

Aiming Wang · Xueping Zhou *Editors*

Current Research Topics in Plant Virology

 Springer

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Preface

Viruses are submicroscopic, obligate intracellular parasites that infect all living organisms and exclusively live and multiply in their host cells. Since the discovery of the first virus tobacco mosaic virus in 1890s, over 5000 virus species have been documented in detail. Viral pathogens virtually infect all living organisms and cause significant losses including mortality, morbidity, and economic losses. In plants, viruses cause many economically important diseases in all parts of the world. Virus infection can reduce crop yields drastically and deteriorate crop quality. In some cases, viral pathogens become a limited factor to crop production. Genetic resistance is the most effective approach to the control of viral diseases. However, natural resistant resources are rare, and, if any, usually only confer resistance to a particular virus species or group of highly related species. Development of novel antiviral strategies relies on knowledge in plant virology research. In the past decade, various breakthroughs have led to the rapid advance of this subject.

This book is aimed to reviewing the advances of major aspects in plant virology. We were fortunately able to recruit over a dozen of international authorities to write this book. Topics covered in this book include RNA silencing and its suppression in plant virus infection (Chap. 1), virus replication mechanisms (Chap. 2), the association of cellular membranes with virus replication and movement (Chap. 3), plant genetic resistance to viruses (Chap. 4), viral cell-to-cell and long distance movement in plants (Chaps. 5 and 6), virus-induced ER stress (Chap. 7), virus diversity and evolution (Chap. 8), virus-vector interactions (Chap. 9), cross protection (Chap. 10), geminiviruses (Chap. 11), negative strand RNA viruses (Chap. 12), and viroids (Chap. 13). As next generation sequencing is revolutionizing the diagnosis of plant viral diseases, the last chapter of this book is specifically dedicated to this topic.

Both the editors are extremely grateful to all the authors for accepting their invitation and making valuable contributions to this book. The editors would like to

thank their families, friends, and colleagues for their encouragement and support, which is essential for the completion of this book. Finally, both the editors wish to express their sincere appreciation to Jacco Flipsen, Mariska van der Stigchel, and the other staff at Springer for their strong support and excellent professionalism during the publication of this book.

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Xueping Zhou

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Editors

Aiming Wang completed his Ph.D. in plant molecular biology and virology from the University of British Columbia in 1999. He is currently Senior Research Scientist at the London Research and Development Centre, Agriculture and Agri-Food Canada (AAFC), and Adjunct Professor of Department of Biology, Western University. Dr. Wang has worked for more than 20 years in the field of crop genetics/breeding and plant virology. He has been the principal investigator of many funded studies. His current research interests include molecular virus-plant interactions, plant virus replication mechanism, cross-protection, novel antiviral strategies, plant biotechnology, and beneficial uses of plant viruses. Dr. Wang has edited four books, and authored or co-authored 22 book chapters, 80 peer-reviewed papers, and over 150 other articles. He is the recipient of various awards, such as a Queen Elizabeth II Diamond Jubilee Medal from Government of Canada in 2013 and a Gold Harvest Award from AAFC in 2010. He has been instrumental in developing novel strategies against potyviruses. Dr. Wang currently serves as associate editor or editorial board member of several scientific journals including *PLoS Pathogens*, *Scientific Reports*, *Journal of Virology*, *Molecular Plant Pathology*, *Molecular Plant-Microbe Interactions*, *Virology*, and *Virology Journal*.

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Chapter 1

Antiviral Silencing and Suppression of Gene Silencing in Plants

Tibor Csorba and József Burgyán

Abstract RNA silencing is an evolutionary conserved sequence-specific gene inactivation mechanism that contributes to the control of development, maintains heterochromatin, acts in stress responses, DNA repair and defends against invading nucleic acids like transposons and viruses. In plants RNA silencing functions as one of the main immune systems. RNA silencing process involves the small RNAs and trans factor components like Dicers, Argonautes and RNA-dependent RNA polymerases. To deal with host antiviral silencing responses viruses evolved mechanisms to avoid or counteract this, most notably through expression of viral suppressors of RNA silencing. Due to the overlap between endogenous and antiviral silencing pathways while blocking antiviral pathways viruses also impact endogenous silencing processes. Here we provide an overview of antiviral silencing pathway, host factors implicated in it and the crosstalk between antiviral and endogenous branches of silencing. We summarize the current status of knowledge about the viral counter-defense strategies acting at various steps during virus infection in plants with the focus on representative, well studied silencing suppressor proteins. Finally we discuss future challenges of the antiviral silencing and counter-defense research field.

Keywords RNA silencing • Virus infection • Antiviral defense • Silencing suppressor strategies • Host-pathogen interaction

1.1 RNA Silencing

1.1.1 Introduction

RNA silencing is a sequence-specific gene-inactivation mechanism conserved from lower eukaryotes to mammals (Shabalina and Koonin 2008; Weiberg and Jin 2015). RNA silencing, also known as RNA interference (RNAi), has diverse functions

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including growth and developmental regulation, DNA repair, biotic and abiotic stress response or host immunity against invading nucleic acids like transposons or viruses (Castel and Martienssen 2013; Martinez de Alba et al. 2013; Pumplin and Voinnet 2013). The trademark molecules of silencing are the small RNAs (sRNAs) (Hamilton and Baulcombe 1999) of 21–24 nt length. These guide the sequence-specific effector steps either at transcriptional or at post-transcriptional levels. During transcriptional gene silencing (TGS) target genes are inhibited by epigenetic modification of chromatin (e.g histone protein post-translational modifications and DNA methylation) (Castel and Martienssen 2013) while during post-transcriptional gene silencing (PTGS) gene inactivation occurs through mRNA cleavage or translational repression (Martinez de Alba et al. 2013). Depending on the sRNA type and effector proteins involved, silencing pathways provide diverse and dedicated functions.

1.1.2 Biochemical Framework of Silencing

RNA silencing process can be partitioned mechanistically into three distinct phases: initiation phase, effector phase and in some specific circumstances amplification phase. Most of the knowledge comes from the model plant *Arabidopsis thaliana*, therefore the nomenclature of components relies on these components.

1.1.2.1 Initiation of Silencing

Initiation of silencing comprises of two main steps: biogenesis of sRNAs and their loading into effector complexes. The trigger of silencing initiation is always a double-stranded RNA (dsRNA) molecule present within the cell: perfect or imperfect dsRNA structures can be formed by single-stranded RNA (ssRNA) transcripts folding into a hairpin secondary structure, may come from the inter-molecular interaction of two partially reverse complementary single-stranded RNAs (ssRNAs) produced either by convergent transcription from the sense and antisense strands of the loci (in cis) or by pairing of homologue regions of transcripts originating from different loci (in trans). Alternatively, perfectly complementary dsRNAs may arise as the product of RNA-dependent RNA polymerases by conversion of ssRNA molecules into dsRNAs. The dsRNA molecules/regions are recognized by a member of the RNase III type enzyme family DICERS, in plants named DICER-LIKE proteins, (DCLs, in *Arabidopsis* DCL1-4) (Bernstein et al. 2001; Hamilton and Baulcombe 1999; Hutvagner et al. 2001). DCLs contain a helicase, a PAZ, two RNase-III and two dsRNA-binding domains. The PAZ and RNA-binding domains position the dsRNA substrate in such a way that the two RNase-III pseudo-dimers catalyzes processing of the dsRNA molecules/regions into sRNA duplexes of 21–24 nt length, with specific 2-nt-long 3' overhangs (having 5'-P and 3'-OH ends).

For the accurate and effective excision of sRNAs from their precursor molecules DCLs require cooperation of DOUBLE-STRANDED RNA BINDING proteins (DRB, in *Arabidopsis* DRB1-5). Sometimes specific DCL-DRB interaction is required for the transfer of sRNA duplex into specific effector complexes (Eamens et al. 2012a, b; Han et al. 2004; Hiraguri et al. 2005). Following processing, the sRNAs are stabilized at their 3' end by the HUA Enhancer 1 (HEN1)-dependent 2'-O-methylation (a process found only in plants and flies so far) (Boutet et al. 2003; Yang et al. 2006) and exported from the nucleus to the cytoplasm by HASTY (HST), the homologue of mammalian exportin-5 (Bollman et al. 2003; Park et al. 2005; Peragine et al. 2004) to be loaded into effector complexes. It is believed that methylation may occur both in the nucleus and cytoplasm (Lozsa et al. 2008).

1.1.2.2 Effector Phase of Silencing

The essential catalytic components of effector complexes of silencing are the Argonaute proteins (AGOs, in *Arabidopsis* AGO1-10), RNase-H type endonucleases (Fagard et al. 2000; Hammond et al. 2001; Hutvagner and Simard 2008; Liu et al. 2004; Mallory and Vaucheret 2010). AGOs together with accessory proteins form the effector complex of silencing: the RNA-Induced Silencing Complex (RISC) that acts during PTGS (Lee et al. 2004; Pham et al. 2004; Tomari et al. 2004), or the RNA-Induced Transcriptional Silencing Complex (RITSC) that acts during TGS (Castel and Martienssen 2013; Ekwall 2004). RISC/RITSC assembly comprises of two clearly distinguishable steps: (i) loading of ds-sRNAs and (ii) unwinding of sRNAs (Kwak and Tomari 2012). Biogenesis and loading of ds-sRNAs seems to be coupled (at least in the case of miRNAs) (Reis et al. 2015). AGO-loading process requires Hsp70-Hsp90 chaperone machinery and ATP hydrolysis to drive AGO conformational changes. The size and the 5' nucleotide type contributes to the sorting of sRNAs into specific AGO partners (e.g 21-nt-long 5' U sRNAs are preferentially loaded into AGO1 etc.) (Mallory and Vaucheret 2010). The strand having less stable 5'-end pairing (within the ds-sRNA molecule) is retained within the AGO while the other, the so-called "star" strand is eliminated (Khvorova et al. 2003; Schwarz et al. 2003). Guided by the sRNA sequence, RISC induces slicing or translational repression of its target RNAs (during PTGS) in a sequence-specific manner (Brodersen and Voinnet 2009; Kim et al. 2014). The cleavage products of RISC are eliminated by the general mRNA decay and quality control machinery present within the cell (Martinez de Alba et al. 2015; Parent et al. 2015b; Ren et al. 2014; Souret et al. 2004; Yu et al. 2015). RITSC complex causes histone and/or DNA methylation, resulting in transcriptional gene silencing (TGS) of the homologous gene (Castel and Martienssen 2013; Creamer and Partridge 2011). AGO1, 2, 3, 5, 7 and 10 have roles in PTGS while AGO4, 6 and 9 are involved in TGS (AGO8 is considered as pseudo-gene) (Mallory and Vaucheret 2010).

1.1.2.3 Amplification of Silencing

Cytoplasmic RNA silencing may be activated also by the presence of RNAs having aberrant features (without CAP-structure, lacking polyA tail etc.) or endonucleolytically cleaved RISC fragments. RNA-DEPENDENT RNA POLYMERASES (RDRs, in *Arabidopsis* RDR1, 2, 3a, 3b, 3c and 6) (Wassenegger and Krczal 2006) protein recognize these molecules as their substrates and convert them into dsRNAs that enter/re-enter into silencing through DCL-mediated sRNA production. RDR6 is the main cytoplasmic enzyme to be involved in this process (Branscheid et al. 2015; Martinez de Alba et al. 2015; Mourrain et al. 2000; Parent et al. 2015b; Sijen et al. 2001; Vaistij et al. 2002; Voinnet et al. 1998). Usage of RISC cleavage products by RDRs results in amplification of silencing response that may have also non-cell-autonomous consequences.

AGO-mediated target cleavage and amplification by RDR enzymes are intimately linked in the nuclear TGS as well. RNA polymerase IV (PolIV, a plant specific polymerase) transcribes short precursor ssRNAs from loci to be silenced. RDR2 physically associates with PolIV to convert its transcripts into dsRNA. DCL3 cleaves the dsRNA to produce sRNAs that are loaded mainly into AGO4 (alternatively AGO6 or 9). AGO4 associates with accessory proteins to form RITS. Guided by the sRNA, RITS is tethered to nascent transcripts synthesized by RNA polymerase V (PolV) and induce silencing of the target loci by recruiting histone and/or DNA modification complexes (Castel and Martienssen 2013).

1.1.3 Endogenous Pathway Diversification

The combined activities of specific (sometimes partially redundant) trans factors of silencing (DCLs, DRBs, AGOs and RDRs) and the involvement of different sRNA precursor molecules result in parallel gene silencing pathways (Bologna and Voinnet 2014; Hiraguri et al. 2005; Mallory and Vaucheret 2010; Wassenegger and Krczal 2006). These pathways rely on various sRNAs like microRNAs (miRNAs), trans-acting small interfering RNAs (ta-siRNAs), natural-antisense RNAs (nat-siRNAs), repeat-associated siRNAs (ra-siRNAs), viral siRNAs (vsiRNAs) and virus-activated siRNAs (vasiRNAs) and provide dedicated functions/roles of silencing (Martinez de Alba et al. 2013).

1.1.4 Systemic Silencing

Amplification of RNA silencing has been implicated in the spread of an RNA silencing signal (Kalantidis et al. 2008; Molnar et al. 2010, 2011; Schwach et al. 2005). Small RNAs of 21–24 nt lengths generated during cell-autonomous

RNA silencing spread from the site of initiation to the neighboring cells through plasmodesmata. Besides this, silencing signal is able to spread systemically over long distances through the phloem. The exact nature of silencing signal is not clear, although sRNAs are known to be involved; sRNAs may be associated with proteins (e.g. AGOs) during translocation that could protect them against cellular nucleases. Mobile sRNAs, similarly to their cell-autonomous counterparts, are able to trigger transcriptional or post-transcriptional silencing. It was shown that silencing signal movement has roles in the formation of patterns within a tissue (e.g. leaf polarity) (Chitwood et al. 2009), contributes to the reinforcement of transposon silencing in generative cells (Borges et al. 2011; Slotkin et al. 2009), initiate epigenetic events during genome defense (Cui and Cao 2014) and respond to external stimuli (Katiyar-Agarwal et al. 2006). Silencing signal movement has also important implication in antiviral defense and plant recovery (Havelda et al. 2003; Szittyta et al. 2002).

1.2 Antiviral Roles of RNA Silencing

1.2.1 Introduction

The antiviral function of RNA silencing was demonstrated in plants and invertebrates (Bronkhorst and van Rij 2014; Pumplin and Voinnet 2013). Recent reports have provided evidence that antiviral silencing also operates in mammals, especially in ESC cells, however its role still remains controversial (Castel and Martienssen 2013; Cullen et al. 2013; Maillard et al. 2013). Is it believed that the ancient function of silencing was the antiviral defense itself (Pumplin and Voinnet 2013; Wang and Metzloff 2005). Specific members of DCL's, DRB's, AGO's, and RDR's contribute to the antiviral pathway during the various host-virus combinations (see Table 1.1 and relevant references within).

1.2.2 Biogenesis of vsRNAs

As one of the first sRNA type discovered, the existence of vsRNAs provided the first hint that silencing may have antiviral roles (Hamilton and Baulcombe 1999). Biogenesis of vsRNAs requires DCL enzymes. Viral substrate molecules for DCLs vary depending on the virus replication strategy. In case of RNA viruses the highly structured fold-back regions of viral single-stranded RNAs (ssRNA) and replicative intermediates (RI) may be the primary source of vsRNA production (Ahlquist 2002; Donaire et al. 2009; Molnar et al. 2005; Szittyta et al. 2010; Kontra et al. unpublished). In case of DNA viruses the overlapping convergent/bidirectional read-through transcripts or fold-back structure of specific regions of RNA

Table 1.1 Plant silencing components with antiviral activity

	Virus	Host	DCLs	DRBs	RDRs	AGOs	References
ssRNA (+)	BMV	Arabidopsis thaliana	DCL2, 4		RDR6	AGO1	Dzianott et al. (2012)
	CMV	Arabidopsis thaliana	DCL1, 2, 3, 4		RDR1, 6	AGO1, 2, 4, 5	Bouche et al. (2006), Diaz-Pendon et al. (2007), Wang et al. (2010, 2011), Morel et al. (2002), Harvey et al. (2011), Takeda et al. (2008), and Hamera et al. (2012)
	CymRSV	Nicotiana benthamiana				AGO1, 2	Scholthof et al. (2011) and Kontra et al. (submitted)
	PVX	A. thaliana, N. benthamiana	DCL2, 4		RDR6	AGO2, 4, 5	Schwach et al. (2005), Bouche et al. (2006), Andika et al. (2015), Brosseau and Moffett (2015), Fatiol et al. (2016), and Bhattacharjee et al. (2009)
	RYMV	Oryza sativa	DCL4				Lacombe et al. (2010)
	TBSV	Nicotiana benthamiana				AGO1, 2	Scholthof et al. (2011), Odokonyero et al. (2015), and Schuck et al. (2013)
	TCV	Arabidopsis thaliana	DCL2, 4	DRB4		AGO1, 2, 7	Qu et al. (2008), Harvey et al. (2011), and Zhang et al. (2012);
	TMV	Arabidopsis thaliana			RDR1, 6		Qi et al. (2009)
	ToRSV	Nicotiana benthamiana				AGO1	Ghoshal and Sanfacon (2014)
	TRV	Arabidopsis thaliana	DCL 2, 3, 4		RDR1, 2, 6	AGO2, 4	Fusaro et al. (2006), Donaire et al. (2008), and Ma et al. (2015)
	TuMV	Arabidopsis thaliana	DCL2, 4		RDR1, 2, 6	AGO1, 2, 5, 7, 10	Garcia-Ruiz et al. (2010, 2015) and Carbonell et al. (2012)
	TYMV	Arabidopsis thaliana	DCL4	DRB4			Jacubiec et al. (2012)
	RSV	Oryza sativa			RDR6	OsAGO1, 18	Jiang et al. (2012) and Wu et al. (2015)
	TSWV	Arabidopsis thaliana	DCL4	DRB4			Curtin et al. (2008)

ssRNA
(-)

dsRNA	OsEV	Oryza sativa	DCL2, 4						Urayama et al. (2010)
	RDV	Oryza sativa			RDR6			OsAGO1, 18	Hong et al. (2015) and Wu et al. (2015)
ssDNA	BCTV	Arabidopsis thaliana	DCL3	DRB3				AGO4	Raja et al. (2014)
	CaLCuV	Arabidopsis thaliana	DCL1, 2, 3, 4	DRB3	RDR1,2,6-indep.			AGO4	Blevins et al. (2006), Aregger et al. (2012), and Raja et al. (2014)
	TYLCV	Nicotiana benthamiana	DCL2, 3						Akbergenov et al. (2006)
dsDNA	CaMV	Arabidopsis thaliana	DCL1, 2, 3, 4	DRB4	RDR1,2,6-indep.			AGO1, 4	Blevins et al. (2006, 2011) and Raja et al. (2014)

Specific plant DCLs, DRBs, RDRs, and AGOs known to contribute to the antiviral pathway in various host-virus combinations. For virus name abbreviations please see the glossary

transcripts contribute to vsiRNA biogenesis (Akbergenov et al. 2006; Aregger et al. 2012; Blevins et al. 2006, 2011; ; Chellappan et al. 2004) (Fig. 1.1). Genetic studies and deep sequencing analysis of vsiRNAs involving *Arabidopsis dcl* mutants revealed that a strong hierarchy exists between DCLs regarding their contribution to vsiRNA production. The main DCL in case of RNA virus infections is the DCL4 while DCL2 becomes critical in its absence (in *dcl4* mutant) (Andika

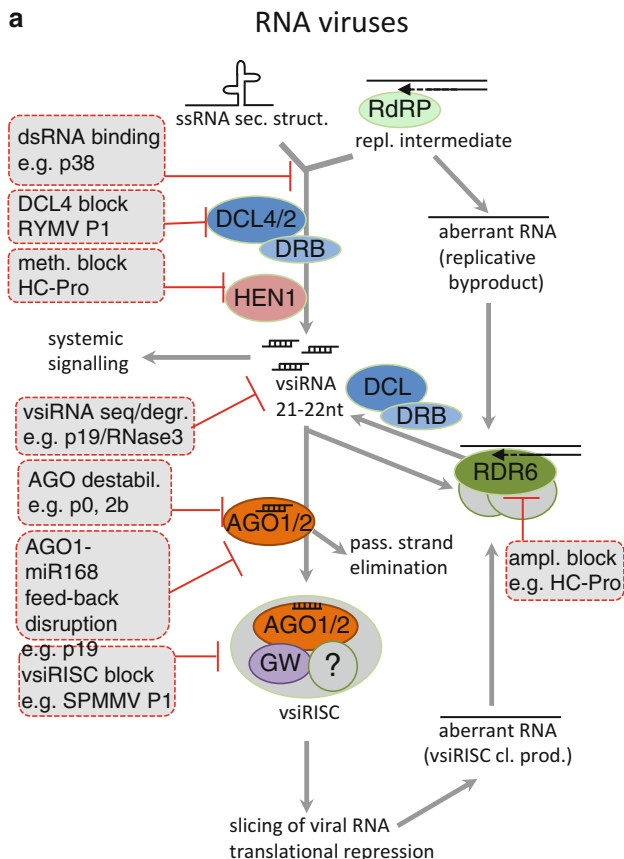


Fig. 1.1 Biochemical framework of antiviral RNA silencing and its suppression by VSRs. Antiviral RNA silencing is initiated by the recognition of viral dsRNA structures (replicative intermediate, partially double-stranded fold-back structures or overlapping RNA transcript pairing), which are processed into viral siRNAs (vsiRNAs) by Dicer-like proteins (DCLs). Subsequently vsiRNAs 21–22 nt or 24 nt long are incorporated into effector complexes RNA-induced silencing complex (RISC) or RNA-Induced transcriptional Silencing Complex (RITSC), respectively. *Question mark* represents unknown cofactors. The vsiRISC targets viral RNAs by slicing or translational inhibition (a), while RITSC induces genome modification (b). Cleavage products and vsiRNA may enter an amplification loop through the actions of RNA-dependent RNA polymerases (RDRs) and cofactors (SGS3 and SDE5) to give rise of secondary vsiRNAs. Antiviral silencing pathway may be halted at various points by viral silencing suppressors (VSRs) (a and b) (dash-line boxes)

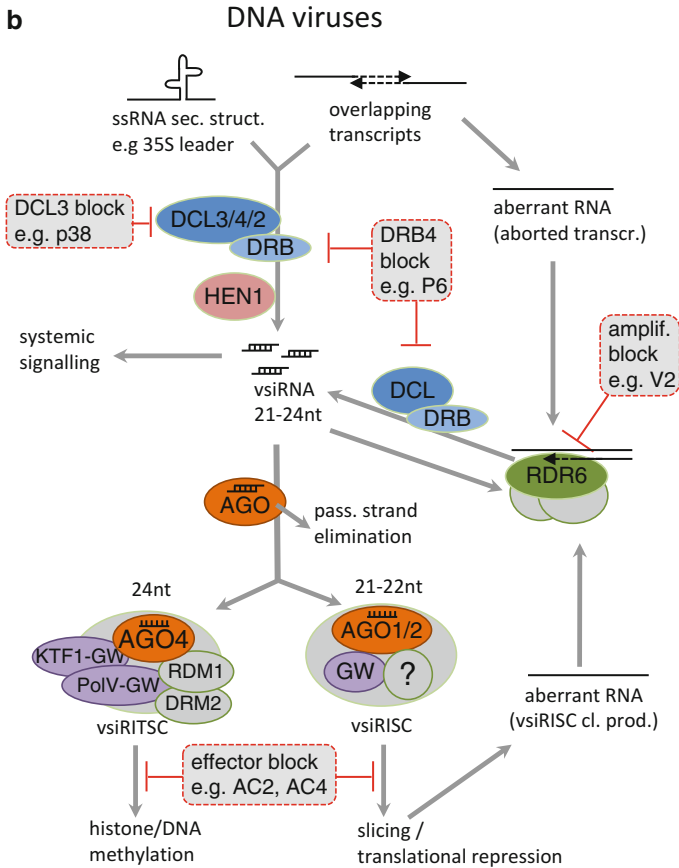


Fig. 1.1 (continued)

et al. 2015; Deleris et al. 2006; Donaire et al. 2009; Dzianott et al. 2012; Garcia-Ruiz et al. 2010; Qu et al. 2008; Urayama et al. 2010). Additional functional diversity between DCL4 and DCL2 has been reported: DCL2 stimulates transitivity and secondary siRNA production, while DCL4 is sufficient for silencing on its own (Parent et al. 2015a). DCL3 has only a minor role against RNA viruses (Qu et al. 2008; Raja et al. 2014). The fact that silencing suppressors of RNA viruses interfere with DCL3 pathway suggests that DCL3 contributes to antiviral silencing (Azevedo et al. 2010; Hamera et al. 2012; Lacombe et al. 2010). During antiviral silencing against DNA viruses DCL3 is essential and works presumably by inducing chromatin modifications (Akbergenov et al. 2006; Blevins et al. 2006; Raja et al. 2014). DCL1 may act as a negative regulator limiting DCL4 and DCL3 through miRNA pathway (Azevedo et al. 2010; Qu et al. 2008).

DCLs' cofactors, the DRB proteins are also required for vasiRNA biogenesis. DRB4, the cofactor of DCL4, takes part in antiviral defense against RNA viruses (Curtin et al. 2008; Jakubiec et al. 2012; Qu et al. 2008). The observation that P6

silencing suppressor of *Cauliflower mosaic virus* (CaMV) inhibits DRB4, strongly suggests that DRB4 is an antiviral factor against DNA viruses as well. DRB3, the cofactor of DCL3, contributes to antiviral defense through chromatin modification against DNA viruses (Raja et al. 2014).

HEN1-mediated vsiRNA methylation is critical for effective antiviral defense (Vogler et al. 2007). *hen1* mutants are more susceptible to *Cucumber mosaic virus* (CMV) and *Turnip crinkle virus* (TCV) virus infections (Boutet et al. 2003; Zhang et al. 2012). vsiRNA-binding viral silencing suppressors were shown to inhibit methylation (Csorba et al. 2007; Lozsa et al. 2008).

1.2.3 Effector Step of Antiviral Silencing

Dicing *per se* is not sufficient for an efficient antiviral silencing response (Wang et al. 2011), suggesting that the DCLs' substrates may be only the byproducts of the viral replication process. vsiRNA-binding VSRs do not compromise dicing, but efficiently inhibit antiviral silencing (Csorba et al. 2015). The downstream AGO-dependent effector step is therefore necessary to restrict virus replication and spread of both RNA and DNA viruses (Azevedo et al. 2010; Carbonell et al. 2012; Harvey et al. 2011; Pantaleo et al. 2007; Qu et al. 2008; Raja et al. 2014; Raja et al. 2008; Wang et al. 2011). The properties of vsiRNAs like 5'-nucleotide, length, thermodynamical properties of sRNA duplex ends and sRNA's duplex structure define loading and sorting into AGO effectors (Khvorova et al. 2003; Mi et al. 2008; Schuck et al. 2013; Schwarz et al. 2003; Zhang et al. 2014; Kontra et al. unpublished). During RNA virus infections AGO1 and AGO2 are the most important effectors, while AGO5, 7, 10 may have additional roles or act during specific host-virus combinations (Carbonell and Carrington 2015) (Fig. 1.1).

