RNA4 was able to trans-complement the movement-deficient potato virus X (PVX) in *N. benthamiana* plants (Xiong et al. 2008). NSvc4 targets to plasmodesmata (PD) and moves cell-to-cell by itself, demonstrating that NSvc4 is the movement protein for RSV (Xiong et al. 2008). The plasmodesmata targeting of NSvc4 is dependent on the ER-to-Golgi early secretory

pathway. Cytoskeleton and myosin VIII-1 were also required for NSvc4 trafficking to PD (Yuan et al. 2011). A transmembrane domain, spanning the amino acids 106–123, in NSvc4 was required for targeting NSvc4 to PD (Rong et al. 2014). In addition to PD localization, NSvc4 was also localized in chloroplast. The N-terminal 73 amino acids of NSvc4 were essential for chloroplast localization (Xu and Zhou 2012).

The nonstructural protein NSvc6 encoded by RGSV corresponds functionally to the nonstructural RSV NSvc4 protein. Replacement of ToMV MP with RGSV NSvc6 will trans-complement the cell-to-cell spread of chimeric ToMV (Hiraguri et al. 2011).

12.3.4 Virus-Derived siRNA and Suppression of RNA Silencing

Small RNAs from RSV-infected *Oryza sativa, N. benthamiana* and the insect vector *Laodelphgax striatellus* were identified by deep sequencing (Xu et al. 2012). The number and size distributions of vsiRNAs from the three hosts were very different (Xu et al. 2012). Xiong et al. (2009) identified that NS3 encoded by RSV was a viral suppressor of RNA silencing (Xiong et al. 2009). Both the N and C terminals of the NS3 protein are essential for silencing suppressor activity. The NS3 protein binds 21-nucleotide siRNA, but not long double-stranded (ds)-RNA. The NS2 protein encoded by RSV also has weak silencing suppressor activity. This activity may be executed via OsSGS3 which interacts with NS2 (Du et al. 2011).

The NS3 protein encoded by RHBV is also an RNA silencing suppressor (Bucher et al. 2003). Interestingly, this protein not only suppresses RNA silencing in plants, but also in mammalian cells (Hemmes et al. 2007; Schnettler et al. 2008). RHBV NS3 is able to substitute the RNAi suppressor function of the Tat protein from the human immunodeficiency virus type 1, suggesting that it also work in mammalian systems (Schnettler et al. 2009). NS3 binds siRNA as well as miRNA molecules (Hemmes et al. 2007). Hemmes et al. (2009) further found that the binding of siRNA by NS3 protein was essential for its RNAi suppressor activity (Hemmes et al. 2009). Crystal structure determination of the N-terminal domain of RHBV NS3 revealed that it forms a dimer structure which binds RNA (Yang et al. 2011).

12.3.5 Insect Transmission

RSV is transmitted by the SBPH, *L. striatellus*, in a persistent-propagative manner. The virus is also transmitted vertically. Confocal analysis of transmission pathways

within the insect showed that RSV firstly infected the midgut epithelium, and then moved into the visceral muscle tissues, through which RSV spread to the entire alimentary canal. Finally, RSV entered into the salivary glands and reproductive system (Yin et al. 2014). Huo et al. (2014) recently reported a mechanism of vertical transmission in insect vectors. Nucleocapsids of RSV were able to bind vitellogenin (Vg) in *L. striatellus*. Vg can help the RSV nucleocapsid cores to migrate from terminal filaments and pedicel areas to the germarium and nurse cells. Knockdown of Vg expression severely inhibits the RSV invasion of ovarioles (Huo et al. 2014).

Xu et al. (2015) found that the ubiquitin/26S proteasome was activated during RSV infection in SBPH. Disrupting the 26S proteasome resulted in a rise of RSV accumulation in the SBPH. RSV NS3 was able to interact with the 26S proteasome subunit RPN3. Silencing of RPN3 resulted in higher accumulation of RSV in the insect vector. Consequently, viruliferous SBPH transmitted the virus more effectively (Xu et al. 2015). Li et al. (2015b) reported that RSV may exert an adverse effect on SBPH. RSV infection causes the hatchability of F1 progeny to decrease significantly. The development of some eggs may also be delayed by RSV infection (Li et al. 2015b).