AGO1 was identified as the main effector against *Brome mosaic virus* (BMV), CMV, TCV, *Turnip mosaic virus* (TuMV) in *Arabidopsis thaliana* (Dzianott et al. 2012; Garcia-Ruiz et al. 2015; Morel et al. 2002). AGO1 participate in removal of TuMV viral RNA through slicing activity (Carbonell et al. 2012). AGO1 translational repression activity was also found to play a role during *Tomato ringspot virus* (ToRSV) infection in *Nicotiana benthamiana* (Ghoshal and Sanfacon 2014). It was shown that during RNA virus infections AGO1 homeostasis (Mallory et al. 2008) is disrupted and AGO1 protein levels are decreased probably through translational repression of AGO1 mRNA by miR168 activity (Varallyay et al. 2010). *Arabidopsis ago1* and *ago2* mutants are hypersusceptible to CMV, TuMV and TCV (Carbonell et al. 2012; Harvey et al. 2011; Morel et al. 2002; Takeda et al. 2008). As AGO1 is the negative regulator of AGO2 through miR403 action, in the absence of AGO1 activity AGO2 levels are elevated (Azevedo et al. 2010; Harvey et al. 2011). AGO2 therefore emerges as a second layer in antiviral pathways. AGO2 was shown to be important in defense against CMV, TCV and *Potato virus X* (PVX) viruses in *A. thaliana* (Brosseau and Moffett 2015;

Harvey et al. 2011; Jaubert et al. 2011). The phenotype of *ago1ago2* double mutant indicates that the two proteins act in a synergistic manner and have non-overlapping functions, as suggested by their phylogenetic distance (Mallory and Vaucheret 2010; Wang et al. 2011).

Our knowledge about the function of AGO proteins during PTGS in species other than *Arabidopsis* is much limited due to lack of genetic tools. In *N. benthamiana* it was shown that AGO2 protects against TBSV, TMV, PVX, *Cucumber necrosis virus* (CNV) and *Cymbidium ringspot virus* (CymRSV) (Fatyol et al. 2016; Odokonyero et al. 2015; Scholthof et al. 2011). Recently, however AGO1 was proposed to be the essential effector against CymRSV (Kontra et al., unpublished) and is also required for recovery during ToRSV infection (Ghoshal and Sanfacon 2014).

In rice there are 19 AGOs categorized into four clades (Nonomura et al. 2007). Genetic and biochemical data suggest that in rice the AGO1 and AGO18 are the main antiviral effectors against *Rice stripe virus* (RSV), *Rice dwarf phyto-reovirus* (RDV) (Hong et al. 2015; Jiang et al. 2012; Wu et al. 2015). AGO18 is induced during virus infection and may confer a broad-spectrum resistance: AGO18 do not bind efficiently vsRNAs, instead, by sequestration of miR168 it interferes with AGO1 homeostasis. This action leads to elevated levels of AGO1 required for antiviral defense (Wu et al. 2015).

Effectors AGO4, 5, 7 and 10 were also proposed to possess additional antiviral roles against RNA viruses. CMV 2b silencing suppressor protein directly interacts with AGO4 and inhibits its slicer activity and methylation and thus creates a favorable niche for CMV proliferation (Hamera et al. 2012). AGO5 (besides AGO2) was shown to be required to inhibit PVX systemic infection (Brosseau and Moffett 2015). AGO7 seems to work as a surrogate of AGO1 but with a preference for the less structured RNA targets (Qu et al. 2008; Takeda et al. 2008). AGO5, 7 and 10 had minor contribution in leaves while AGO10 (alongside AGO1) had antiviral functions in inflorescence during systemic TuMV infection (Garcia-Ruiz et al. 2015).

The nuclear localized AGO4 has been shown to possess important antiviral functions against geminiviruses. *Arabidopsis dcl3*, *drb3* and *ago4* mutants fail to hypermethylate the viral genome that is required for host recovery (Raja et al. 2014). Besides, AGO4 was proposed to be important in transcriptional regulation of host transcriptional response during CMV virus infection (Hamera et al. 2012) or to be involved in PVX virus resistance induced by NB-LRR proteins involving AGO4-mediated translational control (Bhattacharjee et al. 2009).

AGOs loaded with vsRNAs are able to form high molecular weight complexes (Csorba et al. 2010; Pantaleo et al. 2007). The knowledge about RISC (including antiviral RISC, vsRISC) cofactors that cooperate with AGOs in plants is very limited (Omarov et al. 2016). Heat shock protein 70 and 90 (HSP70, HSP90) have been found to be important players in AGO loading by using an *in vitro* cell-free system that recapitulates the loading process (Iki et al. 2010). Further understanding of RISC components, assembly and function may be helped by *in vitro* and transient

sensor systems (Fatyol et al. 2016; Iki et al. 2010; Omarov et al. 2016; Schuck et al. 2013).

1.2.4 Amplification of Silencing

To achieve a robust silencing response RISC cleavage fragments sometimes are channeled back into silencing by RDR-mediated dsRNA synthesis (Bologna and Voinnet 2014; Wassenegger and Krczal 2006). Subsequently to the AGO endonucleolytic cleavage, ssRNA fragments lacking *bona fide* features like cap structure or polyA tail are recognized by RDR polymerases with or without the help of primary vsiRNA and converted into long dsRNAs that are substrates of DCLs (Gazzani et al. 2004; Moreno et al. 2013; Parent et al. 2015b) (Fig. 1.1). RDR1, RDR2 and RDR6 (SDE1/SGS2) were all found to be important factors in vsiRNA production during PVX, CMV, TMV, *Sugarcane mosaic virus* (SCMV), TuMV, *Tobacco rattle virus* (TRV) infections (Diaz-Pendon et al. 2007; Donaire et al. 2008; Garcia-Ruiz et al. 2010; Qu et al. 2008; Schwach et al. 2005). RDR-synthesized dsRNAs are processed by DCL4 and DCL2 into 21–22 nt long vsiRNAs, respectively. Both 21 and 22 nt long vsiRNA were effective in antiviral response against a number of viruses like CMV, *Oilseed rape mosaic virus* (ORMV), TCV, TRV, *Cabbage leaf curl virus* (CaLCuV), CaMV (Blevins et al. 2006; Bouche et al. 2006; Deleris et al. 2006; Donaire et al. 2008). 22 nt long vsiRNAs contribute to secondary siRNA production and mediate systemic silencing (Garcia-Ruiz et al. 2010; Wang et al. 2011). In case of robustly replicating RNA viruses the involvement of RDRs seems to be less important. Upon tobusvirus infection the major part of vsiRNAs derives from the positive RNA strand of the virus genome suggesting that they are primary DCL cleavage products of viral RNA fold-back structures (Aregger et al. 2012; Blevins et al. 2011; Donaire et al. 2008; Molnar et al. 2005; Szittyta et al. 2010; Kontra et al. unpublished). In a similarly RDR-independent manner, massive amount of hairpin-derived vsiRNAs are produced from 35S leader of CaMV (Blevins et al. 2011). The majority of viral siRNAs accumulating during CaLCuV geminivirus infection were RDR1/2/6-independent primary siRNAs generated by pairing of bidirectional read-through transcripts of the circular viral genome (Aregger et al. 2012).

RDR6 activity is facilitated by protein cofactors SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Mourrain et al. 2000), SILENCING DEFECTIVE 5 (SDE5) (Hernandez-Pinzon et al. 2007) and SILENCING DEFECTIVE 3 (SDE3) (Dalmay et al. 2001). SGS3, a plant specific protein associate to RISC complex (Allen et al. 2005; Yoshikawa et al. 2005), stabilizes the RISC-cleavage products following slicing and enhance their conversion into dsRNA by RDR6 (Yoshikawa et al. 2013). Elimination of SGS3 leads to enhanced susceptibility to CMV but not to TuMV or *Turnip vein-clearing virus* (TVCV) infections (Adenot et al. 2006; Yoshikawa et al. 2013). SGS3 was shown to be required for CaLCuV virus induced VIGS of endogenous genes and was further suggested to be involved

in the antiviral response against DNA viruses (Muangsan et al. 2004). This is supported by the fact that *Tomato yellow leaf curl virus* (TYLCV) encodes a silencing suppressor to compromise SGS3 activity (Glick et al. 2008). SDE5 is an RNA trafficking protein homologue of human mRNA export factor. SDE5 acts together with RDR6 to convert ssRNAs into dsRNA. *sde5* mutant plants are hypersusceptible to CMV but not to TuMV infection (Hernandez-Pinzon et al. 2007). Silencing amplification is facilitated by the SDE3, an RNA-helicase like protein. SDE3 was shown to bind to AGOs through its GW domains (Garcia et al. 2012). *sde3* mutant plants are more susceptible to CMV or PVX but not to TRV infections (Dalmay et al. 2001). SDE3 activity occurs downstream to RDR6 and requires AGO1 and AGO2 activities (Garcia et al. 2012). SDE3 was proposed therefore to facilitate the amplification process by unwinding a fraction of RDR6-synthetized dsRNA products using helicase activity.

In rice there are five RDRs annotated, but our knowledge about their involvement in vsiRNA biogenesis is very limited. OsRDR6-silenced transgenic rice plants were shown to be hypersusceptible to RSV and RDV (Hong et al. 2015; Jiang et al. 2012). The rise in viral symptoms was associated with an increase in viral genomic RNA and reduced levels of vsiRNAs. Interestingly, the protein level of the overexpressed OsRDR6 in transgenic rice was reduced during RDV infection, suggesting a negative translational control induced by the virus upon RDR6 expression (Hong et al. 2015).

1.3 Viral Silencing Suppressor Strategies

1.3.1 Introduction

The most common strategy of viruses to protect themselves against antiviral RNA silencing is to express proteins that act as suppressors of silencing. These proteins are the viral suppressors of RNA silencing (VSRs). Discovery of VSRs provided a strong support of RNA silencing being an antiviral mechanism. Available evidences suggest that most viruses encode at least one VSR that, in most cases is essential for successful virus infection. Silencing suppression by VSRs has been described in insect and fungus-infecting viruses as well (Bronkhorst and van Rij 2014). Diversity of VSR's in sequence and structure indicates that they have evolved independently. VSRs were shown to block virtually all steps of RNA silencing like silencing initiation, effector phase, amplification phase, chromatin modification during TGS or modulation of host gene products for a more favorable infection. Here we review the most important strategies employed by presenting the most studied/relevant examples of VSRs (Fig. 1.1).

1.3.2 Blocking Initiation of Antiviral Response

1.3.2.1 Inhibition of DCL's Activities

Initiation of silencing may be blocked by inhibition of dicing itself, either through dsRNA sequestration or through impeding DCLs or their cofactors. *Pothos latent aureusvirus* (PoLV) P14, TCV p38 and CMV 2b have been all shown to bind long dsRNA and thus block vsiRNA biogenesis (Deleris et al. 2006; Goto et al. 2007; Merai et al. 2005). The nuclear localized P6 suppressor of CaMV diminishes dicing through protein-protein interaction: P6 interacts with the nuclear DRB4, a cofactor required for DCL4-dependent vsiRNA processing (Haas et al. 2008). In addition, during CaMV infection massive amounts of vsiRNAs derive from the 35S leader sequence recognized by all four DCLs. 35S leader RNA therefore serves as decoy to divert the effectors of the silencing machinery from more important viral features (Blevins et al. 2011). *Red clover necrotic mosaic virus* (RCNMV) recruits DCL enzymes into its replication complex and therefore deprives them from the silencing machinery. *dcl1* mutant plants are less susceptible to RCNMV infection (Takeda et al. 2005). Similar strategies were described in insect-infecting viruses (Bronkhorst and van Rij 2014).

Viruses may modulate endogenous regulatory pathways in order to alter the strength of silencing in their favor. RNASE THREE_LIKE 1 (RTL1) enzyme was described as an endogenous silencing suppressor: RTL1 is induced during virus infections and prevents vsiRNA production by cleaving viral dsRNAs prior to DCL2/3/4-processing but does not interfere with DCL1-mediated miRNA pathway (Shamandi et al. 2015).

1.3.2.2 vsiRNA Sequestration

Ds-siRNA sequestration is a widespread strategy used by several VSRs originating from diverse genera (P19, Hc-Pro, P21, p15, p122/p126/p130, γ B, NS3, NSs, Pns10 etc.) (Csorba et al. 2007; Harries et al. 2008; Hemmes et al. 2007; Kubota et al. 2003; Lakatos et al. 2006; Merai et al. 2005, 2006; Silhavy et al. 2002). Amongst these, probably the best known is the tombusviral p19 protein (Silhavy et al. 2002). Crystallographic studies have shown that p19 homodimer acts as a molecular caliper to sequester the sRNA duplexes size-specifically (Silhavy et al. 2002; Vargason et al. 2003; Ye et al. 2003). sRNA sequestration prevents RISC assembly as shown by the heterologous *in vitro* Drosophila embryo extract system (Lakatos et al. 2006). It seems that p19-mediated vsiRNA sequestration affects selectively AGO1- but not AGO2-loading in *N. benthamiana* during CymRSV virus infection (Kontra et al., unpublished). It was shown that due to the structural similarity between vsiRNAs and endogenous sRNAs p19 prevents RISC-loading of endogenous sRNA species in transgenic *A. thaliana* and *N. benthamiana* plants (Schott et al. 2012; Kontra et al. unpublished). During

authentic virus infections however, p19-sequestration of endogenous sRNA is not efficient (Lozsa et al. 2008; Kontra et al. unpublished). The tombusviral vsiRNAs bind more efficiently to p19 to outcompete endogenous sRNAs. The basis of vsiRNA competition, besides the massive amount of vsiRNAs, could be the structural preference of p19 for perfect ds-vsiRNAs forms (contrary to the mismatch-containing endogenous sRNAs) (Kontra et al. unpublished).

A consequence of sRNA binding by VSRs is the block of HEN1-dependent methylation of sRNAs (Csorba et al. 2007; Lozsa et al. 2008; Vogler et al. 2007). When sequestered, the methylation of sRNAs is inhibited (Csorba et al. 2007; Lozsa et al. 2008). Whether blocking of vsiRNA methylation leads to a faster decay and this has any biological significance remains a question.

It was shown that *Sweet potato chlorotic stunt crinivirus* (SPCSV) suppressor RNase3 cleaves the 21–24 nt vsiRNAs into 14 bp products rendering them inactive (Cuellar et al. 2009; Kreuze et al. 2005). Although this is a completely different strategy to siRNA-binding, it has a very similar outcome: vsiRNAs are unavailable for AGO-loading.

1.3.2.3 Blocking Systemic Silencing

Although p19 sequesters vsiRNAs very efficiently, its effect to block cell-autonomous silencing and restrict virus replication is mild. The VSR-deficient CymRSV (Cym19stop) replicates as efficiently as the wild type CymRSV in *N. benthamiana* protoplasts (Silhavy et al. 2002). The true strength of p19 lies in blocking systemic silencing through inhibition of vsiRNA mobilization into naive surrounding tissue or long distance (Dunoyer et al. 2010; Havelda et al. 2003; Molnar et al. 2010). RNA binding suppressors NS3 (RSV) and 2b (CMV) were also shown to prevent efficiently the spread of silencing signal (Guo and Ding 2002; Xiong et al. 2009).

1.3.2.4 Interfering with AGO-Loading

An efficient arrest of silencing initiation can be achieved through the block of functional RISC assembly. P0 the suppressor of *Poleroviruses* (Mayo and Ziegler-Graff 1996) was shown to enhance the degradation of effector AGOs (AGO1, 2, 4–6, 9) by inhibition of holo-RISC assembly (Baumberger et al. 2007; Bortolamiol et al. 2007; Csorba et al. 2010; Derrien et al. 2012; Pazhouhandeh et al. 2006) P0-mediated AGO degradation occurs through autophagy pathway (Derrien et al. 2012). ToRSV CP, that acts as a VSR as well, binds to AGO1 to suppress its translational inhibitory activity and to enhance AGO1 degradation through autophagy (Karran and Sanfacon 2014). It was shown that PVX p25 physically interacts with multiple AGOs (AGO1, 2, 3 and 4) to promote their destabilization in a proteasome-dependent manner (Chiu et al. 2010). In the absence of central AGO effector, silencing cannot be programmed/initiated.

VSRs are able to modulate AGO1 availability in a more subtle way. AGO1 homeostasis depends on the miR168-guided AGO1 mRNA cleavage and translational inhibition control (Mallory and Vaucheret 2009; Rhoades et al. 2002). To counteract AGO1-based defense a number of unrelated siRNA-binder VSRs (p19, p122, p38, Hc-Pro and 2b) promote miR168 transcriptional induction that results in miR168-guided AGO1 down-regulation to create a better environment for virus infection. It was shown that (during tombusvirus infection) the miR168 accumulation spatially correlated with the virus localization and was dependent on the presence of p19 (Varallyay and Havelda 2013; Varallyay et al. 2010).

1.3.2.5 Arrest of Programmed RISC Activity

The *Sweet potato mild mottle ipomovirus* (SPMMV) suppressor protein P1 interacts directly with siRNA and/or miRNA-loaded AGO1 present in the high molecular weight holo-RISC but not minimal-RISC through GW/WG-motifs (AGO-hook) and inhibits si/miRNA-loaded RISC activity. The GW/WG-motif containing proteins (GW182 family) were shown to interact with AGOs and support diverse RISC functions (Eulalio et al. 2009). P1 AGO-hook motifs are necessary for both binding and suppression of AGO1 function (Giner et al. 2010; Szabo et al. 2012).

P38 of TCV (Azevedo et al. 2010) and 2b of CMV (Zhang et al. 2006) and *Tomato aspermy virus* (TAV) (Chen et al. 2008) suppressors were proposed to act at multiple steps of silencing (during initiation and effector phase) including RISC activity block through AGO protein interaction.

The block of effector step can be achieved also through targeting the RNA component (the guide vsiRNA) within the vsiRISC. *African cassava mosaic virus* (ACMV) encoded AC4 is able to bind to the ss- but not ds-sRNA forms *in vitro*. Transgenic AC4 decreases accumulation of miRNAs and up-regulates target mRNAs. AC4 acts downstream of the unwinding process by binding miRNAs presumably loaded into AGO (Chellappan et al. 2005; Xiong et al. 2009; Zhou et al. 2006). RSV suppressor NS3 was found to bind to various RNA forms like ss-siRNA, ds-siRNA or long ssRNA (but not long dsRNA). By this, NS3 is able to suppress and revert local silencing but also prevent the long distance spread of silencing signal (Chellappan et al. 2005; Xiong et al. 2009; Zhou et al. 2006). Similarly, *Grapevine virus A* (GVA) p10 suppressor was also suggested to act through both ss- and ds-si/miRNA binding (Chellappan et al. 2005; Xiong et al. 2009; Zhou et al. 2006).

1.3.3 VSR Activities Affecting TGS

Several DNA viruses encode VSRs that have been described to alter the effector step of TGS, the chromatin structure modification. AL2 suppressor of *Tomato golden mosaic virus* (TGMV) and L2 suppressor of *Beet curly top virus* (BCTV)

inhibit adenosine kinase (ADK) activity that plays crucial role in adenosine and methyl-cycle maintenance or cytokinin regulation. AL2 and L2 induce global reduction in cytosine methylation that leads to inactivation and reversal of antiviral silencing (Buchmann et al. 2009; Wang et al. 2003, 2005). *In vitro* methylated TGMV cannot replicate in protoplasts suggesting that viral genome methylation is a *bona fide* defense against geminiviruses (Bisaro 2006). Similarly, β C1 suppressor of *Tomato yellow leaf curl China virus* (TYLCCNV) interacts and inhibits activity of S-adenosyl-homocystein-hydrolase (SAHH) that is involved in methyl-cycle and therefore indirectly affects TGS (Yang et al. 2011).

1.3.4 *Suppression of Antiviral Silencing Amplification*

Blocking RDR activities by VSRs is a very effective strategy employed by viruses since it dampens cell-autonomous silencing amplification and systemic signal movement in distant tissues to facilitate the virus replication and spread (Ren et al. 2010; Schwach et al. 2005). V2 suppressor of TYLCV inhibits RDR6-mediated amplification by direct interaction with SGS3, the cofactor of RDR6 (Glick et al. 2008). Alternatively, V2 may compete with SGS3 for dsRNA having 5' overhang ends that may be an RDR6/SGS3 substrate/intermediate during vsiRNA amplification (Fukunaga and Doudna 2009; Kumakura et al. 2009). Similarly, TRIPLE GENE BOX PROTEIN1 (TGBp1) encoded by PVX was shown to inhibit RDR6/SGS3-dependent dsRNA synthesis (Okano et al. 2014). β C1 suppressor of TYLCCNV DNA satellite interacts with the endogenous suppressor of silencing calmodulin-like protein (rgsCAM) in *N. benthamiana* to repress RDR6 expression (Li et al. 2014). SCMV encoded HC-Pro, TAV 2b and Pns10 of RDV were all shown to downregulate RDR6 to limit amplification and decelerate systemic silencing (Ren et al. 2010; Zhang et al. 2008). Plant RDR1 however, was suggested to have adverse functions: RDR1 is an antagonist of RDR6-mediated sense-PTGS making it an endogenous silencing suppressor (Ying et al. 2010).

1.3.5 *Targeting Multiple Steps of Antiviral Pathways*

Many VSRs have multiple silencing suppressor functions and therefore are capable to act at multiple points to modulate antiviral response. 2b of CMV (CM95R strain) and TAV exhibit high affinity for long dsRNAs and ds-sRNAs (Chen et al. 2008; Duan et al. 2012; Gonzalez et al. 2012; Goto et al. 2007). CMV 2b (Fny and SD strains) was also shown to interact with AGO1 through the PAZ- and partly PIWI domains and blocks RISC slicer activity (Duan et al. 2012; Zhang et al. 2006). Additionally, CMV 2b (SD strain) alters RdDM pathway as well. 2b facilitates cytosine methylation through the transport of siRNAs into the nucleus (Kanazawa et al. 2011). 2b interacts both with AGO4-related siRNAs and with AGO4 protein

through PAZ and PIWI domains. The interaction with 2b reduces AGO4 access to endogenous target loci and consequently modulates endogenous transcription to create a favorable niche for CMV infection (Duan et al. 2012; Gonzalez et al. 2010, 2012; Hamera et al. 2012).

P38 of TCV may also suppress silencing at multiple levels. P38 possesses dsRNA-binding activity (Merai et al. 2006). Since in the presence of p38, siRNAs are undetectable therefore it was proposed that p38 suppress DCLs' activity (Qu et al. 2003). Genetic evidences also supported the role of p38 in inhibiting DCL4 (Deleris et al. 2006). In a later study however, p38 suppressor impact on DCL4 was attributed to an indirect effect of AGO1-mediated DCL-homeostasis and has been shown that p38 blocks AGO1 but not AGO4 activity through its GW-motif binding (Azevedo et al. 2010). P38 is capable to bind and inactivate AGO2 as well (Zhang et al. 2012). Site-directed mutagenesis (GW-to-GA) in the p38 proved that GW motif is absolutely required for both binding and suppression of AGO1 function (Azevedo et al. 2010). *Pelargonium line pattern virus* (PLPV) coat protein p37 (an orthologue of TCV p38) is a GW-containing protein that functions as a VSR as well. It was shown that the mutations within its GW-motif affect p37 localization, interaction with AGO1 and its sRNA-binding ability. Furthermore, GW-mutations also abolished TCV p38 sRNA and long dsRNA-binding capacity (Perez-Canamas and Hernandez 2015). It seems therefore that the domain for different functions may overlap in p37/p38 VSRs. This brings up the possibility that the parallel suppressor functions could cooperate during their interaction with host silencing machinery (e.g. p37/p38 interaction with AGO1 could enhance sRNA duplex sequestration in order to more efficiently prevent RISC programming).

1.3.6 VSRs' Interaction with Host Factors to Modulate Silencing

Besides blocking antiviral silencing VSRs are able to modulate host endogenous pathways in order to fine-tune the host-pathogen interaction. The suppressor of *Tobacco etch virus* (TEV) helper-component protease (HC-Pro) is a multifunctional protein involved in many aspects of virus infection (Anandalakshmi et al. 1998; Carrington et al. 1989; Guo et al. 2011; Kasschau et al. 1997; Lakatos et al. 2006; Mallory et al. 2001). HC-Pro sequesters vsiRNA that leads to inhibition of their methylation and inability to load into vsiRISC (Lakatos et al. 2006; Lozsa et al. 2008). HC-Pro was also found to interact with rgsCAM an endogenous silencing suppressor (Anandalakshmi et al. 2000; Endres et al. 2010; Marquardt et al. 2014). In another study it was shown that rgsCAM counteracts HC-Pro through binding to its positively charged dsRNA-binding surface, prevents HC-Pro siRNA-sequestration and promotes HC-Pro degradation through autophagy pathway (Nakahara et al. 2012). Suppression of silencing by

TuMV HC-Pro requires another host factor, RAV2, a transcription factor. RAV2 targets include *FIERY1* and *CML38*, endogenous suppressors of silencing (Anandalakshmi et al. 2000; Endres et al. 2010; Gy et al. 2007). RAV2 was required for suppression of silencing by *Carmoviral* p38 as well (Endres et al. 2010) suggesting that RAV2 is a cross-talk point between antiviral and endogenous silencing pathways, and may be efficiently used by suppressors to modulate host defense. HC-Pro of another potyvirus, *Papaya ringspot virus* (PRSV), interacts with calreticulin to modulate calcium signaling and thus host defense (Shen et al. 2010a, b). HC-Pro (of *Potato virus A* (PVA), *Potato virus Y* (PVY) and TEV) interacts also with microtubule-associated protein (HIP2) through its highly variable region (HVR). Virus accumulates at lower level when HIP2 is depleted. Mutations affecting HC-Pro HIP2 interaction induces necrosis and hormone (ethylene- and jasmonic acid-) mediated induction of host pathogen-related defense genes (Haikonen et al. 2013a, b).

1.3.7 vsiRNAs May Regulate Host Genes by Exploiting Endogenous Silencing Itself

The high sequence variability of vsiRNAs and the fast evolution of viral genomes, may lead to the production of vsiRNAs that could potentially target endogenous genes/transcripts. By this, viruses may modulate host response to their benefit. There are a few examples to support this idea. vsiRNAs derived from the CMV-Y satellite RNA (Y-Sat) targets magnesium protoporphyrin chelatase subunit I (CHLI), a key component of chlorophyll biosynthesis pathway. vsiRNA-mediated downregulation of CHLI mRNA leads to yellowing of the plant leaves, that was suggested to enhance virus spreading by insects (Shimura et al. 2011). sRNA derived from *Peach latent mosaic viroid* (PLMVd) targets chloroplastic heat-shock protein 90 (cHSP90) in peach. Cleavage of cHSP90 (that participates in chloroplast biosynthesis and plasmid-nucleus signal transduction) induces albinism and may contribute to a more favorable host environment for viroid infection (Navarro et al. 2012). Callose synthase genes encode proteins with role in callose formation during pollen development. *Potato spindle tuber viroid* (PSTVd)-derived sRNAs suppress CalS11-like and CalS12-like mRNAs that greatly affects the severity of disease symptoms (Adkar-Purushothama et al. 2015).

1.4 Perspectives

With the advancement of high throughput technologies the in-depth profiling of vsiRNA generation, their loading into effectors (vsiRISC or RDR complexes) and their involvement in systemic signaling of RNA silencing will lead to a more and

thorough understanding of antiviral defense at cellular, tissue and organism level. In addition to this, the development of novel *in vitro* systems and *in vivo* cellular assays hopefully will make it possible to better understand the mechanistic details at molecular level. The interaction of host with viral pathogens is very complex: the exact details such as how, when, and where in the cell viral RNAs are initially accessed by the RNA silencing machinery and how VSRs counteract silencing response remain elusive. It was recently reported that potyvirus-induced granules (PG) protects PVA viral RNA from antiviral silencing when active viral translation does not occur optimally (Hafren et al. 2015). Antiviral silencing, translation and RNA quality control pathways, alongside with general RNA degradation pathways all compete for endogenous and viral RNAs (Christie et al. 2011). How exactly these pathways share substrates and cooperate during viral infection will be hopefully addressed by further research.

Until recently most studies on antiviral silencing were conducted in the model *Arabidopsis* due to the plethora of genetic tools available. The use of *Arabidopsis*, however, has a major drawback since this plant model hosts only very few plant viruses. Availability of the full genome sequence of the viral model plant *Nicotiana benthamiana* (sensitive to almost all plant viruses) and the development of CRISPR/CAS9 genome editing technology will hopefully allow the study of antiviral RNA silencing during several other virus infections.

An important aim of antiviral silencing research is to gather knowledge in order to be able to design resistant crops. Great advances have been made to develop methods for viral disease control with the expression of artificial sRNAs/miRNAs targeting viral genomes in economically important plants (Kis et al. 2016; Lin et al. 2009; Niu et al. 2006). Similar biotechnological approaches may be very useful to elaborate in the future for economically important crop protection.

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Glossary

RNAi:	RNA interference
TGS:	Transcriptional Gene Silencing
PTGS:	Post Transcriptional Gene Silencing
sRNA:	small RNA
dsRNA:	double-stranded RNA
ssRNA:	single-stranded RNA
DCL:	Dicer-Like enzymes
PAZ:	Piwi/Argonaute/Zwille-domain

AGO:	Argonaute protein
RDR:	RNA-dependent RNA polymerase
RISC:	RNA-Induced Silencing Complex
RITSC:	RNA-Induced Transcriptional Silencing Complex
miRNA	: micro RNA
siRNA:	small interfering RNA
ta-siRNA:	trans-acting small interfering RNA
nat-siRNA:	natural-antisense small interfering RNA
ra-siRNA:	repeat-associated small interfering RNA
vsiRNA:	viral small interfering RNA
vasiRNA:	virus-activated small interfering RNA

(+) *ssRNA Virus*

BMV:	<i>Brome mosaic virus</i>
CMV:	<i>Cucumber mosaic virus</i>
CNV:	<i>Cucumber necrosis virus</i>
CymRSV:	<i>Cymbidium ringspot virus</i>
GVA:	<i>Grapevine virus A</i>
ORMV:	<i>Oilseed rape mosaic virus</i>
PLPV:	<i>Pelargonium line pattern virus</i>
PoLV:	<i>Pothos latent virus</i>
PRSV:	<i>Papaya ringspot virus</i>
PVA:	<i>Potato virus A</i>
PVX:	<i>Potato virus X</i>
PVY:	<i>Potato virus Y</i>
RCNMV:	<i>Red clover necrotic mosaic virus</i>
RYMV:	<i>Rice yellow mottle virus</i>
SCMV:	<i>Sugarcane mosaic virus</i>
SPCSV:	<i>Sweet potato chlorotic stunt virus</i>
SPMMV:	<i>Sweet potato mild mottle virus</i>
TAV:	<i>Tomato aspermy virus</i>
TEV:	<i>Tobacco etch virus</i>
TBSV:	<i>Tomato bushy stunt virus</i>
TCV:	<i>Turnip crinkle virus</i>
TMV:	<i>Tobacco mosaic virus</i>
ToRSV:	<i>Tomato ringspot virus</i>
TRV:	<i>Tobacco rattle virus</i>
TuMV:	<i>Turnip mosaic virus</i>
TYMV:	<i>Turnip yellow mosaic virus</i>

(–) ssRNA Virus

RSV: *Rice stripe virus*
 TSWV: *Tomato spotted wilt virus*

dsRNA Virus

OsEV: *Oryza sativa endornavirus*
 RDV: *Rice dwarf phytoreovirus*

ssDNA Virus

ACMV: *African cassava mosaic virus*
 BCTV: *Beet curly top virus*
 CaLCuV: *Cabbage leaf curl virus*
 TGMV: *Tomato golden mosaic virus*
 TYLCV: *Tomato yellow leaf curl virus*
 TYLCCNV: *Tomato yellow leaf curl China virus*

dsDNA Virus

CaMV: *Cauliflower mosaic virus*
 TVCV: *Turnip vein-clearing virus*
 viroid: non-protein coding infectious RNAs
 PLMVd: *Peach latent mosaic viroid*
 PSTVd: *Potato spindle tuber viroid*

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Chapter 2

Exploration of Plant Virus Replication Inside a Surrogate Host, *Saccharomyces cerevisiae*, Elucidates Complex and Conserved Mechanisms

Zsuzsanna Sasvari and Peter D. Nagy

Abstract Plant RNA viruses are intracellular infectious agents with limited coding capacity. Therefore, these viruses have developed sophisticated ways to co-opt numerous cellular factors to facilitate the viral infectious cycle. To understand virus-host interactions, it is necessary to identify all the host components that are co-opted for viral infections. Development of yeast (*Saccharomyces cerevisiae*) as a host greatly facilitated the progress in our understanding of plant virus, such as brome mosaic virus (BMV) and tomato bushy stunt virus (TBSV), interactions with the host cells. Systematic genome-wide screens using yeast genomic libraries have led to the identification of a large number of host factors affecting (+)RNA virus replication. In combination with proteomic approaches, both susceptibility and restriction factors for BMV and TBSV have been identified using yeast. More detailed biochemical and cellular studies then led to the dissection of molecular functions of many host factors that promote each step of the viral replication process. The development of *in vitro* systems with TBSV, such as yeast cell-free extract and purified active replicase assays, together with proteomics, lipidomics and artificial vesicle-based assays helped to comprehend the complex nature of virus replication. Subsequently, comparable pro- or antiviral functions of several of the characterized yeast host factors have been validated in plant hosts. Overall, yeast is an advanced model organism that has emerged as an attractive host to gain insights into the intricate interactions of plant viruses with host cells. This chapter describes our current understanding of virus-host interactions, based mostly on TBSV-yeast system. Many of the pioneering findings with TBSV are likely applicable to other plant and animal viruses and their interactions with their hosts. The gained knowledge on host factors could lead to novel specific or broad-range antiviral tools against viruses.

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2.1 Introduction

Viruses are the most abundant biological entities on Earth and they largely outnumber all other lifeforms. Regardless of their huge diversity in genome size, coding capacity, or the nature of their nucleic acids, single- or double-stranded, RNA or DNA, they are all molecular parasites that cannot multiply outside of their host cells. Their genomes are relatively small compared to their hosts' genomes. Among plant-infecting viruses, those with RNA genomes are the most widespread, usually coding only for a few conserved replication-associated proteins, coat proteins and plant virus-specific movement proteins and suppressors for gene silencing. Overall, plant viruses inevitably depend on the interactions between the viral components and the surrounding cellular proteins, lipids and metabolites that ensure successful viral multiplication. Accordingly, some cellular factors are essential for both cell propagation/survival and for virus multiplication to complete the infectious cycle. Yet, other host components can be modified, sequestered, retargeted and manipulated by viruses to create subcellular environment suitable for virus replication.

To explore how cellular processes are subverted by the virus after infection and how the viral replication proteins could change subcellular environment as well as how the cells fight back the infection requires systems level approaches. Virologists should identify all the molecular players both from the host and virus sides that participate in the infection process. The gained knowledge could be useful for developing novel anti-viral approaches or might be advantageous to optimize beneficial applications of viruses. We will also learn about the potential repertoire of cellular factors during normal and diseased states. The most feasible way to unveil all the interactions, or networks of interactions, is the utilization of genetically amenable model organisms, such as the baker yeast, *Saccharomyces cerevisiae*. The current chapter will shed light on the amazing complexity of positive-strand (+)RNA virus replication and its dependence on virus-host interactions. We will describe how the facile genetics of *S. cerevisiae* helps to unravel intricate molecular interactions based on molecular mimicry and how the relevance of the intriguing discoveries from yeast could provide deep insights into the natural host-virus interactions.

2.2 Overview of the Infectious Cycle of Positive-Sense RNA Viruses

Research during the last couple of decades established a trend that (+)RNA viruses, which form the largest group among viruses, share several common features in their replication strategies and their interactions with hosts. Briefly, the viral (+)RNA acts as mRNA that is used by the host ribosomes to produce

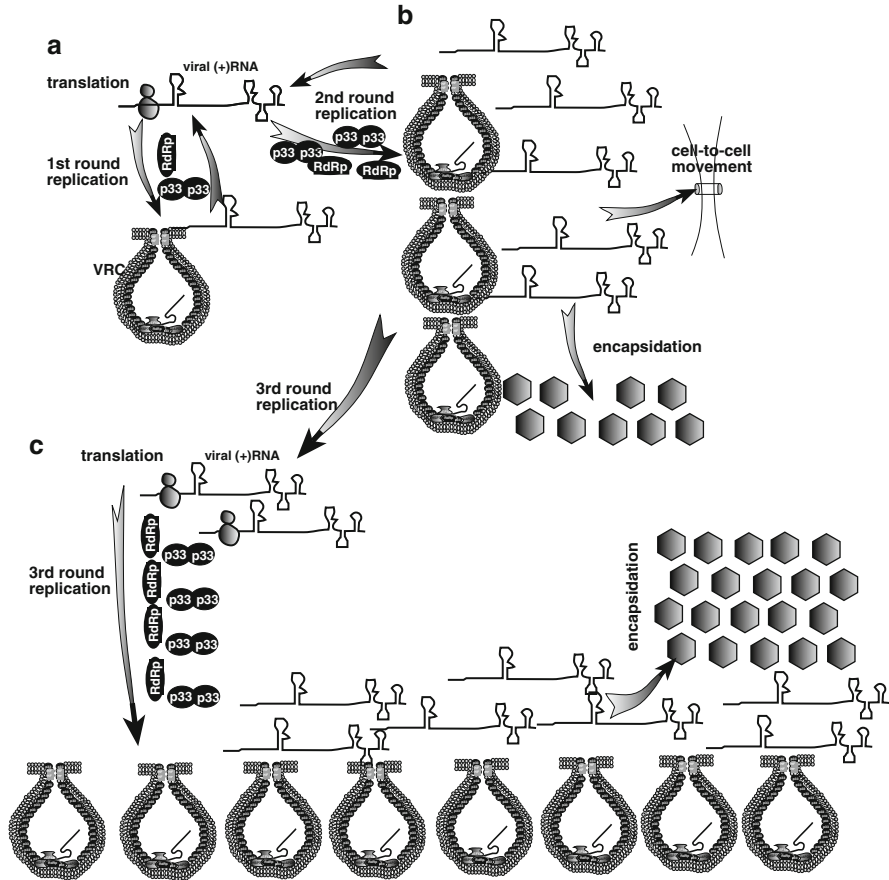


Fig. 2.1 The complex plant (+)RNA virus replication cycles includes the following steps: (a) After the initial translation of the invading TBSV (+)RNA by the cellular ribosomes, the freshly synthesized p33/p92 replication proteins recruit the viral (+)RNA for the assembly of the membrane-bound viral replicase (VRC, represented by a vesicle-like structure) and begins viral RNA replication (1st round). (b) Then, the newly made and released (+)RNA enters a new round of translation, followed by replication (2nd round). (c) The translation/replication cycle is repeated (3rd round). Note that a single infected cell likely perform ~20 sequential translation/replication cycles in 24–48 h that lead to the production of large amounts of viral (+)RNA progeny that participate in cell-to-cell movement and encapsidation

viral proteins at the early stage of infection (Fig. 2.1). This is followed by viral genome replication, then assembly of complete virus particles (virions), cell-to-cell and long-distance movement and spread to other plants. Interestingly, all these steps depend on the availability and functionality of many host factors (den Boon and Ahlquist 2010; Laliberte and Sanfacon 2010; Nagy and Pogany 2012; Wang 2015).

2.2.1 *Genome Organization of Tomato Bushy Stunt Virus*

In this chapter, we will mainly focus on the advancement in plant virus replication made by utilization of yeast as a model host. A more general description of plant virus-plant host interactions could be found in several excellent recent reviews (Laliberte and Sanfacon 2010; Wang 2015). Studies on plant virus-host interactions have been pioneered using bromoviruses and tombusviruses in yeast (den Boon and Ahlquist 2010; Janda and Ahlquist 1993; Nagy 2008; Nagy and Pogany 2006; Nagy et al. 2014; Panavas and Nagy 2003). Here, we will mainly focus on tombusviruses, including Tomato bushy stunt virus (TBSV). TBSV has a small (+)RNA genome (4800 nucleotides), which rapidly multiplies in infected plants, and produces a huge amount of virions. In the last decade, it became clear that TBSV is an excellent model virus to study virus replication and virus-host interactions. The TBSV genome codes for two replication proteins, namely p33 replication cofactor and p92^{pol} RNA dependent RNA polymerase (RdRp), which is a readthrough product of p33 and is expressed at 5 % of p33 level (White and Nagy 2004). Ribosomal read-through of a translational stop codon is a widely utilized strategy for plant RNA viruses to control the expression of downstream open reading frame, which frequently include the viral RdRp or other replication proteins (Nicholson and White 2014). Three other TBSV proteins, which are expressed from two subgenomic RNAs made during TBSV replication, are the capsid protein (p41), the movement protein (p22) and p19 silencing suppressor (White and Nagy 2004). In addition to the protein coding sequences, the TBSV (+)RNA genome contains several regulatory elements, which are present in the 5' or 3' untranslated regions, and even in the coding regions (Nicholson and White 2014). These regulatory RNA elements drive different viral processes, including translation, replication and encapsidation. Interestingly, TBSV (+)RNA genome, which is not capped at 5' end and does not have a 3' poly(A) tail, carries noncanonical translation elements that facilitate efficient translation. For example, a cap independent translation enhancer (3' CITE) is located at the 3' untranslated region (UTR) of the TBSV RNA. The complex interactions between the 3' CITE and the 5' UTR along with another five long-range RNA-RNA interactions in the TBSV (+)RNA were identified (Nicholson and White 2014; Wu et al. 2013). Short and long-distance RNA-RNA interactions within the viral genome also bring *cis*-acting replication elements into close proximity to regulate replication and subgenomic RNA transcription (Nicholson and White 2014; Panavas and Nagy 2005; Pogany et al. 2003; Wu et al. 2009, 2013). Altogether, long-range interactions within the TBSV genome provide mechanisms to regulate a diverse array of viral functions (Nicholson and White 2014).

2.2.2 Functions of cis-Acting Elements in the Genomic RNA During Replication of TBSV (+)RNA

The viral RNA is the master regulator of the replication process, as it serves multiple functions, including the template role, as an assembly platform for the replicase, and the RNA also organizes the replication proteins and host factors (Pathak et al. 2011; Pathak et al. 2012). These activities depend on various *cis*-acting replication elements within the genomic RNAs. Accordingly, the TBSV genomic RNA involves several *cis*-acting sequences that promote different steps of viral replication (Nicholson and White 2014; White and Nagy 2004). For example, the (+)RNA serves as a template for the synthesis of the complementary negative-strand (–)RNA, which then becomes the template for the synthesis of the (+)RNA progenies. Interestingly, (+)RNAs are produced in excess amounts, reaching up to 100 times more than (–)RNA. To tightly regulate this process, TBSV utilizes promoter elements and regulatory elements in both (+)- and (–)RNAs. The unrelated minus-strand and plus-strand initiation promoters are located at the 3' terminus of the (+) and the (–)RNAs, respectively. The former is called genomic promoter (gPR), while the latter is called the complementary promoter (cPR) and they are required for *de novo* (primer-independent) initiation of replication by the viral replicase complex (VRC). The VRC constitutes a membrane-bound large protein complex of p92^{p_{ol}} RdRp, p33 replication protein, the viral (+)- and (–) RNAs and over ten co-opted cellular factors (as discussed below). The main function of the gPR is to interact with and position the viral RdRp over the initiation sequence accurately to ensure the precise initiation of the (–)RNA synthesis. The activity of gPR is regulated by a replication silencer element (RSE), which participates in a five nt-long RNA-RNA interaction with the very 3' end sequence within the gPR. After the (–)RNA synthesis is finished, then the (+)RNA synthesis initiates from the cPR. Interestingly, the (+)RNA synthesis is enhanced by two replication enhancers (REs), one located close to the cPR (termed promoter proximal enhancer, PPE) and the other within the 5' end of the (–)RNA, called RIII(–) replication enhancer. The RIII(–) RE forms a long-range RNA-RNA interaction with the cPR at the 3' end (Panavas and Nagy 2005; Panavas et al. 2006). These viral RE elements ensure the production of excess amounts of infectious (+)RNAs. Overall, the viral RNAs are orchestrating viral replication proteins and a plethora of co-opted host factors to achieve robust and accurate replication.

Because viral replication is a step-wise process, below we will discuss the various steps as they occur in infected cells. Based on our current understanding, we can discriminate six main steps during TBSV replication inside the cell (Nagy and Pogany 2012). These steps are the following: (i) template selection for replication that results in a switch from translation to replication; (ii) recruitment of the RdRp/p33/viral (+)RNA complex to subcellular membrane surfaces; (iii) VRC assembly that also includes the activation of the membrane-bound p92^{p_{ol}} RdRp; (iv) (–)RNA synthesis that leads to the production of dsRNA replication intermediate; (v) (+)RNA

synthesis on the dsRNA template; and (vi) the release of (+)RNA progeny from the VRC into the cytosol to perform additional activities, including new translation/replication cycles, encapsidation or cell-to-cell movement.

2.2.3 (+)RNA Template Selection for Replication and a Switch from Translation to Replication

The genomes of (+)RNA viruses first serve as mRNAs for translation of viral proteins and then, the same viral (+)RNA molecules also act as templates in the subsequent replication process. Therefore, after the production of enough amounts of replication proteins – including the RdRp, the viral (+)RNA has to switch from the translation mode to execute the replication process. These two processes seem conflicting as during translation the ribosome moves from the 5'-to-3' direction on the (+)RNA, while the freshly expressed RdRp is destined to make (–)RNA on the same (+)RNA template, but progressing in 3'-to-5' direction. Although the detailed mechanism of the switch from translation to replication is not yet fully dissected for TBSV, the emerging picture is that multiple regulatory steps are in play at this step. For example, the p92^{pol} RdRp is initially inactive and requires an “activation” step that only takes place in a membrane-bound complex (Pathak et al. 2012; Pogany and Nagy 2012, 2015; Pogany et al. 2008). Therefore, it seems that the translation and the replication processes take place in different subcellular environment, possibly preventing the collision between the ribosomes and the viral RdRp on the same (+)RNA template.

Other (+)RNA viruses likely separate the two processes as well, as indicated for poliovirus, whose genome contains the replication element in close vicinity to the internal ribosome entry site. When cellular factors, namely the poly(C)- and poly(A)-binding proteins bind to the poliovirus (+)RNA, then translation is promoted. However, when the (+)RNA binds to the viral replication protein 3CD, then translation is repressed and replication is launched (Gamarnik and Andino 1998; Walter et al. 2002).

Unlike the cellular mRNAs, which are usually destined for degradation after translation, the viral (+)RNA is rescued by selective interaction with the viral replication protein(s). In case of TBSV, the specific viral (+)RNA template recognition within the heterogeneous pool of host RNAs, is performed preferably in *cis* by the dimerized p33 replication protein. The *cis*-recognition means that the replication protein readily binds to the very same viral (+)RNA that serves as a template for the translation of the viral p33 protein. The TBSV p33 and the p92^{pol} replication proteins interact with each other and they both contain an arginine-rich motif (RPR), that possesses selective viral (+)RNA binding capacity (Monkewich et al. 2005; Panavas et al. 2005a; Pogany et al. 2005; Rajendran and Nagy 2006). During template selection the abundant replication cofactor, p33 binds an internal recognition element (IRE) located within the coding region of the p92^{pol} open reading frame. The specific binding between p33 and the cognate (+)RNA depends on a conserved C-C mismatch present within an extensive RNA helix, called RII(+)-SL.

2.2.4 Recruitment of the RdRp/Viral RNA Complex to Subcellular Membrane Surfaces

The current model predicts that the viral (+)RNA is recruited to the site of replication as a (+)RNA-p33 complex (Monkewich et al. 2005; Pogany et al. 2005). TBSV, similar to other (+)RNA viruses, recruits components of the VRC (i.e., replication proteins, viral (+)RNA, co-opted host factors) from the cytosol to distinct membranous subcellular compartments. The recruitment of the VRC components either occurs into preexisting membranes or in extensively reorganized membranes, such as the TBSV-induced multivesicular bodies (Barajas et al. 2009a; Russo et al. 1994). TBSV facilitates this process by membrane targeting signals located in p92^{pol} and p33 proteins and by two transmembrane domains localized close to the N terminus of these proteins. The scope of the chosen subcellular membrane types is numerous, though mostly specific in case of most viruses. TBSV and the closely related tomosvirus, such as Cucumber necrosis virus (CNV) and Cymbidium ringspot virus, replicate on the cytosolic side of peroxisome membranes (McCartney et al. 2005; Navarro et al. 2006; Panavas et al. 2005a; Pathak et al. 2008), while another tomosvirus, Carnation Italian ringspot virus (CIRV) replicates on the outer mitochondrial membrane (Weber-Lotfi et al. 2002; Xu et al. 2012). Other plant viruses target various subcellular membranes, such as endoplasmic reticulum (ER), chloroplast, or vacuolar membranes for replication (Laliberte and Sanfacon 2010; Wang 2015).

2.2.5 Assembly of the Active Viral Replicase Complex

Recent discoveries using live yeast and yeast cell-free extract (CFE)-based assays revealed three major processes guiding the functional VRC assembly (Nagy and Pogany 2012; Xu and Nagy 2014). The first one utilizes the viral (+)RNA as an assembly platform that binds to p33 and p92^{pol} replication proteins and co-opted host factors. The second process is driven by interactions between p33 replication protein, membrane-bending proteins, such as the co-opted cellular ESCRT proteins, and particular phospholipids in subcellular membranes. These interactions lead to deformation of membranes around the replicase complex. The third process is the activation of the RdRp function of p92^{pol} replication protein within the membrane-bound VRC. *In vitro* experiments with TBSV revealed, that the activation of p92^{pol} replication protein requires two *cis*-acting elements in the TBSV (+)RNA, the p33 replication co-factor as well as cellular co-factors such as heat shock protein (Hsp70) and neutral lipids in the host cell membrane (Pogany and Nagy 2012; Pogany and Nagy 2015).

Many (+)RNA viruses, similar to TBSV, induce membrane invaginations (called spherules) with narrow openings during VRC formation in given membranous subcellular compartments. Other (+)RNA viruses induce double-membrane vesicles or both single- and double-membrane vesicles (Romero-Brey and

Bartenschlager 2014; Wang 2015). TBSV and BMV induce ~70 nm diameter vesicular invaginations both in plant and yeast cells. Spherule induction most likely helps the virus evade from the cellular defense mechanism and protects the viral RNA from degradation. Altogether, the subcellular compartmentalization of the membrane-bound activated VRC prevents not only the collision between the ribosome and the RdRp, but this strategy also avoids viral RNA synthesis in the cytosol that would induce dsRNA-triggered antiviral defense mechanism of the host (Romero-Brey and Bartenschlager 2014; Wang 2015).

2.2.6 Viral (+)RNA Replication Leads to the Production of dsRNA Inside VRC

After the VRC assembly and activation of the p92^{pol} RdRp, (–)RNA synthesis starts from the 3' end of the genomic (+)RNA guided by the gPR promoter sequence. Because the VRC contains both the original (+)RNA and the newly synthesized (–)RNA, the question arises: What is the form of the replication intermediate? Is there any free (–)RNA that can be utilized for new (+)RNA synthesis? It has been shown with the help of *in vitro* experiments that naked (–) RNA does not seem to exist in the VRC at any time during replication. In stead, the (–)RNA is sequestered into double-stranded (ds)RNA, which appears before the robust production of (+)RNA progenies (Kovalev et al. 2014). Interestingly, the dsRNA is used by the RdRp via a strand-displacement mechanism, where the newly made (+)RNA replaces the previously synthesized (+)RNA in the dsRNA intermediate. This strategy ensures the temporal partition of the (–)RNA and (+)RNA synthesis within the VRC and likely provides the means to produce one (–)RNA per VRC and the generation of 20-to-100 (+)RNA progenies (Kovalev et al. 2014). Also, the dsRNA structure might control RdRp activities by supporting only new (+)RNA synthesis with the help of co-opted cellular helicases (Chuang et al. 2015).

2.2.7 Extensive (+)RNA Synthesis in VRCs

As during the (–)RNA synthesis, the viral RNA also regulates (+)RNA synthesis with the help of RNA structure and *cis*-acting elements that bind to protein co-factors. Briefly, the dsRNA structure of the replication intermediate represses the use of *cis*-acting elements on the (+)RNA part of the dsRNA template (Kovalev et al. 2014). However, the *cis*-acting elements in the (–)RNA portion of the dsRNA intermediate become accessible for the RdRp due to interaction with co-opted cellular helicases (Kovalev and Nagy 2014; Kovalev et al. 2012b). To initiate (+)RNA synthesis, the dsRNA intermediate structure must be opened within the cPR sequence. The role of different host factors involved in this process will be discussed below.

2.2.8 Release of (+)RNA Progeny from VRCs

Since (+)RNA virus replication occurs in membranous environment, while other viral processes with (+)RNA take place in the cytosol, there must be mechanism to release the viral (+)RNA from VRCs. Currently not much is known about the release of the viral (+)RNA from VRCs, or whether it is an active or passive mechanism, but it is assumed that VRCs with spherule structures likely use the narrow opening, called neck, to release the new (+)RNA progeny into the cytosol. The release of the (+)RNA through the neck provides a path for the newly synthesized (+)RNA to become encapsidated by the viral coat proteins in the vicinity of the spherules where the virion assembly takes place (Rao et al. 2014).

One full cycle of (+)RNA virus replication from template selection until the release of the new (+)RNA progeny is likely carried out in 2–3 h based on *in vitro* replicase assembly studies (Pogany and Nagy 2008; Pogany et al. 2008). A newly assembled VRC could start releasing new (+)RNA progeny in ~1 h. However, a fraction of the released viral (+)RNAs likely returns to a new round of translation/replication cycle in the infected cells that further enhance the amount of viral progeny. It is estimated that plant (+)RNA viruses might perform as many as twenty replication cycles in a sequential manner [i.e., the (+)RNA product of the previous replication cycle is the template for the new cycle] in single plant cells in ~48 h, resulting in the production of 100,000 to a million progeny (+)RNAs per cell (Miyashita et al. 2015). To achieve this massive production of progeny, many plant RNA viruses convert the host cells into viral replication factories, as explained in the following subchapters.