12.3.6 Pathogenesis and Virus Resistance

RSV SP (NS4) was previously considered as a pathogenesis related protein during RSV infection. Expression of RSV NS4 protein in transgenic plants did not produce visible symptoms, however, symptoms were enhanced in NS4 transgenic plants when compared to wild-type plants following RSV infection (Kong et al. 2014). RSV SP interacts with a 23-kDa oxygen-evolving complex protein PsbP. Silencing of PsbP expression increases RSV symptoms and enhances viral accumulation (Kong et al. 2014).

Naturally resistant rice has been successfully used in controlling RSV disease during the last few decades in Japan and China. One of resistance genes, *STV11*, was recently mapped and cloned. It encodes a sulphotransferase and confers durable resistance to RSV (Wang et al. 2014). RNA interference (RNAi) is also another strategy that is used to generate transgenic rice that provide strong resistance against RSV. Zhou et al. (2012) have generated transgenic rice expressing RNAi constructs targeting all encoded viral proteins of RSV and demonstrated that RNAi constructs specifically targeting the nucleocapsid gene and viral movement protein, were immune to RSV infection (Zhou et al. 2012). When the same strategy was applied for RGSV, the transgenic rice containing RNAi constructs targeting the nucelocapsid protein or movement protein from RGSV also had strong resistance against RGSV infection (Shimizu et al. 2013).

12.4 Ophioviruses

Ophioviruses cause many agriculturally important diseases, such as citrus psorosis disease, big-vein disease and freesia leaf necrosis disease. The name ophiovirus comes from Greek, "ophis", which means snake, due to the snake-like appearance of the virions (Fig. 12.1c). The morphology of the ophiovirus virion resembles those of the tenuiviruses and the RNPs of members from the family *Bunyaviridae*, and do not have enveloped membrane structures (Chen et al. 2013; Ward 1993). The *Ophioviridae* family includes *Citrus psorosis virus* (CPsV), *Freesia sneak virus* (FSV), *Lettuce ring necrosis virus* (LRNV), *Mirafiori lettuce big-vein virus* (MiLBVV), *Ranunculus white mottle virus* (RWMV) and *Tulip mild mottle mosaic virus* (TMMMV). CPsV is the type species of this group.

12.4.1 Genome Structure

Ophioviruses are negative-strand RNA viruses with segmented genomes consisting of three or four RNAs. The 3′ terminus of genomic RNA is of inverted complementation to the 5′ terminus. RNA1 is 7.5–8.2 kb, RNA2 is 1.6–1.8 kb and RNA 3 is 1.3–1.5 kb. CPsV has three genomic RNAs, whereas MiLBVV and LRNV have a fourth RNA segment (RNA4). The negative sense of RNA 1 encodes a 24 kDa protein with movement function and a 280 kDa putative RdRP, separated by a 109-nt intergenic region. RNA2 encodes a polypeptide of 53.7 kDa. RNA3 of CPsV encodes a 48.6 kDa coat protein. The RNA 4 of LRNV encodes a 38 kDa protein, while that of MiLBVV potentially encodes two proteins due to an additional 10.6 kDa ORF overlapping the first ORF by 38 nt (Fig. 12.2).

12.4.2 Viral Movement

Using a microprojectile bombardment assay, a 55 kDa protein encoded by MiLBVV was shown to trans-complement intercellular movement of an MP-deficient ToMV. Subcellular localization analysis showed that the 55 kDa protein, fused with GFP, was localized to plasmodesmata in the epidermal cells of *N. benthamiana* and onion (Hiraguri et al. 2013). Robles Luna et al. (2013) reported that the 54 kDa proteins encoded by RNA2 of both CPsV and MiLBVV localize to plasmodesmata and enhance GFP cell-to-cell diffusion. Moreover, both proteins functionally trans-complement the cell-to-cell movement of movement-defective PVX and TMV mutants. The 54K proteins also interact with virus-specific CP in the cytoplasm, suggesting that this MP may facilitate the cell-to-cell movement of ophiovirus RNPs (Robles Luna et al. 2013).