2.3 Yeast as a Model System to Study (+)RNA Virus Replication

(+)RNA viruses are intracellular infectious agents with limited coding capacity. Therefore, these viruses have developed sophisticated ways to co-opt numerous cellular factors to facilitate the viral infectious cycle. To understand virus-host interactions, it is necessary to identify all the host components that are subverted for viral infections. One major hurdle to implicitly dissect the interactions between a (+)RNA virus and its host is the still scarce availability of powerful experimental tools to manipulate the host's genome or proteome. Yeast with facile genetics is a model cellular eukaryotic organism, which possesses many archetypal aspects of fundamental cellular mechanisms. These include a whole set of eukaryotic chaperones, protein modifying factors, the ubiquitin/proteasome system, the vesicle trafficking and the secretory pathway, the components of mitochondrial and peroxisomal biology as well as the factors of lipid homeostasis and membranous structures. Another advantage is that these cellular processes and the players involved are the best characterized in yeast. Yeast was the first eukaryotic genome fully sequenced. The yeast genome codes for ~6000 genes and more than 75 % of

the genes have assigned functions (<http://www.yeastgenome.org/>). Besides the rapid growth and easy maintenance of the yeast cultures, the availability of wide collections of libraries, such as the gene deletion library, the essential gene knock-down library (Yeast tet promoter Hughes Collection), the GFP-tagged protein expression collection, the protein over-expression library, or the temperature-sensitive library of essential genes (Gelperin et al. 2005; Huh et al. 2003; Janke et al. 2004; Tong et al. 2001, 2004) render the yeast a very attractive model platform. Large-scale and high-throughput approaches and different molecular toolboxes have been developed to tag or delete genes and change promoters in the yeast genome (Hegemann and Heick 2011; Janke et al. 2004; Yofe et al. 2014). GFP and other fluorochrome tags fused to the yeast protein (either expressed from a plasmid or the chromosome) and to the viral proteins enable the simultaneous detection of the subcellular localization of the given proteins by confocal laser microscopy. The images are collected separately for each fluorochrome and then merged to detect whether the localizations of the proteins of interest overlap in the same subcellular compartment. Thus, the redistribution of host proteins due to virus infection or the altered localization of viral proteins in a mutant yeast background or the relative re-localizations of both viral and host proteins can be visualized in live cells. Yeast is a model system for the deduction of functional and mechanistic aspects of proteins, protein networks or lipid homeostasis shared by eukaryotes. Moreover, yeast is useful for the heterologous expression of human or plant proteins for assessment of their functions, which revealed enormous knowledge about various disease states. Examples are amongst defects in DNA mismatch repair (Gammie et al. 2007), pathogenic human mitochondrial gene mutations (Lasserre et al. 2015), defects in RNA processing (Sun et al. 2011) and even neurodegenerative diseases (Braun et al. 2010). The latter sounds surprising, however yeast shares many conserved pathways with higher eukaryotes that are known objects of susceptibility in neurodegenerative diseases.

2.3.1 Development of Viral Replication Systems in Yeast

A plant (+)RNA virus, namely BMV, was the first to be studied in yeast by the Ahlquist group (Janda and Ahlquist 1993; Price et al. 1996). In addition to BMV, the list of viruses studied in yeast includes TBSV and related tombusviruses, such as CIRV, CNV, and Cymbidium ringspot virus and members of alphanodaviruses (Flock house virus and Nodamuravirus) (Panavas and Nagy 2003; Pantaleo et al. 2003; Pogany et al. 2010; Rubino et al. 2007).

To achieve high level of TBSV (+)RNA accumulation in yeast, a small replicon (rep)RNA derived spontaneously from the full-length genomic RNA via multiple deletions was utilized (Panavas and Nagy 2003; White and Morris 1994). The short repRNA retains the collection of *cis*-acting elements essential for replication to ensure efficient multiplication. Interestingly, the repRNA does not code for proteins (also lack the expression of a selection protein), so its replication depends on the replication proteins provided by the helper virus or expressed from plasmids. Consequently, the repRNA is adapted to utilize replication components in *trans*.

When the repRNA accumulates, it slows down the replication of the helper virus as it competes for the same cellular resources as the helper virus. Hence the repRNA is also called defective interfering RNA (DI) (Pathak and Nagy 2009; White and Nagy 2004). When the replication proteins are ectopically expressed from plasmids or a yeast chromosome, then the repRNA replicates and depends on cellular resources in a largely similar manner to the viral genomic RNA as shown in several publications (Panavas and Nagy 2003; Nagy 2008; Nagy and Pogany 2010). Altogether, the use of repRNAs for TBSV or CIRV in replication studies is useful to dissect replication mechanisms, and to understand how these viruses exploit and reconstitute the cellular milieu the same way as it happens in the natural hosts.

2.3.2 Using Yeast to Obtain *In Vitro* Replication Systems

To dissect the mechanism of (+)RNA virus replication and characterize the functions of viral and co-opted host components, it is useful to develop *in vitro* approaches, which allow researchers to control components and conditions. Accordingly, two *in vitro* approaches based on yeast have been developed for TBSV. The first is based on the affinity purification of the assembled active replicase complex containing the viral replication proteins and several host factors after detergent-based solubilization of yeast membranes (Panaviene et al. 2004, 2005; Serva and Nagy 2006). Then, the purified replicase preparations could be tested *in vitro* for the efficiency to synthesize (+)RNA or (–)RNA depending on the external RNA template added. The advantage of using yeast, instead of TBSV-infected plant cells (Nagy and Pogany 2000) is that various yeast mutants can be used for preparation of the replicase, thus easily obtaining replicase preparations with altered/missing cellular components.

The second powerful approach to dissect the molecular mechanisms is based on yeast CFE. The CFE preparations can support one complete cycle of replication of the TBSV repRNA or the genomic RNA if the viral (+)RNA template, purified recombinant p33 and p92^{pol} replication proteins, and ribonucleotides are provided in the *in vitro* assay. The reconstituted CFE-based assay includes all the known replication steps (Pogany and Nagy 2008; Pogany et al. 2008, 2010). Therefore, the yeast CFE-based assay could be used to separately study the roles of membrane and lipid components as well as various host proteins required for RNA template recruitment, replicase assembly, RdRp activation, (–)RNA and (+)RNA synthesis. CFEs prepared from yeasts with different genetic background can help dissect the functions of not only individual components, but protein families, or even series of host factors that mediate a certain subcellular pathway or cellular networks. Importantly, the CFEs prepared from mutant yeast strains can be complemented with purified recombinant proteins or artificial lipids added back to the *in vitro* reaction. Yeast CFEs can also be used to test the antiviral effects of various chemicals or different conditions that may inhibit virus replication. The yeast CFEs can also be fractionated and subcellular organellar membranes, such as ER, mitochondria or peroxisomes and even artificial lipid vesicles in combination with soluble fraction

of yeast CFE could be used for *in vitro* replication studies with TBSV (Xu et al. 2012; Xu and Nagy 2015). Altogether, the combinations of live yeast and CFE-based *in vitro* approaches greatly facilitate the progress towards the complete understanding of a virus-host interaction system at the molecular and cellular levels.

2.4 Insights into the Intricate Virus-Host Interactions

A major advance made with yeast in plant virus-host interaction studies is the identification of host factors based on systematic genome-wide screens with yeast genomic libraries. Accordingly, the highthroughput screens were conducted with BMV and TBSV that led to the identification of over 100 yeast genes affecting either BMV or TBSV replication (Gancarz et al. 2011; Kushner et al. 2003; Panavas et al. 2005b; Serviène et al. 2005). Unfortunately, systematic genome-wide screens have not been conducted with plant RNA viruses in plant hosts.

Additional yeast-based screens with TBSV, including the yeast essential gene library, temperature-sensitive (ts) mutant library, and high-throughput over-expression of ~5,500 yeast genes in wt yeast contributed to the identification of ~250 additional host proteins that could affect TBSV replication (Jiang et al. 2006; Serviène et al. 2006; Shah Nawaz-UI-Rehman et al. 2012, 2013). Global proteomic-based screens with a yeast protein array carrying ~4,100 purified proteins that covers almost all soluble yeast proteins has led to the identification of 57 yeast proteins interacting with tombusvirus p33 replication protein, and 11 host proteins bound to the unique portion of tombusvirus p92^{pol} or to the TBSV repRNAs (Li et al. 2008, 2009). Moreover, yeast membrane-based two-hybrid assay (MYTH) with yeast cDNA libraries also led to the identification of novel set of host proteins interacting with p33 replication protein (Mendu et al. 2010). Altogether, four separate genomics and four proteomics screens with TBSV in yeast have led to the identification of ~500 yeast genes that could be involved in TBSV replication. These systems level approaches make the TBSV-yeast system one of the best characterized pathogen-host systems at the cellular level (Nagy 2011; Nagy and Pogany 2010). Exploiting the above invaluable data sets, detailed mechanistic studies with many of the identified host factors have led to a deeper understanding of plant virus- host interactions, as discussed below.

2.4.1 Membrane Rearrangements and Spherule Formation to Harbor the Viral Replicase Complex

Several plant (+)RNA viruses, including TBSV and BMV, induce the formation of numerous vesicle-like membranous structures that harbor the VRCs (den Boon and Ahlquist 2010; Wang 2015). Most of these virus-induced structures, called

spherules, contain narrow openings toward the cytosol to allow entry of metabolites/ribonucleotides and the escape of the produced viral (+)RNAs (Fig. 2.2a). But how are these intricate structures that are likely stable for several hours formed? Interestingly, genome-wide screens in yeast have identified that both TBSV and BMV subvert cellular membrane bending/remodeling proteins, including the so-called endosomal sorting complex required for transport (ESCRT) machinery (Barajas et al. 2009a, 2014a; Diaz et al. 2015). The ESCRT machinery is conserved across kingdoms of life and is required for the formation of intraluminal vesicles (ILVs) during the generation of multivesicular bodies (MVBs). The ESCRT complex plays a role in the sorting of membrane bound proteins into the MVB pathway to degrade cargo proteins and lipids in the vacuole (Hurley 2015). TBSV co-opts the ESCRT machinery via direct interactions between the viral replication proteins and Vps23 (ESCRT-I member) or Bro1 accessory ESCRT protein, leading to the relocalization of Vps23 and Bro1 to the peroxisome membrane, the site of TBSV replication. This is followed by the sequential recruitment of additional ESCRT proteins that bend the membrane away from the cytoplasm towards the lumen of membranous organelles due to the induction of negative curvatures in the membrane bilayer. Finally, TBSV recruits the ESCRT-associated Vps4 AAA+ ATPase and some auxiliary proteins, which would normally assist the disassembly of the ESCRT complex and leading to membrane scission to create ILVs (Hurley 2015). However, Vps4 function in membrane scission is likely blocked by interaction with p33 replication protein, thus stabilizing the spherule structure (Barajas et al. 2014a). When Vps4 is deleted in yeast, then the neck structure of the spherules remains wide and the replicase complex is no longer protected from the host defense surveillance system (Fig. 2.2b) (Barajas et al. 2014a).

The subversion of the ESCRT machinery by TBSV is critical for replication since in *vps23Δ* yeast, TBSV replication drops dramatically and the ribonuclease sensitivity of the viral (–)RNA is increased when compared to the wt yeast (Barajas et al. 2009a). Another tombusvirus, the mitochondria-based CIRV also recruits Vps23 via direct interaction with the replication protein (Richardson et al. 2014).

The replication of BMV RNA is also dependent on the membrane shaping function of the ESCRT complex in yeast (Diaz et al. 2015). BMV 1a replication protein binds to and recruits Snf7 (ESCRT-III member, also required for TBSV replication) to form spherules. The BMV replicase complex formation also depends on additional membrane shaping proteins, called reticulons, which seem to be dispensable for TBSV. The need of reticulons may seem surprising at first glance as they usually induce and stabilize positive membrane curvatures. BMV could still induce the formation of spherules in reticulon depleted cells, but the spherules are much smaller, ~ 30 nm compared to the original ~70 nm diameter (Diaz et al. 2010). Also protein 1a is not able to recruit the viral RNA template to the site of replication. It seems likely that reticulons are usurped inside the spherule to help expand the negative membrane curve via intercalating short opposing, positive curves from space to space. And also the co-opted reticulons may stabilize the

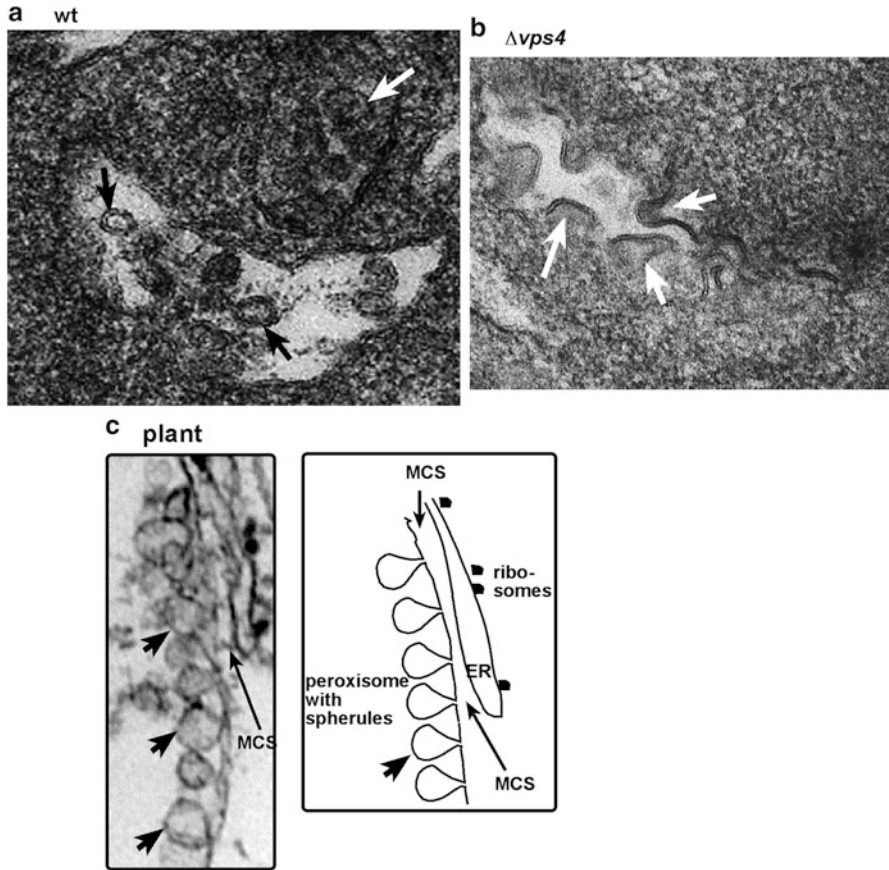


Fig. 2.2 The role of Vps4 ESCRT protein and membrane contact site (MCS) in the formation of spherule-like structures induced by tobusvirus replication proteins. (a) TEM of stained ultra-thin sections of wild type yeast cells replicating TBSV RNA with characteristic membranous compartments with tobusvirus-induced spherules. *Arrows point* to the spherules within the intracellular compartment. (b) *vps4* Δ yeast cells contain crescent-shaped membranes, which face the lumen of the compartment, but apparently fail to complete the spherule constriction since they have wide openings to the cytosol (*white arrows*). (c) The presence of MCS-like structures in the vicinity of tobusvirus-induced spherules in plant cells infected with CNV. Representative electron microscopic images of portion of a *N. benthamiana* cell. Several characteristic virus-induced spherules are marked with *arrowheads* and the MCS-like structures are indicated by *arrows*. These spherules are formed via membrane invagination into peroxisome-derived membranes

positively curved neck region in the spherule (Diaz et al. 2010). Importantly, the requirement for the co-opted cellular membrane-shaping ESCRT proteins has been confirmed in plants for both TBSV and BMV, further justifying the use of yeast as a model to dissect (+)RNA virus replication process (Barajas et al. 2009a, 2014a; Diaz et al. 2015).

2.4.2 (+)RNA Virus Replication Depends on Lipid Biosynthesis and Intracellular Lipid Transport

The genome-wide screens for host factors affecting TBSV and BMV replication also revealed roles for enzymes involved in lipid biosynthesis and intracellular transport (Kushner et al. 2003; Panavas et al. 2005b; Serviène et al. 2005). For example, deletion of yeast genes involved in sterol or phospholipid biosynthesis greatly hinders TBSV replication (Sharma et al. 2010, 2011). Interestingly, TBSV replication induces the upregulation of phospholipid synthesis, especially that of PE (phosphatidylethanolamine), which becomes highly enriched at the sites of TBSV or CIRV replication (Barajas et al. 2014c; Xu and Nagy 2015).

Why are lipids so important for (+)RNA virus replication? Cellular membranes are built from lipid bilayers that contain multitude of different lipids and proteins. Phospholipids, which are the major lipids in the membranes, contain a polar head group and a long hydrophobic chain that points towards each other in a membrane bilayer. The different charges of lipids modify the physical features of the membrane, and may block or promote the assembly and activity of the replicase. Indeed, while neutral lipids are advantageous, negatively charged lipids, such as phosphatidylglycerol (PG) has inhibitory effect on template recruitment and on tombusvirus RdRp activation (Pogany and Nagy 2015; Xu and Nagy 2015). In addition to the phospholipids, the cell membrane is tucked with sterols and covered with glycolipids. Lipids affect the fluidity and thickness of organellar membranes, and affect membrane curvature. Yeast with well-defined lipid metabolism could serve as an outstanding model to dissect the role of various lipids in plant (+)RNA virus replication. Accordingly, yeast and plant lipidomics corroborated that PE content is higher in hosts supporting TBSV replication than in the control, virus-free hosts (Xu and Nagy 2015). An interesting feature of PE is that PE promotes negative membrane curvature that could be beneficial during spherule formation. Hence it is possible that PE enrichment in membranous microdomains is used by other (+)RNA viruses to build spherules.

If lipids are so important for (+)RNA virus replication, then how can the virus subvert those lipids? The emerging picture about TBSV-yeast interaction is that TBSV channels sterols and possibly phospholipids to the site of replication by co-opting lipid-binding proteins. For example, the p33 replication protein binds oxysterol binding protein related proteins (ORPs) and VAP proteins in yeast and in plants and hijacks them to the membranous compartment where VRCs form (Barajas et al. 2014b). VAP proteins are present in all eukaryotes and are known to establish membrane contact sites (MCS), where subcellular membranes are juxtaposed and the microenvironment becomes suitable for sterol transfers (Fig. 2.2c) (Lahiri et al. 2015). Both p33 replication protein and the cellular VAPs bind ORPs and recruit them to MCSs. ORPs deliver sterols from the ER to the acceptor membranes at MCSs to increase sterol concentrations locally and to facilitate membrane bending during VRC formation. *In vitro* experiments with artificial vesicles demonstrated that the activity of the replicase was stimulated by the addition of sterols (Barajas et al. 2014b). The current model predicts that via recruiting VAPs and ORPs, TBSV facilitates the formation of MCSs and triggers

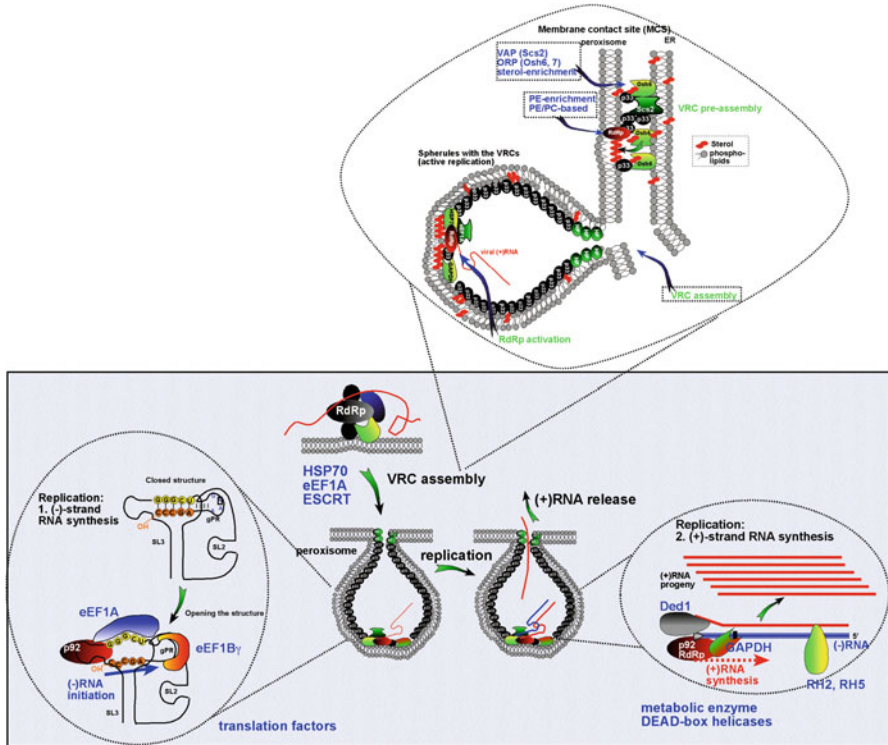


Fig. 2.3 Co-opted proviral host factors facilitate TBSV replication. The assembly of the membrane-bound tobusvirus VRCs is affected by the three shown yeast proteins or protein family (in blue). *TOP*: The VRC formation is facilitated by the stabilization of membrane contact site (MCS) between ER and peroxisome by p33 and the co-opted VAP (the yeast Scs2) and ORPs (members of the yeast Osh family). The function of MCS is to enrich sterols and possibly phospholipids (such as PE) at the viral replication sites (indicated by the vesicle-like spherule structure). *Left*: The minus-strand synthesis by the viral RdRp protein (p92, red oval) is regulated by two translation factors. *Right*: The synthesis of the more abundant (+)RNA (using the dsRNA replication intermediate) is assisted by subverted Ded1 and RH2/RH5 DEAD box helicases and GAPDH metabolic enzyme

sterol enrichment to aid the formation of spherules containing VRCs (Fig. 2.3) (Barajas et al. 2014b).

In addition to the above selective enrichment of sterols at replication sites, TBSV also induces membrane proliferation via generation of new membranes. This is achieved in yeast via interaction of p33 replication protein with the yeast Opi1 and Scs2 (a VAP) proteins, which are phospholipid sensors and Opi1 represses the transcription of phospholipid biosynthesis genes (Barajas et al. 2014c). When p33 binds Scs2 and Opi1 in the ER, then the suppression of phospholipid genes (such as *INO1*, *OPI3* and *CHO1*) is relieved and phospholipids are increasingly synthesized (Barajas et al. 2014c). This observation suggests that TBSV can also utilize *de novo* synthesized phospholipids. Accordingly, deletion of

OPI1 repressor increases TBSV RNA accumulation in yeast and stimulates the activity of the replicase in the CFE assay (Barajas et al. 2014c).

2.4.3 Subcellular Locations for (+)RNA Virus Replication

One of the intriguing aspects of (+)RNA virus replication is the variation of the sites for VRC assembly in spite of the common dependence of these viruses on subcellular membranes. Why does one (+)RNA virus favor a particular subcellular location over the other locations, while another related or unrelated (+)RNA virus prefers a different location? For example, TBSV favors the peroxisomal membrane for VRC assembly in yeast and plants (McCartney et al. 2005; Panavas et al. 2005a), while the closely related CIRV selects the outer mitochondrial membrane (Weber-Lotfi et al. 2002). Yeast and CFE-based studies also help to gain insights into this question. Elimination of peroxisomes via deletion of peroxisome membrane-biogenesis genes, such as *PEX3* or *PEX19*, in yeast has not inhibited TBSV replication, which “switched” to the ER membranes for VRC assembly (Jonczyk et al. 2007). Also CFE-based work with isolated ER or mitochondria from yeast revealed that TBSV could efficiently replicate in the ER membrane and to a lesser extent in the mitochondrial membrane *in vitro* (Xu et al. 2012). Similarly, the insect virus FHV replication can be retargeted from the mitochondrial membrane to the ER without adverse effects at the cellular level (Miller et al. 2003). Thus, (+)RNA viruses seem to be flexible to some extent in their abilities to exploit various subcellular membranes.

However, our understanding of the roles of various organellar membranes in plant (+)RNA virus replication is far from complete. For example, down-regulation of ER resident secretory proteins that play essential role in peroxisome biogenesis affected TBSV replication negatively (Sasvari et al. 2013a). This suggests that the early steps in peroxisome membrane formation are important for TBSV to replicate. Thus, even if the presence of fully matured peroxisome is dispensable and TBSV can assemble VRCs in the ER, it is still important to initiate peroxisome-like membranes for TBSV. It is possible that proximity of various organelles is important for TBSV to reorganize subcellular membranes- accordingly, peroxisome and mitochondria are, in general, in close vicinity to the ER membranes and they regularly transport/exchange metabolic compounds, sterols and lipids (Lahiri et al. 2015).

2.4.4 Co-opted Heat Shock Proteins and Activation of the Viral RdRp

The sophisticated nature of plant (+)RNA viruses is obvious in many subchapters described here, yet one of the unexpected faces of virus replication is the dependence on cellular “house keeping” proteins. A fascinating example is the discovery

of the virus replication-associated role of heat shock protein 70 (Hsp70), which is a molecular chaperone involved in refolding of misfolded cellular proteins. There are three groups of cytosolic Hsp70 chaperones coded in the yeast genome. One group is termed Ssa1-4. Ssa1 and Ssa2 are constitutively expressed and 98 % identical, while Ssa3 and Ssa4 are stress-inducible and 80 % identical to Ssa1/2. Other Hsp70 chaperones in yeast are the ribosome associated Ssb1-4 group, and also the Sse1-4 group. Interestingly, the purified TBSV replicase contained the yeast Ssa1/Ssa2, as determined by 2D-gel electrophoresis and mass-spectrometry analysis (Serva and Nagy 2006). Hsp70 is a highly conserved protein family and it is involved in folding of newly synthesized and refolding of misfolded/aggregated proteins; protein degradation; protein translocation across, or insertion into the membrane; protein complex assembly and disassembly and receptor signaling (Daugaard et al. 2007; Qu et al. 2015). Contributions of Hsp70s to various virus infections were reported, however, characterization of the specific role of Hsp70s in virus replication is far from being straightforward. It is widely observed that at early time of infection, Hsp70 level goes up in response to the affliction of the cell. In general, Hsp70s are mostly involved in co- or posttranslational folding of the viral proteins; however they may also play specialized roles in (+)RNA virus replication (Nagy et al. 2011). Accordingly, specialized pro-viral role of Hsp70s has been discovered in case of TBSV replication. Ssa1-4 are interchangeable for TBSV replication, hence to dissect the mechanism behind the involvement of Hsp70 in the replicase complex, double or triple mutant yeast strains had to be used. It was found that *ssa1Δ ssa2Δ* double mutant yeast supported TBSV replication only marginally, which observation was validated in plants by applying Hsp70 inhibitors to the leaves (Serva and Nagy 2006; Wang et al. 2009a). Further analysis revealed that Ssa1/2 is diverted from its cytosolic distribution to the peroxisome membrane by p33 and p92^{pol} replication proteins. If all the four SSA genes were deleted, yeast cannot grow. However, the simplicity of yeast reverse genetics allows the combinations of diverse mutations. Thus, using a yeast strain harboring ts mutant Ssa1 and lacking *SSA2-4*, the pro-viral function of Ssa1^{ts} can be debilitated or partially debilitated at elevated temperature. Under these circumstances functional VRC could not assemble (Wang et al. 2009b). CFE-based TBSV replication assay also corroborated that Ssa1 (in the absence of the other Ssa members) is essential for VRC assembly and activation of the RdRp function of p92^{pol} (Pogany and Nagy 2015; Pogany et al. 2008). Taken together, using the yeast model platform, distinct functions of Hsp70 chaperones in TBSV replication could be determined. Ssa1/2 proteins are essential for the early steps of TBSV replication: for the recruitment of p33 and p92^{pol} to the membrane, membrane insertion of the replication proteins, VRC assembly and activation of p92^{pol}, while Hsp70s are dispensable for subsequent minus- and plus-strand synthesis.

Besides Hsp70 chaperones, members of the Hsp90 and the J-domain-containing Hsp40 families are often utilized by viruses (Nagy et al. 2011). For example, Ydj1 Hsp40 co-chaperone, which regulates Hsp70 and Hsp90 functions, affected FHV accumulation on the mitochondrial membrane (the native site of replication for FHV) in yeast. Moreover, *in vitro* experiments revealed that Ydj1

is required for the assembly of the FHV replicase complex and for the stability of FHV RdRp. However lack of Ydj1 had little if any effect when FHV replication was retargeted to the ER. This result demonstrates that cellular chaperones may have subcellular membrane-specific differences (Weeks and Miller 2008; Weeks et al. 2010). Ydj1 is also required for BMV RNA replication, though BMV replicates on the ER membrane in yeast. Ydj1 maintains the cytosolic solubility of the BMV 2a polymerase prior to membrane integration but does not affect the recruitment of 1a and 2a proteins to the ER. Despite the correct integration of the BMV replication proteins into the membrane, (–)RNA synthesis is hindered when Ydj1 is mutated in yeast. This suggests that Ydj1 might be needed for the activation of the BMV replicase complex (Tomita et al. 2003) and that Ydj1 has somewhat similar functions in the replication of FHV and BMV. Overall, the above studies on the role of heat shock proteins and their associated J-domain co-chaperones have been greatly facilitated by the facile genetics of yeast, indicating that the challenges with multimember protein families could be overcome in yeast cells.

2.4.5 Complex Roles of Co-opted Host Proteins During Viral RNA Synthesis

The central process in (+)RNA virus replication is RNA synthesis, which generates the new infectious progeny (+)RNAs. This process is driven by the viral-coded RdRp, but co-opted host proteins likely affect RNA synthesis. Accordingly, proteomic-based screens led to the identification of eukaryotic translation elongation factor 1A (eEF1A) as a component of the purified tombusvirus replicase and an interactor with the viral replication proteins as well as the viral RNA (Li et al. 2009, 2010, 2014). eEF1A bears multiple cellular functions, including its canonical role to deliver aminoacyl tRNA to the ribosome. However, other cellular functions, such as quality control of newly produced proteins, ubiquitin-dependent protein degradation, and organization of the actin cytoskeleton were also assigned to this highly abundant protein (Mateyak and Kinzy 2010). Interestingly, eEF1A selectively stimulates TBSV (–)RNA synthesis by acting as a “matchmaker”, via facilitating the interaction between p92^{P₀₁} and the gPR promoter at the 3′ end of (+)RNA (Fig. 2.3) (Li et al. 2010). However, eEF1A does not function alone, but acts synergistically together with another translation factor, called eEF1B γ , in the TBSV replicase complex. eEF1B γ binds to the stem-loop structure of gPR that leads to the opening up the RNA-RNA interaction between gPR and the RSE (Sasvari et al. 2011). This open configuration of gPR and RSE facilitates the binding of eEF1A and p92^{P₀₁} to the 3′ end, and ultimately promotes (–)RNA synthesis (Fig. 2.3) (Sasvari et al. 2011). Thus, the interplay among co-opted cellular translation factors, the TBSV RdRp and the template (+)RNA regulates (–)RNA synthesis within the membrane-bound VRCs.

In addition to the above described role in (–)RNA synthesis, eEF1A plays additional roles in TBSV replication, including enhancing the stability of p33 replication protein and promoting VRC assembly (Li et al. 2010). Therefore, eEF1A is an elegant example that (+)RNA viruses could co-opt cellular protein(s) to perform multiple pro-viral functions. Accordingly, the replications of numerous plant and animal (+)RNA viruses are affected by eEF1A (Mateyak and Kinzy 2010; Thivierge et al. 2008). The detailed role of eEF1A in (–)RNA synthesis was also highlighted in case of West Nile virus (Brinton 2014).

For a long time it was an open question if the same proteins are involved in (–)RNA synthesis as in (+)RNA synthesis. This is because the promoter sequences and enhancer/silencer *cis*-acting elements are different both in sequences and structures in the (+)RNA versus the (–)RNA. How can the same RdRp recognize all these elements and perform the asymmetrical RNA synthesis leading to excess amount of (+)RNA over the (–)RNA during the course of replication? Answers to these questions start to emerge for TBSV based on yeast and CFE replication assays. The high-throughput screens helped identify the essential DEAD-box RNA helicase, Ded1, which selectively affects TBSV (+)RNA level (Kovalev et al. 2012b). Another co-opted cellular protein, GAPDH (glyceraldehyde-3-phosphate dehydrogenase coded by Tdh2 and Tdh3 in yeast), which was identified via a proteomic approach, is also sequestered to the TBSV replicase complex and affect (+)RNA level (Wang and Nagy 2008). The identification of these cellular proteins in the tombusvirus VRCs and their effects mostly on (+)RNA levels strongly suggested that host proteins involved in (–)RNA and (+)RNA synthesis are not the same.

Although many (+)RNA viruses code for helicases that likely facilitate unwinding of RNA structures or remodeling protein-RNA complexes, small (+)RNA viruses, like TBSV, do not code for helicases. However, the emerging picture is that TBSV recruits several cellular helicases to facilitate (+)RNA synthesis. The first subverted helicases characterized were Ded1 and Dbp2, which have partially redundant functions during TBSV replication. Both Ded1 and Dbp2 bind to the 3' end of the (–)RNA and, in an ATP-dependent manner, and facilitate (+)RNA synthesis (Kovalev et al. 2012a, b). The major function of Ded1/Dbp2 is to open up the dsRNA intermediate only at one of the ends, which harbors the cPR [i.e., 3' end of the (–)RNA]. This then allows the loading of the p92 RdRp onto the 3'-end of the (–)RNA, followed by initiation of (+)RNA synthesis guided by the cPR sequence (Fig. 2.3). Interestingly, Ded1 also facilitates the release of the p92 RdRp from the (+)RNA when the RdRp is paused (usually at the end of the template when complementary RNA synthesis is accomplished) (Chuang et al. 2015). Therefore, these functions of co-opted Ded1 help the RdRp switch from (–)RNA to (+)RNA synthesis. The Arabidopsis homolog of Ded1/Dbp2, called AtRH20, also promotes (+)RNA synthesis in a yeast CFE-based assay, suggesting that plant helicases with corresponding functions are present in plant hosts (Kovalev et al. 2012a).

Although the formation of dsRNA intermediate during (–)RNA synthesis (Kovalev et al. 2014) prevents new (–)RNA synthesis due to “burying” the gPR and other *cis*-acting sequences within the dsRNA structure, while allowing (+)RNA

synthesis with the help of Ded1/Dbp2, it seems that this strategy is not robust enough to guarantee 20-to-100-fold excess of (+)RNA synthesis over (–)RNA synthesis. Indeed, TBSV recruits a second group of cellular helicases, which consist of Fal1 and Dbp3 in yeast and AtRH2 and AtRH5 in plant, to “boost” (+)RNA synthesis (Fig. 2.3). The members of this group of helicases have redundant functions and they open up the dsRNA intermediate within the RIII(–) RE sequence located close to the 5′ end of (–)RNA. Since the opening of dsRNA only takes place locally within the RE sequence, the actual 5′ end of (–)RNA and thus the 3′-end of (+)RNA carrying the gPR are still buried in dsRNA form. Interestingly, opening of RIII(–) RE brings the 5′- and the 3′-ends of (–)RNA into proximity via long range base-pairing and enhances multiple rounds of (+) RNA synthesis via repeatedly “recycling” the RdRp from termination to new round of (+)RNA initiation from the cPR sequence. Thus, the current model predicts that the coordinated actions of these co-opted cellular helicases are needed for the asymmetric accumulation of (+)RNA (Fig. 2.3) (Kovalev and Nagy 2014). Ded1 helicase was also shown to play a role in BMV replication in yeast, albeit in a different role. Ded1 was shown to selectively inhibit the translation of the viral 2a RdRp to down-regulate 2a protein level compared with the 1a replication protein (Noueiry et al. 2000).

Another RNA-binding cellular protein, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is also sequestered to the TBSV replicase complex (Serva and Nagy 2006). This protein has ample functions unrelated to its well-known glycolytic function (Sirover 2014). Yeast has two copies of GAPDH, Tdh2 and Tdh3, and at least one must be functional for viability. The replicase complex was purified from a wild type and from a mutant strain (*tdh2Δ* and down-regulated TDH3) and it was found that in the absence of sufficient amount of GAPDH, the asymmetric nature of TBSV replication was abolished, the synthesis of (+)RNA has dramatically dropped (Huang and Nagy 2011; Wang and Nagy 2008). Down-regulation of GAPDH in plant also decreased TBSV replication. Hence it seems that a very neat choreography involving the viral RNA, p92 RdRp and p33 RNA chaperone in concert with co-opted cellular helicases and a metabolic enzyme is at work to maintain the required over-production of viral (+)RNAs during infections.

2.4.6 Discovery of Cell-Intrinsic Viral Restriction Factors in Yeast

The cells are not passive “hosts” of viruses, but recognize viral components or the damage caused by the viral infection and launch various cellular responses. Moreover, cells likely have antiviral factors that guard against viruses and limit the infection process. These cellular factors are termed cell-intrinsic restriction factors (CIRFs) (Diamond and Gale 2012; Sasvari et al. 2014). The yeast-based genome-wide screens and proteomics approaches can also lead to identification of CIRFs, as

demonstrated for TBSV. For example, certain members of the Cyp40 cyclophilin family, which are peptidyl-prolyl-*cis-trans*-isomerases, strongly inhibit TBSV replication in yeast. Cyclophilins work by binding to client proteins and performing isomerisation of peptidyl-prolyl bonds. Interestingly, the yeast Cpr1 (the human orthologue is called CypA) and Cpr7 (Cyp40-like) bind to the RNA binding motif (RPR) of p33 replication protein (Kovalev and Nagy 2013; Lin et al. 2012; Mendu et al. 2010). This interaction leads to inhibition of p33-driven (+)RNA template selection and viral (+)RNA recruitment to the replicase complex (Fig. 2.4). The corresponding cyclophilins from Arabidopsis are the strongest inhibitors by reducing TBSV genomic RNA accumulation by 90 %. This result verified the anti-viral effect of Cyp40-like cyclophilins in plants.

In addition to the antiviral cyclophilins, ~70 other CIRFs were also identified by yeast library screens that impede TBSV replication. These include the WW domain proteins carrying a highly conserved structure responsible for protein-protein interactions. For example, the yeast NEDD40-like Rsp5 E3 ubiquitin ligase possesses WW domain and was identified as a very potent inhibitor of TBSV replication in yeast (Barajas et al. 2009b). Several plant derived WW domain proteins also had strong negative regulatory effect on tombusvirus genomic RNA accumulation. Interestingly, replication of FHV and NoV are also refrained by certain WW proteins in yeast (Barajas et al. 2015; Qin et al. 2012). Over-expression of certain WW-domain proteins in yeast also reduces the quantity of several host-factors co-opted in the VRCs. The amount of subverted cellular ESCRT proteins, eEF1A, GAPDH and Pex19 were found the most reduced. The current model predicts that certain proteins with WW domain prevents new VRC assembly when the availability of pro-viral proteins becomes limited (Fig. 2.4). This late-stage regulation of replication may trigger the switch from progeny RNA synthesis to virion assembly (Barajas et al. 2015).

CIRFs will likely have multiple functions and they may interact with tens or hundreds of other proteins probably manifesting diverse roles in seemingly unrelated pathways. Moreover, even if physical interaction cannot be detected between given proteins, these proteins can be genetically connected. These interactions usually are visualized as a network. The main nodes in the network, the Hub genes, have an extraordinary number of connections that interact with many unrelated pathways. To gain insight into the function of the identified CIRFs of TBSV replication, a protein network, including the identified restriction factors, has been built based on the yeast interaction map (SGD database, <http://www.yeastgenome.org>). Three Hub proteins were unveiled, Xrn1p 5'-3' exoribonuclease (Fig. 2.4), Act1p actin protein and Cse4p centromere protein (Sasvari et al. 2014). Protein network analysis of orthologous plant genes revealed three strongly connected groups, similar to those found in the yeast network. In summary, CIRFs seem to function as either direct antagonists of viral components through binding and blocking viral functions, or they may inhibit the pro-viral functions of other co-opted host proteins. Others, like cyclophilins may also act as 'guardians' by protecting cellular chaperones, like Hsp70 through inhibiting their subversion by the virus (Sasvari et al. 2014).

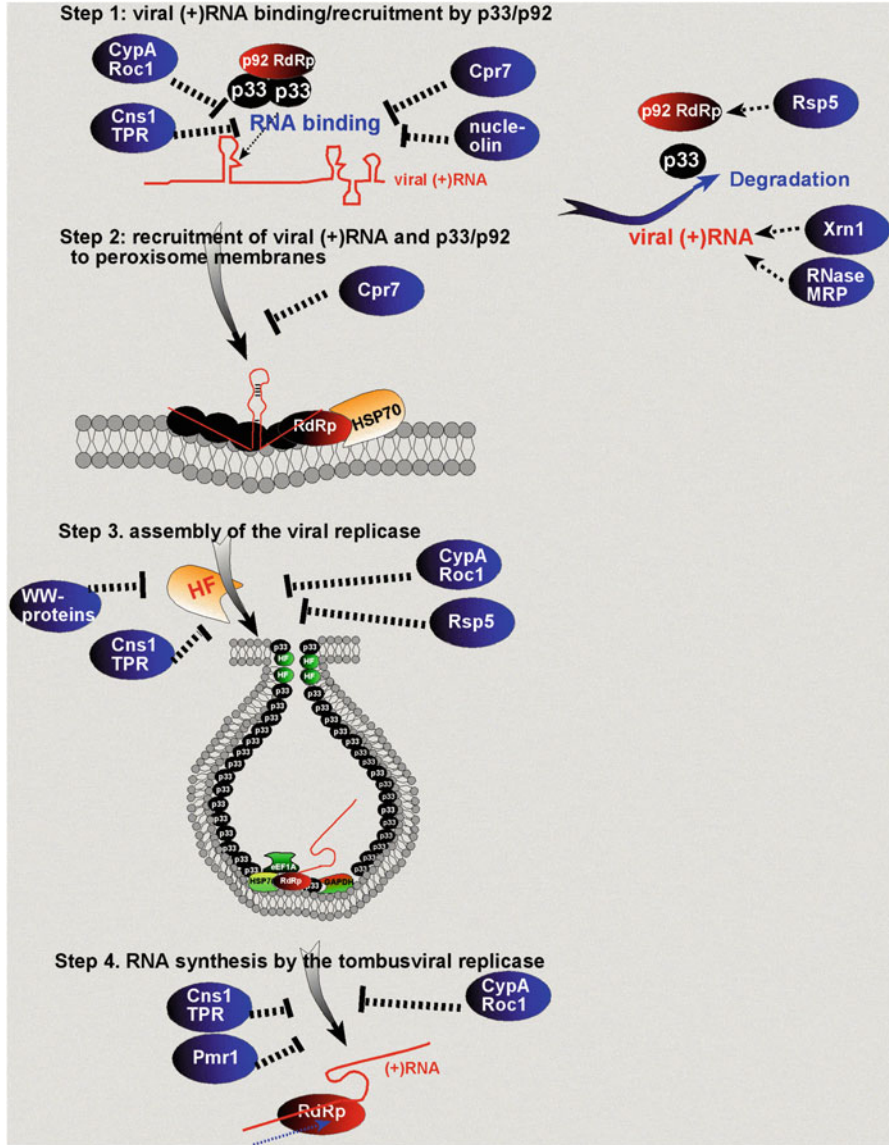


Fig. 2.4 Targets and antiviral functions of CIRFs in tombusvirus replication. The sequential TBSV replication steps and degradation of viral components (p33/p92 and the viral RNA) are shown. “HF” indicates pro-viral host factors co-opted by TBSV. The virus induced spherule (vesicle-like structure) harboring the membrane-bound VRC is shown. The detailed functions of CIRFs are described in Sasvari et al. (2014)

2.4.7 Additional Aspects of Viral Processes Dissected in Yeast: Viral Sensing of the Subcellular Environment

Overall, yeast provides a powerful platform to identify and dissect the molecular functions of cellular factors exploited by viruses throughout their infectious cycles. Because host factors are recruited to assist every step during replication and several of the host factors are kept permanently in the VRCs, therefore (+)RNA viruses should likely “sense” the molecular environment, especially the availability/accessibility of host factors as virus replication progresses with incredible speed and efficiency. If host factors became scarce/limited due to their robust exploitation during previous rounds of VRC assembly, then (+)RNA viruses will likely halt new VRC assembly. Accordingly, based on TBSV and yeast, it has been shown that the availability of several pro-viral host proteins versus the regulatory WW-domain proteins determine if new VRC assembly continues or halt (Barajas et al. 2015). The pro-viral host factors bind with higher affinity to p33 and these pro-viral host proteins will be sequestered first for VRC assembly. Then, when the pro-viral host factors become limited, the yeast WW-domain proteins, which bind to p33 with lower affinity, could bind to the TBSV p33/p92^{P_{ol}} proteins, resulting in a complex that hinders the assembly of new VRCs, blocking p33-viral (+)RNA interactions and promoting the degradation of p92^{P_{ol}} (Barajas et al. 2015). TBSV might also be able to “sense” the availability of suitable membranes for new VRC assembly through p33/p92^{P_{ol}} binding to PE versus PG phospholipids in the subcellular membranes. Binding to PE is more favoured due to its higher amount and the induction of PE synthesis and membrane proliferation by TBSV (Xu and Nagy 2015), leading to the activation of p92 RdRp and VRC assembly. However, at the late stage of infection, the availability of “free” PE might be limited, and p33/p92^{P_{ol}} could bind to the accessible PG in membranes that would block new VRC assembly and inactivate p92 RdRp (Pogany and Nagy 2015). These interactions would free the new viral (+)RNA from replication cycle to facilitate robust encapsidation.

2.4.8 A Major Effect of Cellular Ion Homeostasis on TBSV Replication in Yeast

An unexpected outcome of global screens is the identification of cellular factors involved in maintaining ion homeostasis in cells. The lipid bilayer in the subcellular membranes is impermeable for ions and polar molecules. Permeability is conferred by ion pump- or channel-proteins embedded in various subcellular membranes. Interestingly, the inactivation of PMR1, which codes for a Ca²⁺/Mn²⁺ pump, greatly increases TBSV replication and also viral RNA recombination in yeast (Jaag et al. 2010). This surprising effect by Pmr1 is due to regulation of Mn²⁺ concentration in the cytosol, which increases when pmr1 is deleted, leading to the

utilization of Mn^{2+} ions instead of Mg^{2+} by the viral replicase, which renders the replicase more active, but error prone (Jaag et al. 2010).

The deletion of another ion transporter, Gef1, strongly inhibits TBSV replication in yeast (Sasvari et al. 2013b). Gef1 is the only proton-chloride exchanger in yeast and it is responsible for the maintenance of cytosolic and organelle pH. Deletion of GEF1 transporter affects Cu^{2+} homeostasis and Cu^{2+} may replace the Mg^{2+} in the active center of viral RdRp, rendering the RdRp inactive. Indeed, deletion of Ccc2 copper ion pump also changes Cu^{2+} concentration and hampers TBSV replication in yeast (Sasvari et al. 2013b). The above discoveries show that the yeast-based TBSV replication system is highly suitable to explore the effect of ion homeostasis on (+)RNA virus replication, further contributing to our growing understanding of cellular factors affecting virus-host interactions at the cellular level.

2.5 Conclusions and Prospects

Development of yeast as a host greatly facilitated the progress in our understanding of TBSV and BMV plant viruses' interactions with the host cells. Systematic genome-wide screens using yeast genomic libraries have led to the identification of a large number of host factors affecting (+)RNA virus replication. More detailed biochemical and cellular studies then led to the dissection of molecular functions of many host factors that promote each step of the viral replication process. The development of *in vitro* systems with TBSV, such as yeast CFE and purified active replicase assays, together with proteomics, lipidomics and artificial vesicle-based assays helped to comprehend the complex nature of virus replication. Despite of the rapidly emerging details on host-virus interactions, our knowledge is far from complete. Dissection of the molecular features of viral components and their interrelationship with cellular factors may reveal non-canonical roles of host components or new features of these molecules that are only "invented" by viruses.

In a nutshell, using yeast platform can bring various cellular conditions on the same page and give an opportunity to compare the effects of viral infection-caused cellular perturbations, genetic variations, genetic disorders, protein malfunctions, and environmental factors at the systems and molecular levels. Once the processes have been characterized in yeast, then the discoveries can be further explored and applied to native organisms. Detailed knowledge on interactions from the highest resolution to the most complex systems facilitates targeted anti-viral drug design in many ways. This is because host components are less prone to genetic variations than viruses, thus drugs that block pro-viral functions of host factors are less sensitive to the threat of drug resistance. Furthermore the gained knowledge may advance virus-mediated strategies to combat debilitating genetic diseases in various organisms.

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Chapter 3

Membrane Association for Plant Virus Replication and Movement

Jun Jiang and Jean-François Laliberté

Abstract Plant viral genomes encode a limited number of proteins. These viral proteins are required for viral RNA (vRNA) replication, movement and encapsidation. At least one of these viral proteins invariably associates with cellular membranes. These membrane-associated viral proteins have multifunctional domains, notably trans-membrane domains (TMDs) and/or amphipathic helices for membrane insertion or association. Expression of these membrane-associated viral proteins induces extensive intracellular membrane remodeling that leads to the formation of viral factories. These viral factories are either static spherules within organelle membranes (e.g. mitochondrion, chloroplast), or endoplasmic reticulum (ER) – derived motile vesicles. These membranous viral factories, especially the ER-derived vesicles, not only support vRNA synthesis, but also deliver their viral infectious content to the neighboring non-infected cells, and establish the virus systemic infection. Biogenesis of these vesicles very often involves the hijacking of components of the cellular secretory pathway, and association with ER/actin network.

3.1 Introduction

Plant viruses are obligate intracellular parasites that rely on the host cell for their replication. A successful plant virus infection is a multi-step event that includes vRNA replication, movement and encapsidation. For instance, the viral infectious unit needs to build up within the primary permissive host cell. It then moves intracellularly and intercellularly through the symplasmic channel called plasmodesmata (PD) into the neighboring non-infected cells for a new round of infection. This process is repeated until the viral infectious unit finally loads into, and uploads from, the vascular tissues (phloem and/or xylem) for systemic infection (Harries

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and Ding 2011). The viral infectious unit can be in the form of virions or yet-to-be defined viral ribonucleoprotein (vRNP) complexes.

Plant viruses with a positive sense RNA [(+) RNA] genome account for the majority of plant pathogens. One property of these viruses is that viral protein synthesis is initiated as soon as the vRNA is exposed in the cytoplasm of the invaded host cell. The genome of this group of plant viruses encodes a limited number of proteins, from three to slightly more than ten proteins. The minimum set of viral proteins includes the viral RNA dependent RNA polymerase (vRdRp) and associated replication proteins (e.g. helicase), the movement protein (MP) and the coat protein (CP), exemplified by the *Tobacco mosaic virus* (TMV) (Zaitlin 1999). This limited number of proteins is enough to co-opt important cellular processes, to the point of causing on some occasions the death of the plant. vRNA replication invariably associates with cellular membranes, and takes place within virus-induced quasi-organelles known as ‘viral factories’ (den Boon and Ahlquist 2010; Laliberte and Sanfacon 2010). It is believed that these viral factories function to increase the local concentration of components required for vRNA replication. They provide a platform for the anchorage of the viral replication complexes (VRCs) and confine the replicating vRNA to a specific location that prevents the activation of host defense functions (Laliberte and Sanfacon 2010).

Several review articles published recently have elaborated on the cellular remodeling that takes place during animal and plant virus infections (Miller and Krijnse-Locker 2008; den Boon and Ahlquist 2010; den Boon et al. 2010; Laliberte and Sanfacon 2010; Laliberté and Zheng 2014). The field is, however, moving rapidly. More and more studies are providing molecular mechanisms on how viral proteins co-opt host proteins for the biogenesis of viral factories. In this chapter, we will focus on the recent findings on cellular membrane modifications during (+) RNA plant virus infections. We will explore the architecture of the virus-induced viral factories, which are either spherule-shaped or vesicle-shaped. The different cellular pathways and host proteins that lead to their biogenesis will also be presented.

3.2 Plant Virus-Induced Cellular Remodeling

Extensive endomembrane reorganization is taking place during infection by viruses. This leads to the formation of viral factories that house the VRCs. The architecture of these viral factories differs among different viruses, although similarities exist.

3.2.1 *Spherule-Shape Viral Factories*

Some (+) RNA plant virus infections lead to the formation of spherules, which are 50–400 nm sized membranous invaginations of the limiting membrane of organelles, such as the peroxisome, the mitochondrion and the chloroplast (Prod’homme

et al. 2003; McCartney et al. 2005; Hwang et al. 2008). One common characteristic of these different spherules is that they are static, in opposition to the motile, ER-derived, vesicular-shape viral factories (see below).

Infection by *Tomato bushy stunt virus* (TBSV) or *Cucumber necrosis virus* (CNV) causes the formation of multivesicular bodies (MVBs) (Fig. 3.1a) (Russo and Martelli 1972; Rochon et al. 2014). These MVBs contain spherical to ovoid compartments of 80–150 nm in diameter that are progressively formed by inward invagination of the boundary membrane of the peroxisome (Russo et al. 1983). The interior of the spherules is connected to the cytoplasm through pores (Fig. 3.1a) that allow the import of replication proteins and vRNA templates and the export of progeny vRNAs. Other viruses, such as the *Carnation Italian ringspot virus* (CIRV) and the *Melon necrotic spot virus* (MNSV), also induce the production of MVBs, but from the mitochondrial membrane (Hwang et al. 2008; Gomez-Aix et al. 2015).