12.4.3 Resistance

CPsV cause the psorosis disease of citrus trees in many countries. No sources of natural resistance have been identified for this virus. Virus-derived resistance provides an alternative to control viral diseases. Zanek et al. (2008) have generated transgenic sweet orange expressing the coat protein gene of CPsV, however, all transgenic lines showed symptoms of psorosis following virus challenge. Although the sweet orange transformant failed to protect against CPsV, it was found that the CP gene in the transgenic plant was methylated, suggesting that PTGS inhibited the production of a CP transcript (Zanek et al. 2008). Reyes et al. (2009) further generated transgenic *N. benthamiana* plants expressing hairpin RNA from the CP gene and 54K genes, respectively. Those lines expressing the CP gene of CPsV successfully conferred resistance against CPsV, showing that transgenic plants could confer resistance to CPsV within the context of serious citrus disease (Reyes et al. 2009).

12.5 Emaraviruses

Emaravirus is a new established genus. Phylogenetic analysis showed that emaraviruses have large similarity with TSWV. *European mountain ash ringspot-associated virus* (EMARV), *Fig mosaic virus* (FMV), *Pigeonpea sterility mosaic virus* (PPSMV), *Raspberry leaf blotch virus* (RLBV), *Rose rosette virus* (RRV) and *Maize red stripe virus* (MRSV) are now included in this genus. They are grouped in three different clusters (Elbeaino et al. 2013). EMARV is the type species in the genus *Emaravirus*. Emaraviruses cause severe economic losses in trees and fruits.

12.5.1 Particle Morphology and Genome Structure

Viruses in the genus *Emaravirus* have double-membraned particles (DMBs) with 80–200 nm diameter (Fig. 12.1d). DMBs resemble large tospoviral particles, therefore, emaraviruses were previously thought to be tospoviruses, but later study excluded this possibility. EMARV, FMV, RRV and PPSMV have spherical virions, but RLBV only has filamentous particles. DMBs of these viruses were detected in the cytoplasm, especially near the ER and Golgi cisterns, much like the virions of TSWV (Tatineni et al. 2014).

Emaraviruses have multipartite single-stranded negative RNA genomes (Fig. 12.2). They have different segments among different species. Each segment contains a single ORF. RNA1 encodes an RdRP. RNA2 encodes a putative

glycoprotein precursor. RNA3 encodes a putative nucleocapsid protein. RNA4 encodes a putative movement protein, while other genomic segments encode proteins with unknown functions. EMARaV has four RNA fragments, however, FMV has two additional RNA segments. The protein functions encoded by FMV RNA5 and RNA6 are not presently clear. PPSMV may have 5–8 RNA segments. Deep sequencing has identified PPSMV RNA 1–5 and confirmed its classification status in the genus *Emaravirus* (Elbeaino et al. 2014) . RRV has four RNA segments. Genome organization and RNA sequences of RRV show striking similarities to EMARaV and FMV. RLBV has five RNA filaments and the P5 encoded by RNA5 is unique to RLBV. Three genomic RNAs of MRSV have been identified, but more segments are likely present.

12.5.2 Transcription and Movement

FMV mRNA from genome segments 2 and 3 contain 12–18 nt length of heterogeneous nucleotide sequences at their 5′ termini. Using GST-tagged recombinant $eIF4E_{K119A}$, which has high affinity for cap binding, FMV mRNAs were confirmed as having a 5′ cap. The 5′ cap of FMV mRNAs were most likely to have been generated by cap snatching (Walia and Falk 2012).