In few cases, (+) RNA plant virus-induced spherules are derived from the ER membrane. *Brome mosaic virus* (BMV) induces the invagination of the perinuclear ER membrane, leading to the formation of 50–70 nm spherules in its surrogate host yeast cell (Fig. 3.1b) (Schwartz et al. 2002). Under certain situations, the large, karmellae-like, multilayer stacks of appressed double membrane structures are formed instead of the spherical compartments (Fig. 3.1c) (Schwartz et al. 2004). Replication of *Beet black scorch virus* (BBSV) is also confined within the ER-derived spherules (Fig. 3.1d) (Cao et al. 2015). A three-dimensional electron tomographic reconstruction shows the presence of multiple ER-derived vesicle packets (Fig. 3.1e). Each vesicle packet contains few to hundreds of independent spherules that result from the invaginations of the ER membrane. Each packet is connected to other packets through the formation of tubules, a rare rearrangement event among virus-induced membrane reorganizations. The interior of the spherules contains condensed or fibrillar materials that are presumably nucleic acids.

The morphology of these spherules is well characterized, and it is acknowledged that vRNA replication is taking place within these structures. However, not much is known about how viral proteins and vRNA templates are targeted to these sites, and how progeny vRNAs leave these compartments. Furthermore, how other virus replication processes, such as vRNA translation, encapsidation and intracellular movement are linked to the spherules remain elusive.

3.2.2 Vesicle-Shape Viral Factories

More frequently, (+) RNA plant virus infection leads to the reorganization of the ER for the production of motile vesicles, which range from 30 to 300 nm in diameter. These ER-derived vesicles have been observed during infection by *Turnip mosaic virus* (TuMV), *Grapevine fanleaf virus* (GFLV), *Cowpea mosaic virus* (CPMV), *Tomato mosaic virus* (ToMV), *Bamboo mosaic virus* (BaMV), *Potato virus X* (PVX) and TMV (Carette et al. 2000; Ritzenthaler et al. 2002; Mitra et al. 2003; Kawakami et al. 2004; Cotton et al. 2009; Wu et al. 2011).

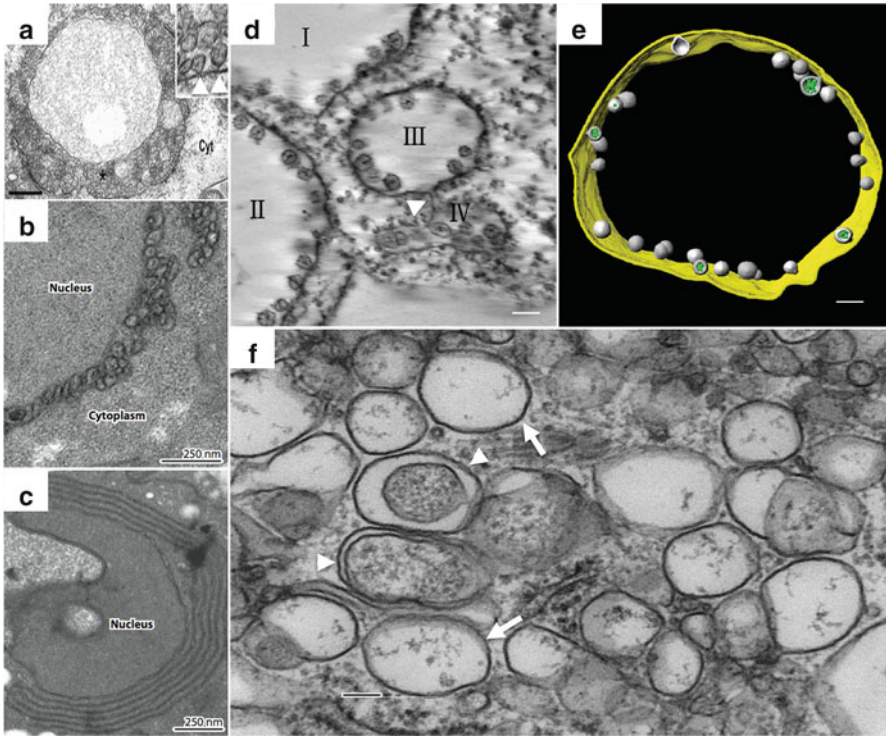


Fig. 3.1 Plant virus-induced membrane remodeling. (a) Electron micrograph of TBSV-induced MVBs in *N. benthamiana* mesophyll cell. The arrowheads in the inset denote the pores that connect the interior of the spherules with the cytoplasm. (b) The perinuclear ER-derived spherules induced by the membrane-associated viral 1a protein of BMV, in its surrogate host yeast cell. Similar spherules are seen in the cells expressing the 1a protein plus low level of 2a^{pol} protein. (c) In the presence of 1a and high level of 2a^{pol} protein, the double membrane multilayer structures are formed. (d) The ER-derived vesicle packets (indicated by I, II, III, IV) induced by BBSV in *N. benthamiana* cell. The arrowhead points the tubule that connects the vesicle packets. (e) The three dimensional view of a single vesicle packet is shown. Gold color denotes the ER membrane, gray the BBSV-induced spherules, and green the fibrillar materials within the spherules. (f) The single membrane vesicles (indicated by arrows) and double membrane vesicles (indicated by arrowheads) induced by TuMV in *N. benthamiana* cell. (a) is reproduced from (McCartney et al. 2005) with permission of the American Society of Plant Biologists; (b) is adapted, with permission, from (Schwartz et al. 2002) © 2002, Elsevier; (c) is adapted, with permission, from (Schwartz et al. 2004) © 2004 by the National Academy of Sciences. (d) and (e) are adapted, with permission, from (Cao et al. 2015) © 2015 by the American Society for Microbiology

TuMV-induced ER-derived vesicles have been well characterized. TuMV infection leads to the formation of numerous vesicles, sometimes accumulating into a large amalgam of ER and Golgi bodies near the nucleus (Grangeon et al. 2012). At the ultrastructural level, ER dilation is observed early in infection, which is followed by the formation of single membrane vesicles (SMVs) and double membrane vesicles (DMVs) (Grangeon et al. 2012) (Fig. 3.1f).

These ER-derived vesicles contain several viral and host proteins. In the case of TuMV-induced vesicles, the hub viral protein VPg (short for *Virus Protein, genome linked*) locates within the vesicles, and, by binding several proteins, controls many virus replication processes (Cotton et al. 2009; Jiang and Laliberte 2011). These viral proteins are the vRdRp and the helicase, which are required for vRNA synthesis (Cotton et al. 2009). Components of the host protein translational machinery, such as the eukaryotic translation initiation factor (iso) 4E [eIF(iso)4E], the translation elongation factor 1A (eEF1A) and the poly(A)-binding protein (PABP), are present in these vesicles. These factors are likely required for virus multiplication, and some of them determine the host susceptibility to the virus infection (Dufresne et al. 2008b; Cotton et al. 2009; Kim et al. 2014). Similarly, the TMV- and ToMV-induced vesicles also comprise host factors (Nishikiori et al. 2006; Yamaji et al. 2006; Hwang et al. 2013). Other host factors, such as heat shock proteins, RNA-binding proteins and membrane-shaping proteins, are incorporated into these ER-derived vesicles (Nishikiori et al. 2006; Dufresne et al. 2008a; Lee et al. 2010; Verchot 2012).

These vesicles are motile and are involved in the intracellular movement of the vRNA. The trafficking of ER-derived vesicles requires the ER/actin network (Heinlein et al. 1998; Cotton et al. 2009; Genoves et al. 2009; Cui et al. 2010). In particular, the motility of these vesicles highly depends on myosin XI-K (Kawakami et al. 2004; Amari et al. 2011; Peremyslov et al. 2012; Agbeci et al. 2013). Different steps of virus infection need distinct myosins. For instance, class XI myosins contribute mainly to TMV intracellular propagation and trafficking, whereas class VIII myosins are specifically required for MP targeting and moving the virus infectious unit across the PDs (Amari et al. 2014). In contrast, the intracellular vRNA movement is more often microtubule-based for animal viruses (Dohner and Sodeik 2005).

3.3 Lipids for Virus Replication

Being fundamental building blocks of cellular membranes, lipids play an important role in plant virus infection. The deprivation of lipid synthesis dramatically impairs virus production (Carette et al. 2000; Ritzenthaler et al. 2002; Sharma et al. 2011). Accordingly, virus infection enhances lipid biosynthesis (Lee and Ahlquist 2003; Barajas et al. 2014a, b, c). Furthermore, virus infection changes the host cell lipid composition (Fernández-Calvino et al. 2014). For instance, the amount of unsaturated fatty acid determines the fluidity and plasticity of membranes, thereby governing the number and the morphology of the viral factories (Lee et al. 2001; Lee and Ahlquist 2003). To ensure efficient replication, viruses preferentially hijack certain kinds of lipids. For example, the phospholipid phosphatidylethanolamine (PE) is relocated to vRNA replication sites in order to build a PE-enriched microenvironment for the replication of TBSV (Xu and Nagy 2015). The presence

of PE would promote vRNA binding to the vRdRp, thus facilitating vRNA replication (Pogany and Nagy 2015).

The question is how are lipids redirected to viral factories. For animal viruses, such as *Coxsackievirus B3* (CVB3), the lipid kinase phosphatidylinositol-4-kinase III β (PI4KIII β) is recruited to membranes during infection, which leads to the formation of PI4P lipid-enriched viral factories (Hsu et al. 2010). Although it is not known whether plant viruses can modify the microenvironment in a similar way, a direct lipid transfer from the cellular membrane to the viral factories has been proposed. For instance, TBSV co-opts the host VAP (vesicle-associated membrane protein-associated proteins) to facilitate the formation of membrane contact sites between the sterol biosynthetic ER membrane and viral factories (Barajas et al. 2014b). TBSV further recruits the oxysterol-binding protein-related proteins that are host lipid transfer proteins, likely channeling the sterols to the viral factories.

3.4 Membrane-Associated Viral Proteins

Plant viral genomes encode at least one membrane-associated protein that triggers membrane rearrangement (Table 3.1). These viral proteins, which are part of VRCs, associate with the membrane either through TMDs and/or amphipathic helices (Zhang et al. 2005; Li et al. 2009). Host membrane proteins may further mediate the association of VRCs with cellular membranes. For instance, the membrane association of the ToMV 180K replication protein is strengthened by interaction with two host membrane proteins, TOM1 and ARL8 (Nishikiori et al. 2011).

Ectopic expression of these membrane-associated proteins very often induces membranous structure assemblies similar to virus-induced viral factories (Schwartz et al. 2002; Wei and Wang 2008). In some instances, viral proteins by themselves may alter cellular membranes, inducing membrane proliferation and dilation, but they are not able to produce spherule-shape or vesicle-shape viral factories, suggesting that other viral proteins and possibly vRNA are involved in viral factory biogenesis (McCartney et al. 2005; Cao et al. 2015). These membrane-associated viral proteins contain functional domains that interact with host membrane-shaping factors for the formation of viral factories (see below).

A frequent observation is that these membrane-associated viral proteins, such as the p33 protein of TBSV, the 1a protein of BMV and the 6K₂ protein of *Plum pox virus* (PPV), form oligomers (Rajendran and Nagy 2004; Zilian and Maiss 2011; Diaz et al. 2012). Protein self-interaction is one way to induce membrane curvature (McMahon and Gallop 2005; Miller and Krijnse-Locker 2008). It has been suggested that the 1a protein of BMV induces replication vesicles by forming a capsid-like interior shell within the spherules (Diaz et al. 2012).

Table 3.1 The functions of membrane-associated viral proteins of plant virus TBSV, BMV, TuMV and BaMV/PVX

Virus	Membrane-associated viral protein	Membrane targeted	Functions	References
TBSV	p33	Peroxisome (switch to ER in the absence of peroxisome)	Upregulates phospholipid biosynthesis; recruits ESCRT factors for VRCs assembly; selectively vRNA recruitment; interacts with the p92 ^{pol} ; binds eEF1A to promote VRCs assembly and (–) vRNA synthesis.	Rajendran and Nagy (2004) Pogany et al. (2005), Jonczyk et al. (2007), Li et al. (2009), and Barajas et al. (2014a)
	p92 ^{pol}	Peroxisome	vRdRp; interacts with p33; recruits GAPDH to the VRCs.	Rajendran and Nagy (2004) and Huang and Nagy (2011)
BMV	1a	ER	Induces the formation of viral factories; recruits the vRNA to the viral factories; hijacks reticulons for membrane curvature.	Schwartz et al. (2002), Wang et al. (2005), Liu et al. (2009), and Diaz et al. (2010)
	2a ^{pol}	ER	vRdRp; interacts with the capsid protein maybe for genome packaging.	Chaturvedi and Rao (2014)
TuMV	6K ₂	ER	VRCs assembly; virus intracellular, intercellular and long distance movement.	Agbeci et al. (2013), Grangeon et al. (2013), and Wan et al. (2015)
	P3	ER	Virus pathogenesis; symptom and avirulence determinant; genome amplification.	Jenner et al. (2003) and Cui et al. (2010)
BaMV/ PVX	TGBp1		RNA binding; suppresses host gene silencing; virus movement; regulates the size exclusion limit of the PD; induces the formation of X-body.	Wung et al. (1999), Howard et al. (2004), and Tilsner et al. (2012)
	TGBp2	ER	Induces VRCs formation; interacts with TGBp3.	Ju et al. (2007) and Samuels et al. (2007)
	TGBp3	ER	Associates with the virions for virus delivery; interacts with TGBp2.	Samuels et al. (2007) and Chou et al. (2013)

3.5 Host Proteins in Viral Factory Formation

Although membrane-associated viral proteins directly target cellular membranes, this targeting further involves the participation of host proteins that have key roles in membrane dynamics. These host factors are the host reticulon homology proteins (RHPs), the endosomal sorting complexes required for transport (ESCRT) factors, and the early secretory pathway components.

3.5.1 RHPs and ESCRT Factors

The RHPs are members of a family of membrane-associated proteins that principally localize to the ER (Yang and Strittmatter 2007). These proteins contain two hydrophobic segments that associate with the outer leaflet of membranes, thus facilitating membrane curvature (Voeltz et al. 2006). In yeast cell, the depletion of RHPs (Rtn1p, Rtn2p and Yop1p) does not affect the BMV viral proteins 1a and 2a^{pol} localizing to the perinuclear ER membrane, but the viral proteins fail to induce the formation of spherules, indicating the importance of the RHPs in viral factory biogenesis (Diaz et al. 2010). The RHPs were shown to interact with the viral protein 1a within the spherules. The abundance and the morphology of the virus-induced spherules are regulated by the RHPs. In particular, the diameter of spherules induced by BMV decreases from 50 to 70 nm to an average of 27 nm when the Rtn2p and Yop1p are deleted. Meanwhile, the virus produce twofold more spherules in this double-knockout yeast cell (Diaz et al. 2010). The RHPs are also co-opted by several animal viruses to the replication site. The viral protein 2C encoded by Enterovirus 71 and Poliovirus interacts with the host reticulon 3 (RTN3). The RTN3 is important for virus replication, as the reduced expression of RTN3 impairs vRNA synthesis and viral protein translation (Tang et al. 2007).

ESCRT factors are involved in various cellular membrane bending and separation processes, including cytokinesis and formation of MVBs. These processes require the sequential assembly of ESCRT complexes (ESCRT-0, -I, -II, -III) on the membrane, and an accessory protein complex to disassemble the ESCRT complexes from the membrane for recycling (Schmidt and Teis 2012). The membrane-associated viral proteins can interact with the ESCRT components (Barajas and Nagy 2010; Diaz et al. 2015). For example, the p33 protein of TBSV interacts with the ESCRT-I Vps23p factor and the accessory factor Bro1p in its surrogate host yeast cell. This interaction leads to the recruitment of Vps23p to the replication site (peroxisome-derived), and the disruption of this interaction results in an increase sensitivity of the vRNA to nuclease activity (Barajas et al. 2009). Interestingly, CIRV, which modifies the mitochondrion for the formation of MVBs, hijacks the same ESCRT Vps23p factor for its replication (Richardson et al. 2014). In the case of BMV, the viral protein 1a associates with the ESCRT-III Snf7p factor, but the factor Vps23p is not required (Diaz et al. 2015).

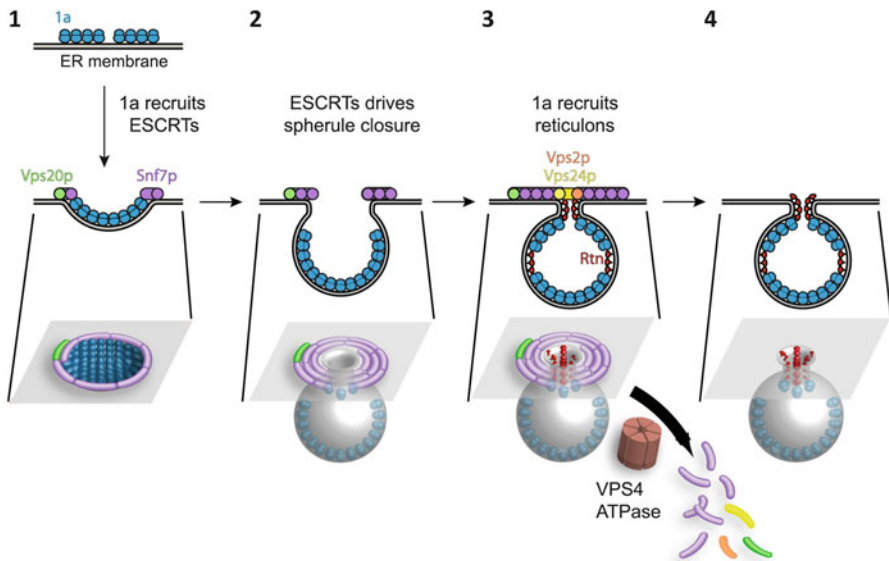


Fig. 3.2 Model for the contribution of RHPs and ESCRT factors for BMV spherule formation. The membrane-associated 1a protein associates with the ER membrane through its amphipathic helix, and the oligomerization of 1a protein initiates the membrane curvature. The ESCRT factors, Vps20p and Snf7p, are recruited to the deformed ER membrane site (step 1). The membrane rim is constricted by the ESCRT factors, to drive the spherule closure with the progressive formation of a pore (step 2). The other ESCRT factors (e.g. Vps2p, Vps24p) and the RHPs localize to and maintain the stability of the spherule (step 3). The ESCRT ATPase Vps4p disassembles the ESCRT machinery for the recycling, resulting the formation of the BMV spherule with the pore to its final size (step 4) (Reproduced, with permission, from Diaz et al. (2015))

Potentially, these ESCRT factors may be involved in viral factory formation for other viruses. The model shown in Fig. 3.2 highlights the contribution of the RHPs and the ESCRT factors for the BMV-induced spherule formation.

3.5.2 Early Secretory Pathway Components

The early secretory pathway, which is composed of the ER and Golgi bodies, functions in the vesicular transport of proteins and lipids (Brandizzi and Barlowe 2013). The pathway anterograde trafficking initiates with the cargo being synthesized on the rough ER, and then incorporated into coat protein complex II (COPII) vesicles for Golgi targeting, where the cargo is sorted to its proper cellular destination. The retrograde trafficking, which goes in the reverse direction, occurs via the COPI complex vesicles and maintains the membrane integrity of the cellular compartments. The guanine nucleotide exchange factor Sec12 recruits the GTPase Sar1 to the ER exit sites (ERES), where the COPII coatomers Sec23-Sec24 and Sec13-Sec31 are gathered to initiate the ER-Golgi vesicular transport (Brandizzi

and Barlowe 2013). In parallel, the GTPase Arf1 facilitates the assembly of COPI coatomers on the Golgi membrane (Beck et al. 2009). Other proteins, such as the soluble N-ethylmaleimide-sensitive factor activating protein receptors (SNAREs), mediate the fusion of COPII- and COPI-vesicles with their target membranes.

The secretory pathway has been shown to be important in the biogenesis of vesicle-shape viral factories (Ribeiro et al. 2008; Wei and Wang 2008; Hyodo et al. 2013; Sun et al. 2014). The selective uptake of viral proteins into COPII vesicles is mediated by an ER export signal, and several of them have been characterized. For the 6K₂ protein of TuMV, the N-terminal 12 amino acid stretch is required for the vesicle formation (Jiang et al. 2015). However, no clearly defined ER export motif has been identified for plant viral proteins (Wu et al. 2011).

Several secretory pathway components have been shown to directly interact with viral proteins. The GTPase, such as the Arf1, preferentially binds to the C-terminal region of the viral protein p27 of *Red clover necrotic mosaic virus* (RCNMV) (Hyodo et al. 2013). The COPII GTPase Sar1 interacts with the P2 protein of *Wheat yellow mosaic virus* (WYMV) (Sun et al. 2014). The COPII coatomer Sec24a recognizes the N-terminal cytoplasmic tail of the TuMV 6K₂ protein, thus facilitating the incorporation of the viral protein into COPII vesicles (Jiang et al. 2015). Furthermore, as the regulators of the secretory pathway, the SNAREs proteins can be hijacked by the viruses for the formation of vesicles. For example, the ER localized SNARE-like protein VAP27 can interact with the 60K helicase of CPMV (Carette et al. 2002). The TuMV 6K₂ protein also can interact with VAP27 protein, and by binding VAP27, 6K₂ associates also with Syp71, which is involved in vesicle fusion (Wei et al. 2013). The model shown in Fig. 3.3 illustrates the early secretory pathway involvement in viral factory formation.

3.6 The Motile Vesicle – Mediator of vRNA Replication and Movement

It was traditionally believed that vRNA replication and vRNA movement were separate events. It is now becoming clear that vesicles are not only used for vRNA synthesis, but also for vRNA movement. In other words, by inducing membranous vesicle, virus replication and movement are coupled. This tight coupling may explain why certain viruses quickly establish the systemic infection of the plant.

The PVX genome encodes three viral proteins (TGBp1, TGBp2 and TGBp3) from overlapping open reading frames, termed the triple gene block. The membrane-associated TGBp2 reorganizes the ER membrane for the formation of motile vesicles, to which the membrane-associated TGBp3 is also recruited (Schepetilnikov et al. 2005; Samuels et al. 2007). TGBp1 is recruited to modify PDs and to facilitate the deposition of CP for binding and moving progeny vRNAs across into non-infected cells (Howard et al. 2004). All three proteins have been

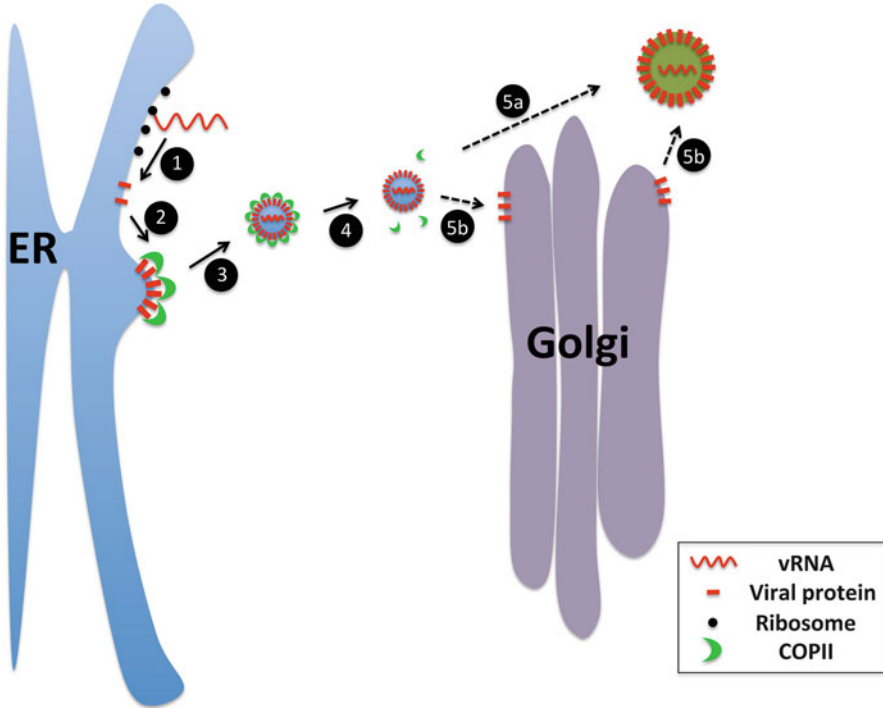


Fig. 3.3 Model for the early secretory pathway dependent vesicle-shape viral factory formation. vRNAs are translated on ER-associated ribosomes, and the synthesized viral membrane-associated proteins integrate to, or associate with, membranes (step 1). The COPII components are then recruited to initiate vesicle formation (step 2). The pre-formed vesicles bud from the ER membrane (step 3), and are shielded by COPII components (step 4). These vesicles may bypass the Golgi bodies and mature into vesicle-shape viral factories (step 5a), or may fuse with the Golgi membrane, which then exit from Golgi bodies to form the vesicle-shape viral factories (step 5b)

defined as MPs before it was found out that TGBp2/3-tagged vesicles support vRNA synthesis also. These replicating vesicles are located at the cytoplasmic orifices of PDs. This arrangement suggests a coreplicative model for intercellular movement of vRNA (Tilsner et al. 2013).

A long-standing question is what is the viral infectious unit that goes through PDs into the neighboring healthy cells? In the case of icosahedral viruses, the infectious unit is the virion itself (Halk and McGuire 1973; Ritzenthaler et al. 1995). Very often, the genome of these viruses encodes MPs that oligomerize to form tubules that go through PDs. Within these tubules are the virions that are moving into healthy cells. The situation is different for filamentous viruses, where it is presumed that a vRNP complex is the infectious unit. The full nature of this complex still needs to be defined for most viruses, but observations suggest that for TMV, the intercellular movement of vRNP complexes is carried out in the form of

vesicles containing VRCs (Kawakami et al. 2004). Movement of such vesicles has been captured by live cell imaging in a single TuMV-infected cell (Grangeon et al. 2013). The whole process, which includes the production and the movement of the VRCs from the infected cell into non-infected cells, takes 2–4 h (Kawakami et al. 2004; Agbeci et al. 2013). Intercellular movement of membrane-bound VRCs might be a very efficient way of rapidly spreading the infection throughout the whole plant. The number of viral genomes entering and replicating within a cell has been called the multiplicity of cellular infection (MOI) (Gutiérrez et al. 2012). In the case of TuMV, the MOI has been estimated to be close to one (Gutiérrez et al. 2012).

3.7 Lipids and Systemic Virus Movement

Virus intercellular movement within different types of cells results in the systemic infection of the plant. Initially, the virus replicates in the epidermal cells that are the outmost cell layer of the plant leaf tissue. The infectious unit, which is internalized, enters the mesophyll cells that are specialized for photosynthesis, crosses sequentially the bundle sheath and the vascular parenchyma cells to reach the transport conducting tubes (phloem and xylem) of the plant. At this point, viruses spread rapidly throughout the plant.

Most plant viruses utilize the phloem for their systemic movement (Cruz et al. 1998; Silva et al. 2002). The sieve element, which is the conducting tube of the phloem, is enucleated when it becomes mature and depends on the associated companion cell for the maintenance of its function. The viral infectious unit moves through the specialized PD termed pore plasmodesmal unit (PPU) for the phloem loading. The xylem, despite being overlooked, can also support the virus systemic movement (Verchot et al. 2001; Moreno et al. 2004). The vessel element is the conducting unit of the xylem. The perforation plate, which is formed during the programmed cell death of the vessel element, is the opening for the viral infectious unit systemic movement through the xylem. The pit, which is located on the side wall of the mature vessel element, is the presumed place for the viral infectious unit to load into the xylem (Opalka et al. 1998).

In the case of TuMV, Wan et al. have found that replicating vesicles are present both in the phloem and xylem (Wan et al. 2015). Interestingly, these $6K_2$ -induced vesicle aggregates contain large amount of lipids, revealing their importance for plant virus systemic infection. The membrane-containing aggregates in the phloem are suspected to be the site for virion assembly, and the assembled virions are then released for phloem transport (Fig. 3.4a). Individual $6K_2$ -induced vesicle movement for phloem transport is also possible. On the other hand, $6K_2$ -induced vesicles are thought to enter the xylem vessel through pit membranes, and to replicate in the xylem vessel. Once the xylem vessel becomes a hollow vessel, the vesicles then move upward along the flow of water (Fig. 3.4b).

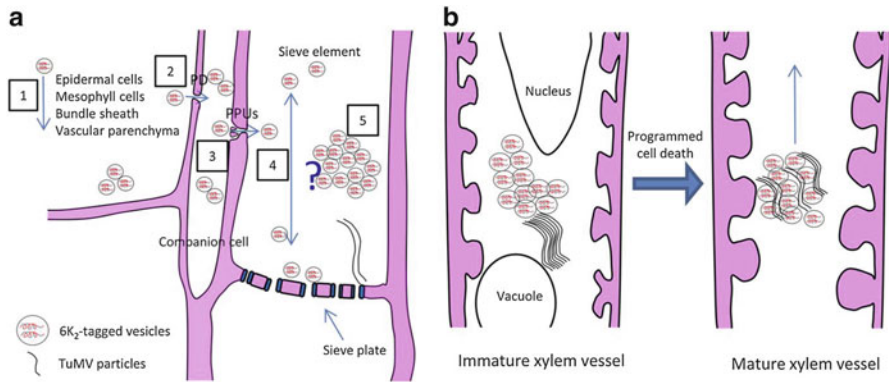


Fig. 3.4 TuMV systemic movement. **(a)** Schematic model for TuMV moving through the phloem. The viral membrane-associated 6K₂ protein-induced replication vesicles move from the infected epidermal cells to mesophyll cells, and then to bundle sheath and to vascular parenchyma (step 1), and further reach the companion cells through the PDs (step 2). The virus replicates in all these cells, and the newly synthesized replication vesicles move across the PPUVs for phloem loading (step 3). Once in the sieve element, the individual replication vesicles move through the sieve elements (step 4), or form aggregates for the assembly of the virions involved in systemic movement (step 5). **(b)** Schematic model for the TuMV moving through the xylem. The replication vesicles reach the immature xylem vessel through the pits, and replicate in the cell. After programmed cell death, the immature xylem vessels become hollow vessels, in which the infectious units then move upward along the flow of water (Reproduced from (Wan et al. 2015) with permission © 2015 the American Society of Plant Biologists)

3.8 Conclusions

Plant viruses encode membrane-associated viral proteins to actively modify cellular membranes for the production of viral factories, thus promoting virus replication and movement. Although the morphological characterization of these virus-induced changes are being carried out, the biogenesis of these structures still needs to be defined. For instance, how do the proliferated membranes convert from SMVs to DMVs? What is the full set of host proteins co-opted for the production of the viral factories? Despite that more and more host factors are being identified, the interaction network and their biological significance are still unknown. For instance, what are the factors that mediate the association of vesicle-shape viral factories with the actomyosin motors? We also need to know how progeny vRNAs leave the lumen of spherules to be transported into neighboring non-infected cells. Finally, the relationship of viral factories with other cellular processes, such as the unfolded protein response and the autophagy, remains elusive.

On a practical aspect, the high dependence of plant viruses on membranes raises the possibility of whether this property can be targeted for a broad-spectrum approach for developing crop plants that are resistant to viruses. Since lipids are vital for plant virus infection, it will be exciting to identify potential antiviral chemicals that selectively target the lipid-binding site of these membrane-associated viral proteins in order to block either vRNA synthesis or movement.

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Chapter 4

Plant Genetic Resistance to Viruses

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Abstract Plants have evolved a variety of active and passive mechanisms to defend themselves against viral pathogens, and disease resistance genes have been incorporated into crop plants to protect against diseases caused by viruses. The specificity of resistance genes is usually limited to a virus species or group of highly related species, and there is not natural resistance available to all viruses of economic importance. For these reasons, there has also been great interest in developing or engineering novel virus resistance traits based on our knowledge of plant antiviral immune systems such as RNA silencing. With the recent emergence of technologies based on site-specific nucleases that can be used to manipulate the sequence of genes within crop plant genomes, there are now opportunities to further exploit our knowledge of plant-virus interactions to develop plants with novel forms of resistance. However, viruses have the potential to rapidly evolve to overcome resistance whether it is natural or engineered. Therefore, it is essential that we understand factors that influence the durability of resistance traits to maximize their longevity. In this chapter, we briefly highlight different forms of natural and engineered virus resistance mechanisms, discuss approaches to use genome editing for developing virus resistant plants, and explore the issue of durability as it relates to both natural and engineered resistance traits. Finally, we consider future research prospects that will continue to expand our knowledge of host-virus interactions and provide a solid foundation for understanding and possibly predicting resistance durability.

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4.1 Introduction

The deployment of genetic resistance is the most effective, environmentally friendly, and economically sound approach to manage diseases of crop plants that are caused by viruses (Fraser 1986). Plants have evolved a variety of mechanisms to defend themselves against viral pathogens, and human kind has learned how to harness and manipulate plant genetic resistance to a certain extent. Virus resistance mechanisms range from programmable antiviral RNA silencing pathways to specific gene-for-gene interactions in which cytoplasmic receptors specify recognition of individual viral proteins. Resistance mechanisms may also be viewed from the perspective of whether they are active or passive (Fraser 1990). The term active resistance implies that specific recognition of a virus precedes a programmed response that limits viral replication and spread. Passive resistance mechanisms are not necessarily accompanied by induced defense responses, but rather, they generally result from the lack of essential plant susceptibility factors that are required for viral replication and/or movement. We typically consider active or induced defenses to be dominant or semi-dominant traits, because resistance responses occur in plants that are homozygous or heterozygous for the resistance gene. However, passive resistance is generally recessive, because only plants homozygous for the resistance alleles are phenotypically resistant.

There have been a number of recent and excellent reviews on the topics of virus resistance genes and engineered resistance, and these are cited in the appropriate contexts below. Therefore, we do not attempt to present a comprehensive review of the broad topic of genetic resistance to viruses. Here, we briefly review general aspects of different genetic virus resistance strategies in plants, and we use this as a platform to discuss a major challenge associated with deploying genetic resistance for crop protection, which is the issue of durability. In this context, we also explore recently emerging genome editing technologies that are anticipated to lead to the development of crop plants with novel virus resistance traits.

4.2 Virus Resistance Mechanisms

4.2.1 Dominant Resistance

The majority of dominant resistance (*R*) genes, which are well characterized, mediate specific recognition of a virus species or group of related species within a single genus. Subsequently, host defenses are activated at the site of inoculation that takes the form of the hypersensitive response (HR). HR is characterized by the local induction of cell death accompanied by massive changes in gene expression, phytoalexin production, oxidative burst, and activation of defense hormone signaling. Necrotic localized lesions that typically appear within a few days after inoculation are the hallmark of HR. Viruses that induce HR are, in

most cases, confined to the site of lesion formation. However, the exact mechanism(s) by which the HR acts to limit viral replication and spread are still not known. Macroscopic HR lesions are observed, because the virus is able to initiate replication and local movement that results in multicellular infection foci in which the HR is activated.

Dominant *R* genes also confer other resistance phenotypes such as extreme resistance (ER), inhibition of replication, and inhibition of long-distance movement. In ER, there are no macroscopic signs of a host response, because the virus is effectively restricted to the inoculated cells. ER and HR appear to be mechanistically related for two primary reasons. First, it is possible to express a viral elicitor of ER in a plant and induce HR (Bendahmane et al. 2002). Second, viral mutants or isolates that partially evade ER can cause HR or systemic HR that is *R* gene dependent (Hajimorad and Hill 2001). In addition, activation of HR and possibly ER leads to the production and long-distance transport of signals from the inoculated leaves to systemic tissues that induce systemic acquired resistance (SAR) (Liu et al. 2010). *R* genes that specifically interfere with replication or long-distance movement do not activate HR and there are typically no other accompanying markers of associated defense responses other than the virus is restricted to the inoculated leaf (Mahajan et al. 1998).

Many viral *R* genes have been identified and several have been cloned. Reviews have presented comprehensive lists of these genes and their encoded proteins (Kang et al. 2005; Revers and Nicaise 2014; de Ronde et al. 2014). In general, genes that confer HR and ER encode proteins belonging to the nucleotide binding site-leucine-rich repeat class (NBS-LRR), which is an abundant class of R proteins that endow plants with abilities to recognize a wide range of pathogens and pests (Sekhwal et al. 2015). NBS-LRR proteins are further sub-classified into the Toll/Interleukin-1 receptor NBS-LRR (TNL) and non-TNL/coiled-coil NBS-LRR (CNL) depending on the domain present at the amino terminus. Resistance to viruses is conferred by both CNLs and TNLs in dicots. Although relatively few virus *R* genes have been cloned from monocots (Mandadi and Scholthof 2013), the rarity of the TNL proteins in monocot genomes that have been sequenced to date (Kim et al. 2012; Tarr and Alexander 2009) suggests that CNLs have primary responsibility for conferring resistance to viruses and other pathogens (Meyers et al. 2002).

Cloned viral *R* genes, such as *Tm-1* from tomato and *RTM1*, *RTM2*, and *RTM3* from Arabidopsis, which specifically restrict replication or movement do not correspond to any typical class of R protein. These R proteins function by mechanisms that are not effective against other types of pathogenic microorganisms. *Tm-1* directly interacts with the helicase domain of the *Tomato mosaic virus* (ToMV) replication protein and interferes with its nucleotide triphosphatase activity (Ishibashi et al. 2014). The virus can evade this interaction through mutations in its helicase domain that impair interactions with *Tm-1* (Ishibashi and Ishikawa 2014). Genetic data suggest that as many as five loci are required for the *RTM* virus resistance trait that restricts long-distance movement of *Tobacco etch virus*, *Plum pox virus*, and *Lettuce mosaic virus* in resistant ecotypes of Arabidopsis. The three cloned *RTM* genes encode unusual proteins that have similarity to the lectin

jacalin (RTM1), small heat shock proteins (RTM2), and MATH domain-containing proteins (RTM3) (Chisholm et al. 2000; Whitham et al. 2000; Cosson et al. 2010). RTM resistance blocks viral movement at the long-distance step and it bears no resemblance to active defense associated with HR or SAR (Mahajan et al. 1998). The encoded proteins appear to function in the phloem where the RTM1 and RTM2 proteins are detected (Chisholm et al. 2001). Interestingly, another lectin-domain protein conferring resistance to a virus, Jacalin-type lectin required for potexvirus resistance 1 (JAX1), was recently cloned from *Arabidopsis* (Yamaji et al. 2012). The similarity of JAX1 to RTM1 suggests that lectin binding proteins represent a new and important mechanism mediating resistance to plant viruses (Ouibrahim and Caranta 2013).

NBS-LRR proteins conferring virus resistance possess no secretion signals or transmembrane regions that would indicate an extracellular localization. Therefore, they recognize, directly or indirectly, pathogen-produced molecules that accumulate inside the cell, which is consistent with the location of viral proteins. Viral proteins recognized by *R* genes are referred to as elicitor or avirulence proteins. The specific recognition of viral proteins by NBS-LRR proteins is consistent with effector-triggered immunity, which historically had been referred to as gene-for-gene resistance (Gassmann and Bhattacharjee 2012). Due to their compact genomes, viral proteins all have essential and often overlapping functions in replication, gene expression, host defense suppression, movement, encapsidation, and transmission (Hull 2014). There does not appear to be a particular function that is preferentially targeted by *R* proteins, and so, it may be proper to think of all viral proteins as effectors. Following recognition of the elicitor protein, the *R* protein initiates signaling events that result in defense responses. The complex signaling networks that are activated involve phytohormones, ions, ROS, transcription factors, kinases, etc. (for a detailed review see Carr et al. 2010).

4.2.2 Priming and Systemic Antiviral Defenses

Plants can be primed so that they are less susceptible to challenge by viral pathogens. This is known to occur through at least two major pathways, SAR and induced systemic resistance (ISR) (Faoro and Gozzo 2015). ISR is induced when roots are colonized by plant growth-promoting rhizobacteria, and it has been shown to reduce virus titers and disease symptoms in some hosts. Because the mechanisms of antiviral ISR have not been investigated in detail, we focus on SAR. SAR is induced systemically following the onset of the HR and ER (Ross 1961; Liu et al. 2010). When SAR is activated, plants are less susceptible to infection by viruses and a variety of other pathogens for extended periods of weeks or months. Decreased susceptibility to viruses results from inhibition of replication, cell-to-cell movement, and/or long-distance movement (Murphy and Carr 2002; Wong et al. 2002; Faoro and Gozzo 2015). SAR is also accompanied by coordinated biochemical and molecular changes such as cell wall strengthening, phytoalexin

production, and increased levels of reactive oxygen species, callose, and pathogenesis-related (*PR*) gene expression (Ryals et al. 1996).

The phytohormone salicylic acid (SA) is a key signaling molecule that activates SAR, either through endogenous accumulation or exogenous application. The activation of SAR is regulated by the non-expresser of PR proteins (NPR1, NPR3, and NPR4) that mediate cellular responses to changing levels of SA (Yan and Dong 2014). However, SA also induces antiviral defenses independent of its ability to induce *PR* gene expression through NPR1 (Wong et al. 2002), which implies that SA activates other signaling mechanisms and/or that it might have direct effects on viruses within cells. In support of these ideas, an increasing number of plant proteins have been shown to bind SA (Tian et al. 2012), and roles in antiviral defenses have been investigated for two of them. One SAR-independent signaling mechanism involves the activation of the alternative oxidase pathway that functions to limit viral infection (Lee et al. 2011). Liao et al. (2015) recently showed in tomato that SA binds to and inhibits α -keto glutarate dehydrogenase subunit E2 (*S* α kGDH), which induces the alternative oxidase pathway. In another study, SA was shown to have a direct effect on viral replication through its binding to a host factor glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Tian et al. 2015). Cytosolic GAPDH promotes asymmetric synthesis of *Tomato bushy stunt virus* (TBSV) plus-strand RNA by its greater binding affinity to the negative strand (Huang and Nagy 2011; Wang and Nagy 2008). When GAPDH binds to SA, replication of TBSV is suppressed, because GAPDH binding to the negative strand is inhibited. In addition to roles in signaling and direct antiviral activities, SA itself is modified in rice plants carrying the *STV11* gene, which confers resistance to *Rice stripe virus*. *STV11* is a sulfotransferase that converts SA to sulphonated SA that is a more potent inhibitor of viral replication than SA.

The importance of SA in defense signaling and other antiviral mechanisms suggests that viruses may have ways to interfere with SA functions. Indeed, the coat protein (CP) of *Tobacco mosaic virus* (TMV) strain Cg (TMV-Cg) was recently demonstrated to interfere with SA-induced signaling (Rodriguez et al. 2014). TMV-Cg CP binds to and stabilizes DELLA proteins in the presence of gibberellic acid, which in turn negatively modulate the expression of SA-inducible genes. The *Cucumber mosaic virus* (CMV) 2b protein (CMV-2b) protein, which is a viral suppressor of RNA silencing (VSR), also interferes with SA-inducible antiviral defense (Ji and Ding 2001). Interestingly, it appears that CMV-2b may also induce the accumulation of SA (Lewsey et al. 2010).

4.2.3 Recessive Resistance

Recessive virus resistance genes are generally thought to confer passive resistance, because there is no activation of defense responses. Plants are resistant to infection, because they simply lack a host factor or appropriate form of the factor necessary for viral infection. The best characterized and by far the most numerous form of

recessive resistance is based on variants of the elongation initiation factor (eIF) proteins, eIF4E and eIF4G and their isoforms eIFiso4E and eIFiso4G (Nicaise 2014; Sanfacon 2015). Nearly one-half of the known virus resistance genes are recessive alleles of these proteins, which have essential functions in initiating translation of capped and polyadenylated host mRNAs. Initially, this resistance was demonstrated to be effective against several potyviruses, and subsequently, it was extended to members of other virus families including bymoviruses, cucumoviruses, ipomoviruses, sobemoviruses, carmoviruses, and waikiviruses. EIF4E binds the 5' cap of host mRNAs, and eIF4G acts as a scaffold by interacting with eIF4E and polyA binding protein (PABP) to circularize mRNA during initiation of host translation. Mutations in these proteins affect their interactions with viral proteins and RNAs resulting in the inhibition of replication and movement, likely because the viral RNAs cannot be translated or properly trafficked in the cell. Other translation factors such as eIF4A-like helicases, eIF3, eEF1A and eEF1B have been shown to interact with viral proteins and RNAs, and they likely represent potential new targets for recessive virus resistance in plants (Sanfacon 2015).

4.2.4 Antiviral RNA Silencing

RNA silencing systems function in different ways to defend plants from virus infections. They have direct antiviral activities, and they produce regulatory small RNAs that control the expression of *R* genes (Li et al. 2012a). The basic RNA silencing pathways are initiated by Dicer-like proteins (DCL) that cleave double-stranded RNA (dsRNA) templates into 21–24 nucleotide (nt) fragments known as small interfering RNAs (siRNAs) (recently reviewed in Zhang et al. 2015). The source of dsRNA could be double-stranded viral replication products, secondary structures within single stranded RNA such as stem-loops, or overlapping regions in viral mRNAs produced by convergent transcription. There are different size classes of small RNAs that are produced depending on the DCL protein that cleaves the viral RNA. DCL4, DCL2, and DCL3 produce 21, 22, and 24 nt small RNAs, respectively. These DCLs have hierarchical functions in antiviral defense (Garcia-Ruiz et al. 2010). For example, DCL4 and DCL2 are the most important in defense against plus-stranded RNA viruses, but in their absence, DCL3 becomes more active in producing 24 nt RNA derived from the viral genome although it does not appear to have much effect on RNA virus accumulation.

The virus-derived siRNA (vsRNA) generated by DCL proteins are chemically modified, then bound by Argonaute (AGO) proteins, and then one of the strands is discarded. The resulting protein-RNA complex is known as the RNA-induced silencing complex (RISC). The endonuclease activity in RISC is provided by AGO proteins that cleave RNAs that have complementarity to the bound vsRNA. The production of vsRNA by DCL proteins, therefore, programs the RISC to recognize and cleave any other viral RNA in or entering the cell that shares sequence complementarity to the bound vsRNA. Once a virus is detected and

dsRNA is processed by DCL proteins and RISC is programmed, the signal can be amplified by RNA-dependent RNA polymerases (RDRs). RDRs use the cleavage products from RISC to generate more dsRNA template that can be cleaved by DCLs to produce vsRNA. In turn, these vsRNA form more RISC that can specifically cleave viral RNA targets. This amplification is mediated by the RDR1, RDR2, and RDR6 proteins in Arabidopsis.

Antiviral RNA silencing is effective against both RNA and DNA viruses. The importance of RNA silencing as an antiviral defense mechanism is underscored by the presence of one or more VSR encoded by the genomes of most plant viruses (Csorba et al. 2015; Nakahara and Masuta 2014). These VSR can block antiviral RNA silencing at any number of steps including biogenesis of small RNAs resulting from interfering with DCL proteins. The assembly of functional RISC can be prevented by sequestering small RNA or inhibiting the accumulation or function of proteins, such as AGO (Giner et al. 2010; Garcia-Ruiz et al. 2015). The amplification and movement of small RNAs can also be prevented to promote viral infection. As viruses suppress these steps in RNA silencing pathways, they also interfere with host gene expression, because many of the components also have activities in pathways that control gene expression via small RNAs, such as miRNAs (Mallory et al. 2002; Kasschau et al. 2003; Chapman et al. 2004; Shibolet et al. 2007). This leads to mis-expression of plant genes that in part contribute to the symptoms commonly observed in virus-infected plants.

4.3 Engineering Viral Resistance

There are a growing number of ways that plants can be engineered for resistance to viruses, and many of these have been covered in recent reviews (Cillo and Palukaitis 2014; Galvez et al. 2014). Here, we briefly summarize engineered resistance based on transgenic plants that prime the RNA silencing system for antiviral defense, and then we discuss, in more detail, recent developments related to the applications of site-specific nucleases for developing virus-resistant plants.

4.3.1 *Engineered Resistance Based on RNA Silencing*

Over the past 10–15 years, most examples of engineered resistance have exploited plant antiviral RNA silencing systems. Because RISC first requires production of small RNA by DCL, RNA silencing is programmable and can be manipulated in a variety of ways to produce a virtually unlimited array of small RNA species (siRNA or micro RNA (miRNA)) to target viral genomes and transcripts. There are several strategies for generating small RNA species, but regardless of their biogenesis, the resulting small RNAs direct RISC to degrade viral RNA species. A major and

efficient strategy for producing dsRNA that can be processed by DCL is the inverted-repeat RNA (irRNA) or hairpin RNA (Smith et al. 2000). These constructs typically contain an inverted repeat of the viral target sequence with an intervening loop or intron sequence. The resulting siRNA are distributed from across the inverted repeat sequence. The irRNA can be designed to target a sequence within a single virus, or sequences can be combined together so that a single transgene mRNA can target multiple viruses (e.g. Zhang et al. 2011).

Endogenous miRNA directly and indirectly regulate the expression of plant mRNA transcripts. MiRNA are 21 nt small RNA processed from an inverted repeat contained within a pre-cursor miRNA (pre-miRNA) transcript. The native inverted repeats can be replaced with virus-derived sequences to produce a miRNA complementary to viral sequences. As opposed to the irRNA strategy, which results in a population of siRNA, the processing of the artificial miRNA (amiRNA) precursor results in the production of a single amiRNA (Schwab et al. 2006). The amiRNA has greater specificity than irRNA, so it is possible to specifically target a virus without concern for potential off target effects on plant gene expression (Niu et al. 2006). However, this specificity is also a disadvantage, because it is possible for a virus to evade amiRNA-based resistance with one or a few mutations (Lafforgue et al. 2011). The amiRNA approach can be improved by targeting different sites in a virus genome by combining multiple amiRNA precursors into a single polycistronic transcript (Fahim and Larkin 2013; Fahim et al. 2012; Kung et al. 2012). Another approach to initiate production of small RNA is the use of artificial trans-acting siRNA (atasiRNA) (Singh et al. 2015). As with miRNA, endogenous tasiRNA also regulate plant gene expression. However, their biosynthesis occurs in a hierarchical process. A specific miRNA directs an AGO to cleave the tasiRNA transcript, the cleaved transcript is used by RDR6 to produce dsRNA, and then the dsRNA is cleaved into phased 21 nt siRNA by a DCL. This system can be engineered to produce transcripts that contain the viral target sequences and the miRNA binding sites in the appropriate position. Potential advantages of the tasiRNA approach are that it is not necessary to produce the irRNA constructs and it is straightforward to target multiple viruses by concatenating sequences. However, this system is dependent on the expression of endogenous miRNA to initiate production of the vsRNA.

4.3.2 Use of Site-Specific Nucleases for Engineering Virus Resistance

Site-specific nucleases (SSN) are proteins that can be designed or programmed to specifically bind nucleic acid sequences and then cleave target sequences at or near the binding sites. Four different types of SSN have been used in plants: meganucleases, zinc-finger nucleases (ZFN), transcription activator-like effector

nuclease (TALEN), and clustered regularly interspaced palindromic repeats-CRISPR associated (CRISPR-Cas) (Voytas and Gao 2014). Since the discovery of TALEN and subsequently CRISPR-Cas, there has been a lot of excitement over the application of these technologies for improving crop plants. This excitement has been sparked by the relative ease with which it is now possible to target specific sites in plant genomes for modification. This is particularly true for the CRISPR-Cas system in which delivery of a guide RNA directs the Cas9 protein to cleave a double-stranded DNA at a specific sequence (Jinek et al. 2012). The specificity of Cas9 is thus readily modified by simply expressing different guide RNA, and it can also simultaneously target multiple locations by co-expressing multiple guide RNA (Cong et al. 2013).

SSN technologies are used to create double-strand breaks at precise positions in genomes. These breaks can then be repaired by one of two mechanisms: non-homologous end joining (NHEJ) or homologous recombination (Voytas and Gao 2014). The most straightforward application of SSN is to create gene knock-outs through NHEJ. Repair of double-strand breaks by NHEJ is frequently imprecise leading to small deletions or insertions. These insertion/deletion (INDEL) mutations can disrupt coding or regulatory sequences of the target gene resulting in loss-of-function. Repair of double-strand breaks by homologous recombination is more complex, because it requires the simultaneous delivery of a DNA repair template that carries the desired change(s) to be incorporated into the repaired locus. Homologous recombination can be used to alter a single DNA base or to insert a novel gene into a specific position in the genome.

The potential for using SSN to improve genetic resistance to viruses is tremendous. Here, we discuss three strategies for utilizing SSN to improve crop plant resistance to viruses: directly targeting viral genomes, introducing recessive resistance through modification of host factors, and modification of dominant resistance genes. The current SSN used in plants operate on DNA templates, and so, they can be used to directly target DNA viruses, such as geminiviruses. In some cases, artificial zinc finger protein (AZP) and artificial TALE (ATALE) have been used to demonstrate the potential of these technologies to confer resistance by directly targeting sequences in geminivirus genomes. The AZP and TALE differ from ZFN and TALEN, because they lack the FokI nuclease that is used to create precise double-strand DNA breaks.

Initial studies using AZP focused on resistance to the genus *Begomovirus*, which contains about 89 % of recognized species of geminiviruses, and the replication (Rep) protein or its site of action were targeted. One of the essential Rep functions is to bind to the viral origin of replication, a stem-loop structure in the common region, and introduce a nick in the loop. Two different AZP were designed to bind the origin of *Beet severe curly top virus* (BSCTV) or *Tomato yellow leaf curl virus* (TYLCV) with an affinity at least 1000 times greater than Rep (Mori et al. 2013; Sera 2005). Transgenic Arabidopsis plants expressing the AZP targeting the BSCTV origin were reported to be completely resistant to BSCTV (Sera 2005). Although the TYLCV AZP has a similar binding affinity for the TYLCV replication

origin, there is so far no report of its ability to confer resistance in transgenic tomato.