Ishikawa et al. (2013) identified that the P4 protein of FMV is involved in cell-to-cell movement. P4 protein was localized to plasmodesmata (Ishikawa et al. 2013), indicating that it is a movement protein. Yu et al. (2013) found that RLBV P4 rescued the cell-to-cell movement of a defective PVX which had the triple gene block 1 deleted (Yu et al. 2013). Immunogold labeling electron microscopy revealed that the nucleocapsid protein of FMV forms agglomerates and localized in the ER. Nucleocapsid bodies moved rapidly within the ER in an actomyosin-dependent manner. This movement is dependent on the XI-1, XI-2, and XI-K myosins (Ishikawa et al. 2015).

12.5.3 Resistance

Natural resistance to PPSMV was investigated in Pigeonpea (Daspute et al. 2014). Comparison of resistant and susceptible genotypes revealed that inheritance of PPSMV resistance was controlled by two genes functioning through inhibitory gene interaction (Daspute et al. 2014). From a F2 mapping population developed by crossing Gullyal white and BSMR, a set of 32 DNA markers associated with the allele of a gene conferring resistance to PPSMV was identified in Pigeonpea (Daspute and Fakrudin 2015).

12.6 The Plant-Infecting Rhabdoviruses

Rhabdoviridae are taxonomically classified in the order *Mononegavirales*. Members in the family *Rhabdoviridae* collectively infect invertebrates, vertebrates and plants, including fish, humans, livestock and agricultural important crops (Jackson et al. 2005). Plant rhabdoviruses are classified into two genera, *Cytorhabdovirus* and *Nucleorhabdovirus*, primarily according to their sites of replication, morphogenesis, and virion maturation. *Cytorhabdovirus* has nine recognized species, with *Lettuce necrotic yellows virus* (LNYV) as the type species. *Nucleorhabdovirus* has ten recognized species. *Potato yellow dwarf virus* (PYDV) is the type species, while *Sonchus yellow net virus* (SYNV) is the best-studied species in this genus. Additionally, there are more than 90 tentative species in the plant rhabdoviruses. Plant rhabdoviruses infect a wide variety of host species across both monocot and dicot families. Many of them infect agriculturally important crops including lettuce, wheat, barley, rice, maize, potato and tomato.

12.6.1 Virus Structure and Genomic Organization

Rhabdoviruses are negative-strand RNA viruses with a bilayer lipid envelope. The basic virion structure and morphology of plant rhabdoviruses is similar to that of vertebrate rhabdoviruses. The virions are bullet-shaped particles of approximately 100–430 nm length and 45–100 nm diameter (Fig. 12.1e). Glycoproteins (G) form spikes extending from the surface of the lipid membrane. Inside the envelope is a helical ribonucleoprotein core consisting of negative-sense, single-stranded RNA wrapped by nucleoprotein. The ribonucleoprotein core is associated with smaller amounts of two other proteins: the phosphoprotein (P) and the large protein (L). Matrix protein (M) forms an inner layer under the helical ribonucleoprotein.

The genome of rhabdoviruses is approximately 11–16 kb in length with a leader sequence at 3′ end and a trailer sequence at 5′ end (Fig. 12.2). Plant rhabdoviruses genomes are somewhat larger than most of vertebrate rhabdovirus genomes and encode homologs of the five structural rhabdoviral genes found in the prototypical vesicular stomatitis virus (VSV) and rabies virus (RABV) genomes which are organized in the conserved order of 3′-N-P-M-G-L-5′ (Jackson et al. 2005). The N protein is the major component of the ribonucleoprotein core and associates with negative and positive sense genomic RNAs, but not mRNAs. The L protein associates with the ribonucleoprotein core and plays essential roles in transcription and replication. The P protein is a cofactor of the viral polymerase. The M protein is an inner component of the virion. The G protein trimerizes to form virus surface peplomers. Plant rhabdovirus genomes also encode diverse accessory genes between N-P, P-M and/or G-L genes. These proteins are likely to have functions associated with enhancing replication efficiency, blocking host innate immune defenses, and allowing effective cell-to-cell transportation (Walker et al. 2011).

12.6.2 Replication and Transcription

Replication and transcription of rhabdoviruses is similar across different viruses in the family. After rhabdoviruses enter cells, the nucleocapsid core is released by fusing viral membranes with the endosome and polymerase recognizes the 3′ end of the negative-strand genome. The virus firstly transcribes the leader RNA, and subsequently transcribes each gene from 3′ to 5′ with progressively decreasing yield. The transcription is initiated with a specific consensus sequence and terminated at each intergenic region by iterative slippage on a poly-U tract that generates the poly-A tail. The transcripts are capped and methylated at their 5′ end and polyadenylated at their 3′ end by the L protein. Following the accumulation of sufficient N proteins, the viral polymerase complex switches from transcription to replication. During replication, the nascent genomic RNA is immediately encapsidated by the N protein to form the nucleocapsid complex.

Cytorhabdoviruses replicate in the cytoplasm of plant cells. Plant cytorhabdoviruses first localize to the ER to induce viroplasms. The replication of viral genomic RNA and assembly of nucleocapsid complexes occurs within the viroplasm. When RNPs are forming, G protein in the plasma membrane localizes to sites that are favorable for budding initiation and RNP condensation by M protein. The condensation of RNPs occurs at regions of the plasma membrane via interactions with M protein which contain locally high concentrations of G protein, and this results in formation of bud sites. The enveloped virions buds into the cytoplasm (Mann and Dietzgen 2014).

Plant nucleorhabdoviruses replicate in the nuclei. Nucleorhabdoviruses import the nucleocapsid complex into the nucleus. The RdRP transcribes viral mRNAs followed by exportation into the cytoplasm for translation. The N/P/L proteins are then imported into the nucleus to form viroplasms. In the later stage of replication, the nucleocapsid core forms a complex with matrix protein and accumulates at the site of G protein on the inner nuclear envelope membranes. Maturation of virions occurs by budding through inner nuclear envelope membranes into perinuclear spaces. A mini-replication reverse genetic system has recently been established for SYNV, providing a powerful platform to study nucleocapsid assembly, transcription and replication (Ganesan et al. 2013).

12.6.3 Cell-to-Cell Movement

Scholthof et al. (1994) reported that sc4 encoded by SYNV, the predicted movement protein, is a membrane associated protein (Scholthof et al. 1994). Goodin et al. (2007) showed that sc4 accumulated primarily at punctate loci on the periphery of cells (Goodin et al. 2007). Potato yellow dwarf virus (PYDV) Y protein was also found to target plasmodesmata (Bandyopadhyay et al. 2010), whereas lettuce necrotic yellows virus (LNYV) 4b and maize fine streak virus

(MFSV) P4 proteins localized to both plasmodesmata and the nucleus (Martin et al. 2012; Tsai et al. 2005). Ectopically expressed rice yellow stunt virus (RYSV) P3 was shown to complement the cell-to-cell movement of a movement-defective PVX mutant in *N. benthamiana* and bind to ssRNA *in vitro*. RYSV P3 can also bind specifically to the nuclecapsid protein and, thus, it may facilitate the movement of RYSV RNPs (Huang et al. 2005). High-resolution protein interaction screens have been conducted to identify host factors involved in the cell-to-cell movement of SYNV. Yeast two hybrid screening identified the motor protein sc4i21 as the sc4 interactor, which localizes on microtubules. Screening also identified the nucleocapsid interactor Ni67, which localized on the endoplasmic reticulum. Two host proteins are homologues of transcription activators. This data fits into a model in which transcription activators tethered in the cytoplasm aid the nucleocapsids of SYNV that are transported from the nucleus and move from cell-to-cell (Min et al. 2010).

12.6.4 Suppression of RNA Silencing by Plant Rhabdoviruses

Two nucleorhabdovirus proteins (SYNV P protein and RYSV P6 protein) and one cytorhabdovirus protein (LNYV P protein) were shown to have RNA silencing suppressor activity. The SYNV P could maintain 16c transgenic GFP expression when co-expressed with the GFP silencing suppression construct (Jackson et al. 2005). RYSV P6 protein enhances the virulence of PVX, represses the production of secondary siRNAs and inhibits systemic GFP RNA silencing. Suppression of secondary siRNA production is probably achieved through an interaction with RDR6 accumulation (Guo et al. 2013). LNYV P protein has relatively weak RNA silencing suppression activity and delays systemic silencing of GFP in plants, however, P protein does not have RNA silencing suppression activity in insect cells (Mann et al. 2015).

12.6.5 Transmission

Plant rhabdoviruses are transmitted by aphids, leafhoppers or delphacid planthoppers (Hogenhout et al. 2003). The viruses replicate in both plants and their insect vectors. Rhabdoviruses systemically infect insect hosts, replicating in nearly all organs and transmit in a persistent manner. Specificity of vector transmission is determined by recognition events between gut cell receptors and rhabdovirus G proteins (Ammar et al. 2009). The route of infection begins with virus entry into the midgut, the first barrier to acquisition. To be transmitted, rhabdoviruses must reach salivary glands via the hemolymph, nervous system, or other

routes. Successful rhabdovirus transmission ultimately depends on virus exiting the salivary glands (Ammar et al. 2009).

12.7 Varicosavirus

Varicosavirus is two-segmented, negative-sense, single-stranded RNA virus and is distantly related to members in the family of *Rhabdoviridae* (Kormelink et al. 2011). The virion is rod-shaped with a particle length of 320–360 nm and is 18 nm in diameter (Fig. 12.1f). Virion particles consist of a coat protein only and do not contain an envelope, resembling the inner nucleocapsid core of rhabdoviruses. *Lettuce big-vein associated virus* (LBVaV), previously designated *Lettuce big-vein virus* (LBVV), is the only confirmed species in the genus *Varicosavirus*.

LBVaV has two RNA segments. The larger genome segment (RNA1) is 6.8 kb in length and the smaller genome segment (RNA2) is 6.1 kb (Fig. 12.2). The genome structure of LBVaV is similar to that of rhabdoviruses. The 3′- and 5-′-terminal sequences of the two RNAs are similar, but do not exhibit inverse complementarities. RNA1 encodes a 5 kDa protein and a 232 kDa putative RdRP. RNA2 contains five major ORFs. The first ORF at the 3′ end of the viral RNA encodes the coat protein (44.5 kDa) and the second to the fifth ORFs code for proteins with unknown functions (36, 32, 19 and 41 kDa respectively). The CP, but not the other four proteins, shares significant sequence similarity to the nucleocapsid protein of rhabdoviruses.

The LBVaV genome has transcription termination/polyadenylation and initiation signal sequences at gene-junction regions, similar to those in rhabdoviruses, especially the plant-infecting cytorhabdoviruses. The capped and polyadenylated monocistronic mRNAs are transcribed from individual LBVaV genes. It is possible that varicosaviruses and rhabdoviruses use a similar mechanism to differentially express individual genes from a contiguous virus genome.

12.8 Conclusion

Much progress has been made in understanding the replication, transcription, particle morphogenesis, movement, suppression of RNA silencing and vector transmission for the negative-stranded plant RNA viruses in recent years. However, compared to the positive-stranded RNA viruses, much more effort is needed to further understand the molecular mechanisms of different aspects in viral life cycle for negative-stranded plant RNA viruses. A reverse genetic system hasn't yet been established for any of negative-strand plant viruses. Although a mini-replicon system has been developed for SYNV, the full length infectious clones for non-segmented or segmented plant viruses still need to be established in the future. These reverse genetic systems will provide powerful platforms to further

understand molecular mechanisms of the negative-stranded RNA viruses infecting plants. Further studies will help us to develop a better understanding of how the negative-strand plant viruses have evolved through acquisition of movement protein to facilitate their cell-to-cell and long distance movement through plasmodesmata in plants, and by obtaining viral RSS proteins to overcome the innate immune systems in plants and insects. In these future studies, more efforts will need to focus on the virus-host interactions that facilitate viral replication, transcription etc.

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