Chen et al. (2014) acknowledged that the approach to use AZP to target the origin of BSCTV and TYLCV was effective. However, they expressed concern that the resistance would not be durable because the AZP were designed to specific viruses, and thus would not be broadly effective. They argued that targeting conserved regions would be expected to confer more durable resistance. To that end, three ZFN pairs were developed that targeted sequences within the *AC1* gene (Rep) of *Tomato yellow leaf curl China virus* (TYLCCNV) that were conserved across most monopartite begomoviruses. One ZFN pair recognizing a 25 bp target sequence was determined to have the highest DNA binding affinity, and it was selected for transient assays to test its effect on TYLCCNV and *Tobacco curly shoot virus* (TbCSV) accumulation. TbCSV is 80.01 % identical to TYLCCNV at the nucleotide level. Both viruses were inhibited to a similar level indicating that the ZFN pair worked well in this transient assay, but transgenic plants were not tested.

ATALE has been used in a similar approach to generate resistance to begomoviruses (Cheng et al. 2015). ATALE were designed to target two highly conserved 12 nucleotide motifs, the conserved hairpin in the common region (all begomoviruses) and within the *AC1* ORF (98 % of begomoviruses). ATALE was used instead of TALEN, because it was not possible to identify a highly conserved site that would facilitate the binding of a TALEN pair, which is necessary for DNA cleavage. The two ATALE were co-expressed in transgenic *N. benthamiana* plants after their DNA binding affinities were confirmed. Co-expression of the ATALE reduced symptoms, increased time to systemic infection, and decreased accumulation of viral DNA in plants challenged with three different begomoviruses that share between 75 and 82 % sequence identity with one another. Similar to the AZP approach, the partial resistance to the begomoviruses was likely conferred by the ATALE interfering with Rep function or possibly through inhibition of *AC1* transcription.

The CRISPR-Cas system has been used to create plants with resistance to the begomoviruses, BSCTV and *Bean yellow dwarf virus* (BeYDV) (Ji et al. 2015; Baltes et al. 2015). In both papers, the authors first selected candidate sites, designed guide RNA, and then tested them in transient assays for the ability to reduce virus infection. The ability of guide RNA-Cas9 to act directly on viral genomes as opposed to the T-DNA that served as the source of inoculum was tested by inoculating the virus at a separate location from the site where the guide RNA and Cas9 were expressed. As the viruses moved through the cells expressing guide RNA-Cas9, their genomes acquired small INDELS at the targeted sites as expected. Unexpectedly, some guide RNA did not induce INDELS even though they suppressed viral replication (Baltes et al. 2015). This results suggests that some guide RNA-Cas9 complexes may function similar to the AZP and ATALE to interfere with the expression or function of the Rep protein. The guide RNA that reduced virus levels the most were then selected for co-expression with Cas9 in transgenic plants. Transgenic Arabidopsis and *N. benthamiana* supported

significantly less BSCTV accumulation (Ji et al. 2015), and transgenic *N. benthamiana* had much reduced levels of BeYDV (Baltes et al. 2015). In addition, Baltes et al. (2015) showed that the combination of two guide RNA reduced virus levels more than either guide RNA alone, but that guide RNA targeting sequences near the hairpin in the common region were the least effective.

The use of guide RNA-Cas9 for geminivirus resistance has significant advantages over other biotechnology approaches. The specificity of the guide RNA reduces likelihood of off-target effects, and the ability to multiplex guide RNA will improve the durability by targeting multiple sequences within a virus and by providing the ability to target any additional geminiviruses as well (Baltes et al. 2015). Targeting multiple viruses is important for controlling disease complexes that are caused by mixed infections of geminiviruses and their satellites (Baltes et al. 2015). The limitation of this direct approach is that guide RNA-Cas9 can only be used to target DNA viruses, but that could change in the future, because Cas9 can be programmed to cleave RNA (O'Connell et al. 2014) and the Type III-B CRISPR-Cas system mediates programmable cleavage of RNA sequences that are complementary to a guide RNA (Hale et al. 2012; Hale et al. 2009).

In addition to directly targeting viral genomes, SSN have the potential to modify plant genes that affect plant responses to viral infection through modification of host factors to create recessive resistance or by modifying *R* genes to modify their specificity (Ilardi and Tavazza 2015). Currently, there are only two specific examples of using SSN in either approach to engineer virus resistance, and so we primarily discuss the future prospects here. SSN may be introduced as transgenes to create the genome edits, and then progeny plants can be selected that carry the desired edits and that have lost the SSN transgene locus through segregation. Alternatively, the SSN protein and other reagents, like guide RNA, may be introduced directly into cells, which would not involve their incorporation into the genome. The resulting plants would be indistinguishable from plants carrying naturally occurring alleles or those identified from screens following random mutagenesis (Bortesi and Fischer 2015; Voytas and Gao 2014). These approaches are applicable to viruses with RNA or DNA genomes.

As discussed in 2.3, the best characterized examples of recessive virus resistance come from translation initiation factors, such as eIF4E and eIF(iso)4E (Sanfacon 2015). Natural allelic diversity and mutagenesis screens have identified viral resistance based on eIF4E/(iso)4E. For example, Arabidopsis mutants that were resistant to *Turnip mosaic virus* were identified from screening an EMS-mutagenized population (Lellis et al. 2002). Characterization of the mutant determined that it was recessive and carried a mutation in the *eIF(iso)4E* gene. This form of recessive resistance could be exploited with the aid of SSN to create novel resistance alleles in crop plants to protect them against problematic viruses that utilize host translation initiation factors. The translation initiation factors are prime candidates for host genes that can be targeted, but any host gene encoding a factor on which the virus is dependent is a potential target for modification. Recently, the CRISPR/Cas9 technology was used to create mutations in the cucumber *eIF4E* gene and the Arabidopsis *eIF(iso)4E* gene that conferred resistance to potyviruses (Chandrasekaran et al. 2016; Pyott et al. 2016).

4.4 Durability of Resistance to Viruses

Durability has been a matter of concern for natural and engineered resistance traits. This concern is mainly due to the potential of viruses, in particular RNA viruses, for rapid adaptability, which is a consequence of high mutation rate, high accumulation level, and rapid replication cycles (Drake and Holland 1999). Indeed, in the majority of cases, virulent variants emerged simply as a consequence of single point mutations in viral encoded proteins serving as avirulence determinants (Hull 2014). Despite this concern, resistance against plant viruses mediated by the majority of naturally occurring resistance genes has proven to be remarkably durable (Harrison 2002). It appears that rapid break-down of resistance, as was observed for the *Tm-1* gene of tomato and TMV (Pelham et al. 1970), is an exception rather than a rule.

4.4.1 Factors Influencing Durability

Development of elite cultivars expressing resistance to viruses is an expensive and time consuming endeavor. As such, attempts have been made to identify parameters influencing durability of resistance in order to make knowledge-based decision regarding durability prior to deployment. Most published studies, however, are focused on naturally occurring resistance possibly because of a history of deployment under field conditions. In general, durability can be measured only after a long period of cultivation under different environmental conditions and in the presence of pathogen pressure. Unlike naturally occurring resistance, engineered resistance has not been widely deployed in the field on a large scale. One notable exception is transgenic papaya resistant to *Papaya ring spot virus* (PRSV) with over a decade-long history of deployment (Tripathi et al. 2008). Although no resistance-breaking isolate of PRSV has evolved under Hawaiian conditions to date, resistance break-down could occur with the emergence of new viral strains locally or by introduction of diverse strains from other parts of the world (Tripathi et al. 2008). It is known that transgenic papaya plants grown in Hawaii have a varying degree of resistance to non-Hawaiian isolates of PRSV (Gonsalves 1998). It should be noted that in general, engineered resistance to viruses is sequence homology dependent, but divergence of sequences as a consequence of mutation, or recombination, is not the only factor responsible for breakdown of resistance mediated by RNAi (Tripathi et al. 2004). Isolates of a virus with strong silencing suppressor activity can overcome RNAi-mediated resistance even when they share 100% identity with the sequence of the transgene transcript; however, experimental evidence in support of this possibility is limited (Li and Ding 2006; Tripathi et al. 2008). Nevertheless, it has been shown experimentally that resistance mediated by RNAi can be overcome simply as a consequence of point mutations in viral genomic sequence complementary to the transgene transcript sequences (Lafforgue et al. 2011).

Strategies for improving durability of RNAi-mediated resistance have been suggested, but none has been tested under field conditions (Lafforgue et al. 2013).

For naturally occurring virus resistance genes, a number of parameters have been identified that influence durability. These include the role and significance of avirulence determinants in the virus infection cycles, the number of mutations required for gain of virulence, the genetic context of viral genomes expressing avirulence genes, background of plants expressing the resistance genes, and fitness costs to the viruses in susceptible hosts as a consequence of gain of virulence (Harrison 2002; Janzac et al. 2009; Leach et al. 2001; Mestre et al. 2000; Palloix et al. 2009; Quenouille et al. 2013; Vera Cruz et al. 2000; Wang and Hajimorad 2015). Thus, knowledge on these aspects of virus-host interactions allows predictions to be made in regard to the durability of the resistance genes prior to deployment. Identification of viral determinants interacting directly or indirectly with the product of resistance genes, however, is the essential first step.

4.4.2 Identification of Avirulence/Virulence Determinants

We define “virulence” here as the genetic ability of a virus to overcome resistance mediated by a dominant or recessive gene (Shaner et al. 1992). To identify and map the avirulence/virulence determinants, virologists have taken advantage of naturally occurring resistance-breaking isolates combined with reverse genetic approaches (Hull 2014). In the absence of naturally occurring resistance-breaking isolates, other creative approaches including experimental evolution of avirulent viruses or transient gene expression of viral encoded proteins, have been utilized (Abbinck et al. 1998; Fellers et al. 2002; Hajimorad et al. 2003, 2011; Kiraly et al. 1999; Mestre et al. 2000, 2003). In some instances, closely related viruses have been used to identify the avirulence/virulence determinants (Padgett and Beachy 1993; Padgett et al. 1997). These efforts have shown a positive correlation between number of mutations required for evasion of resistance and durability, which has been discussed in detail by Harrison (2002). Furthermore, a positive correlation between durability and the role and significance of avirulence determinants in the infection cycle of viruses have also been observed (Janzac et al. 2009; Mestre et al. 2003). Thus, knowledge of avirulence/virulence determinants and the role(s) they play in virus infection cycles allows predictions to be made in regard to durability of the corresponding resistance genes prior to deployment.

4.4.3 Fitness Penalty as a Consequence of Gain of Virulence

In contrast to the relatively large number of studies on identification of viral avirulence/virulence determinants, only limited studies have been done on fitness cost as a consequence of gain of virulence (Table 4.1). It should be noted that “fitness”

Table 4.1 Fitness loss in susceptible hosts associated with gain of virulence by avirulent viruses on resistant hosts

R gene	Host	Virus ^a	AVR factor ^b	References
<i>Rx</i>	Potato	PVX	CP	Goulden et al. (1993)
L, L2, L3	Pepper	PMMoV	CP	Fraile et al. (2011)
<i>Rsv4</i>	Soybean	SMV	P3	Wang and Hajimorad (2015)
<i>Rsv1</i>	Soybean	SMV	HC-Pro, P3	Khatabi et al. (2013)
<i>TuRB01</i>	Turnip	TuMV	CI	Jenner et al. (2002b)
<i>TuRB04</i>	Turnip	TuMV	P3	Jenner et al. (2002a)
<i>TuRB04</i>	Turnip	TuMV	CI	Jenner et al. (2002a)
<i>Tm-1</i>	Tomato	ToMV	Pol	Fraser (1992)
<i>Pvr4</i>	Pepper	PVY	Pol	Janzac et al. (2010)
<i>N'</i>	Tobacco	TMV	CP	Taraporewala and Culver (1996)
<i>Rz1</i>	Sugar beet	BNYVV	P25	Bornemann and Varrelmann (2013)
<i>pvr2</i> ³	Pepper	PVY	VPg	Ayme et al. (2006)
<i>rymv1-2</i>	Rice	RYMV	VPg	Poulicard et al. (2010)

^aViruses names: *Potato virus X* (PVX); *Pepper mild mottle virus* (PMMoV); *Soybean mosaic virus* (SMV); *Turnip mosaic virus* (TuMV); *Tomato mosaic virus* (ToMV); *Potato virus Y* (PVY); *Tobacco mosaic virus* (TMV); *Beet necrotic yellow vein virus* (BNYVV); *Rice yellow mottle virus* (RYMV)

^bCoat protein (CP); protein 3 (P3); helper component-proteinase (HC-Pro); cylindrical inclusion protein (CI); RNA dependent RNA polymerase (Pol); 25 kilodalton protein (P25); viral protein genome-linked (VPg)

is defined as the ability of a plant virus to replicate and propagate in susceptible hosts and accumulate to a high level (Holland et al. 1991). Interestingly, based on theoretical considerations, a higher cost on pathogenicity has been predicted for gain of virulence on resistant plants for viruses compared to cellular pathogens (Sacristan and Garcia-Arenal 2008). This is presumably because of the multifunctional roles that viral encoded proteins play in virus infection cycles (Hull 2014).

Loss of virus fitness has been assessed in competition experiments where a susceptible host is co-infected with both virulent and avirulent viruses (Jenner et al. 2002b). Alternatively, the susceptible host plants are infected individually with virulent or avirulent viruses for comparison (Wang and Hajimorad 2015; Khatabi et al. 2013). Fitness loss as a consequence of gain of virulence can be inferred from indirect evidence as well (Mestre et al. 2003; Murrant et al. 1968). These studies show that virulent variants accumulate at lower levels compared to avirulent variants, which may negatively affect their transmission in the field. This may be more applicable to viruses that have persistent relationships with vectors (Banik and Zitter 1990; Gray et al. 1991). For non-persistent, stylet-borne viruses, a low level of virus particles in tissues may not have significant negative impact on uptake and transmission (Moury et al. 2007). Reduced fitness may influence other steps in a virus infection cycle. A notable example is TMV that cannot easily overcome the *N'* gene of tobacco, because the alteration of TMV CP required for evasion of *N'*-mediated recognition destabilizes the virions, and consequently inhibits systemic movement (Taraporewala and Culver 1996).

4.4.4 *Highly Durable Antiviral Resistance Genes*

There is not much public information on the level of deployment of resistance genes against viruses in commercial cultivars. However, tobacco *N* against TMV, potato *Ry* against PVY, and potato *Rx* against PVX have all been deployed over a long period of time and on a large scale. Interestingly, these three *R* genes have proved durable. The viral avirulence factor corresponding to each of these genes has been identified, but the number of mutations required for gain of virulence has only been determined for the PVX/*Rx* system (Goulden et al. 1993). Only a single virulent strain of PVX overcoming *Rx*-mediated resistance has been identified globally to date, which was isolated from a potato germplasm collection from Bolivia (Moreira et al. 1980). To some extent, the durability of *Rx*-mediated resistance can be attributed to the requirement for two simultaneous mutations in CP as well as associated fitness loss in susceptible potato as a consequence of gain of virulence. However, PVX does not have an active vector and it is mechanically or vegetatively transmitted. Thus, it is possible that lack of an active vector affects its durability under field conditions. In the TMV/tobacco *N* system, no resistance-breaking isolate of TMV has been detected globally. However, a related *Tobamovirus*, formerly called TMV strain Ob, was used to identify the TMV helicase as the avirulence factor, but it remains unknown how many mutations are required for acquisition of virulence by an avirulent TMV. It also remains unknown whether there is any fitness penalty to TMV for acquisition of virulence on *N*-genotype tobacco. It should be noted that TMV, similar to PVX, lacks an active vector, and sanitary practices by growers reduce pathogen pressure in the field. Interestingly, no resistance-breaking isolate has been identified worldwide for the PVY/*Ry* system, but unlike PVX and TMV, PVY is transmitted efficiently by aphids. Because there is no resistance-breaking strain of PVY, the number of mutations required for acquisition of virulence has not been identified. Similarly, fitness cost to PVY as a consequence of gain of virulence remains unknown. Nevertheless, durability of *Ry* has been attributed to constraints on evolution of its cognate avirulence factor, which is the nuclear inclusion a-protein (NIa) (Mestre et al. 2000, 2003). NIa plays a key role in the replication of potyviruses (Revers and Garcia 2015).

In soybean three dominant genes confer resistance against SMV, but we lack information about the extent of their deployment under field conditions. Studies on SMV isolates from different parts of the world have shown widespread presence of virulent strains on *Rsv3*-genotype soybeans (Ahangaran et al. 2013; Khatabi et al. 2012; Seo et al. 2009b; Viel et al. 2009). In contrast, a limited number of *Rsv4* resistance breaking isolates have been reported (Ahangaran et al. 2013; Chowda-Reddy et al. 2011; Gunduz et al. 2004; Khatabi et al. 2012). Virulent isolates on *Rsv1*-genotype soybeans have been reported only from Korea as well as one strain in North America that was isolated originally from imported soybean germplasm (Cho and Goodman 1979; Choi et al. 2005). Currently, the reason behind widespread presence of *Rsv3*-breaking isolates of SMV remains unknown. The strain-specific cytoplasmic inclusion (CI) protein acts as the avirulence

determinant (Seo et al. 2009a; Zhang et al. 2009). However, it appears there is no fitness cost to SMV for gain of virulence on *Rsv3* (Khatabi et al. 2013; Wang and Hajimorad 2015). In contrast, gain of virulence on both *Rsv1* and *Rsv4* results in fitness loss (Khatabi et al. 2013; Wang and Hajimorad 2015). Interestingly, two SMV-encoded proteins, HC-Pro and P3, are targeted for recognition by the *Rsv1* locus and simultaneous mutations in both is a requirement for gain of virulence (Eggenberger et al. 2008; Hajimorad et al. 2011; Wen et al. 2013).

4.4.5 Strategies to Enhance Durability

A requirement for multiple mutations in a virus genome combined with a high fitness penalty for gain of virulence would confer the highest durability to a resistance gene. Thus, stacking resistance genes targeting different viral encoded proteins is one approach to enhance durability. Indeed complementary actions of two recessive genes conferred durable resistance against PVY in a tobacco genotype (Acosta-Leal and Xiong 2008). The *Rsv1* locus in soybean conferring extreme resistance against SMV also contains multiple resistance genes perceiving two different SMV encoded proteins as avirulence factors (Wen et al. 2013). Plant breeders can also develop cultivars containing more than one resistance gene targeting different viral encoded proteins for recognition. Soybean plants expressing multiple *R* genes against SMV have been developed, but the durability of these genotypes under field conditions is unknown (Saghai Maroof et al. 2008; Shi et al. 2009). It has also been shown that combining the effect of a single resistance gene with quantitative resistance, controlled by the genetic background of a host, also lead to durable resistance (Palloix et al. 2009; Quenouille et al. 2013). To further enhance durability, one can also combine RNAi-based resistance with naturally occurring resistance. However, it appears that generation of such genotype is a challenging endeavor in some pathosystems (Beyene et al. 2015).

4.5 Conclusion

The study of genetic resistance to viruses has provided important insights into plant immunity mechanisms. Some of the mechanisms, such as NBS-LRR *R* genes, are broadly applicable to other kinds of pathogens while others, such as eIF4E, are specific to viruses or even specific genera of viruses. Researchers have also revealed the basis of antiviral RNA silencing, and they have demonstrated that this manipulable mechanism can easily be programmed to confer highly effective resistance to important plant viruses. However, viruses are continually evolving and new variants emerge, which continually forces development of new sources of genetic resistance. The following research areas focus on increasing our understanding

the factors that influence host-virus interactions in order to improve the longevity of virus resistance traits whether they are found in nature or newly engineered.

1. Mutation(s) in viral genomes is essential for evasion of resistance, but the functional roles of virulence mutations and the mechanisms of evasion remain to be understood. Coupled with this, more information is needed on the functional constraints that such mutations place on viral pathogenesis.
2. A number of viral suppressors of gene silencing have been targeted for recognition by resistance genes in different pathosystems (Choi et al. 2004; Kobayashi and Hohn 2004; Li et al. 1999; Scholthof 2006; Wen et al. 2013). However, to date a link between avirulence activity and suppression of gene silencing activity has been documented only in two pathosystems (Ishibashi et al. 2011; de Ronde et al. 2013).
3. Environmental conditions or interactions with other unrelated viruses or organisms may impact the efficacy of resistance. These factors need to be understood in more detail to guide deployment of natural and rationally engineered resistance traits.
4. Understanding the structural basis for recognition of viral avirulence determinants by R proteins. This information coupled with artificial evolution is beginning to be used to expand the recognition specificity of viral R proteins, such as Rx (Harris et al. 2013; Farnham and Baulcombe 2006), and it could eventually be used in combination with SSN to expand the virus R gene repertoire in crop plants.
5. Precise information on how host factors and viral proteins interact would increase our ability to modify host proteins to evade interaction with viral proteins without disrupting their normal cellular functions. Knowledge of recessive resistance traits and host factors has already been used as the basis to create SSN-directed mutations in barley and rice genes to generate recessive resistance to powdery mildew and *Xanthomonas oryzae* pv. *oryzae*, respectively (Wang et al. 2014; Li et al. 2012b).

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Chapter 5

Cell-to-Cell Movement of Plant Viruses: A Diversity of Mechanisms and Strategies

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Abstract Plant viruses have the capacity to replicate in most individual cells, but only those that can egress from these initially infected cells and move, cell to cell via plasmodesmata (PD), can establish a productive infection. A critical step in this process is the capacity of the virus to encode one or more movement proteins (MPs) that interact with PD, increase the PD size exclusion limit (SEL) and mediate the cell-to-cell movement of viral nucleic acids (DNA or RNA) or virions. Numerous distinct cell-to-cell movement mechanisms are known for plant viruses. Moreover, the fact that different types of viruses (e.g., families or genera) share similar cell-to-cell movement mechanisms suggests convergent evolution. Here, we will review the types and properties of major cell-to-cell movement mechanisms of plant viruses. The first include a large number of positive-sense single-stranded (ss) RNA viruses that are not phloem-limited and subtly modify PD. These move cell-to-cell either as a vRNA-MP complex, not requiring the capsid protein (CP), and are exemplified as *Tobacco mosaic virus* (TMV); or as a vRNA-MP-CP complex, most likely a non-virion form, and are exemplified as *Potato virus X* (PVX). The second type is a diverse group of non-phloem-limited viruses, which include double-stranded (ds) DNA and positive-sense ssRNA viruses, and have evolved a mechanism to drastically modify PD into MP-lined tubules through which intact virions move cell to cell. The third type includes phloem-associated and -limited viruses that include the positive-sense ssRNA closteroviruses and luteoviruses and the circular ssDNA geminiviruses. These viruses utilize the specialized PD that interconnect sieve elements (SEs) and companion cells (CCs) for cell-to-cell movement.

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Geminiviruses can move cell-to-cell via PD or cell division, induced by viral infection or associated with normal plant development. These phloem-limited viruses may or may not require virions for cell-to-cell movement. Although the precise mechanism(s) by which any virus moves, cell to cell, is not known, considerable progress has been made in understanding the cell biology of the process. The endoplasmic reticulum (ER) plays a critical role in cell-to-cell movement, especially for viruses that do not use tubules; the cytoskeleton and myosins mediate trafficking of the movement competent form of the virus to the plasma membrane (PM) and PD; and considerably more is known of the host factors involved. As new and powerful tools for cell biology, microscopy and genomics/proteomics continue to be developed, integrated studies should allow for a comprehensive picture of the viral and host factors involved in cell-to-cell movement.

5.1 Introduction

Successful infection by a plant virus generally involves three basic steps: replication, cell-to-cell movement and long distance transport through the phloem. First, the virus (usually virions) is introduced into a wounded cell that is competent for replication. These wounds are most commonly made by the feeding of insect vectors (e.g., aphids, leafhoppers, thrips and whiteflies), but are also made by other types of vectors (e.g., fungi and mites), physical damage (e.g., plant-to-plant contact), and pollen and seeds. The fact that the majority of plant viruses (~75 %) are delivered into plant cells via the feeding of insect vectors reflects their importance in allowing viruses to circumvent the ~200 nm cell wall barrier. Having gained access into a wounded living cell, the virus is unencapsidated and the steps in replication proceed.

Most viruses can replicate in most individual plant cells, but only a subset have the capacity to move cell to cell and develop a productive infection. The capacity for a virus to move cell to cell depends on the expression of movement proteins (MPs), which interact with and modify plasmodesmata (PD), the plasma membrane-lined pores that interconnect plant cells into a symplasm (Lucas et al. 2009) (Table 5.1). MPs are essential for viral cell-to-cell movement because the PD pores are typically far too small (diameter of ~3 nm) to allow diffusion of virus nucleic acids and virions (Kumar et al. 2015). Indeed, the constraint imposed on virus infection by the dimensions of the PD is reflected by the fact that nearly all viruses have acquired or evolved one or more genes that express a MP(s) that allows for modification and increase of the size exclusion limit (SEL) of PD. The action of these MPs mediates the cell-to-cell movement of an infectious form of the virus. In general, MPs have the following properties: binding with the viral nucleic acid, usually not in a sequence-specific manner but

Table 5.1 List of terms for cell-to-cell movement of plant viruses

Plasmodesmata
Intercellular junctions of cytoplasmic continuity in plant cells. Allows for cell-to-cell trafficking of macromolecules.
Movement protein (MP)
A viral-encoded protein that mediates cell-to-cell movement. It may increase the size exclusion limit of the PD, allowing for large macromolecules to move cell-to-cell.
Endoplasmic reticulum (ER)
An organelle that consists of a membranous system continuous to the nuclear membrane. It is the site of production and processing of lipids and proteins.
Endosomal system
A system that allows for the internalization, trafficking and sorting of molecules through membranous compartments (endosomes) for degradation or delivery to the trans-Golgi network for recycling.
Secretory system/pathway
A secretion pathway utilized by the cell to export proteins or other metabolites to different compartments and organelles like the plasma membrane, Golgi complex, plasmodesmata or the extracellular environment.
Microtubules
Long tubular components of the cytoskeleton that have essential roles in cell shape definition, transport of organelles and cell division.
Microfilaments
Actin-based polymers that are a component of the cytoskeleton, and are involved in maintaining cell shape and providing a structure that functions as “rails” over which cell components can be transported.
Myosin
Family of ATP-dependent motor proteins involved in short-range cargo (e.g., vesicles, RNA) transport on actin microfilaments.
Vesicles
Closed sac-like structures usually formed by a lipid bilayer membrane and enclosing fluid.
Host factors
Proteins of the host cell that can have a function in non-host processes, such as viral infection.
Host receptors
Internal or cell-surface host-encoded proteins that have affinity for specific molecules and can trigger cell responses upon binding these molecules. Viruses may use receptors to facilitate infection.
Chemical inhibitor
A chemical compound that disrupts or hinders the proper functioning of cell components or processes, e.g., latrunculin.
Gene silencing
A system that regulates gene expression through the specific degradation of RNA. It is one of the most important plant defense mechanisms against viral infection.
Endogenous non cell-to-cell autonomous proteins (NCAPs)
Proteins that act in cells distant from where they were translated and typically are involved in gene regulation. NCAPs may traffic through PD and play a role in processes such as the determination of cell fate in the plant meristem.

according to the type of viral nucleic acid; localizing to PD; modifying and increasing the PD SEL; and facilitating cell-to-cell movement of viral nucleic acids or virions through altered PD. Moreover, the critical importance of this step in the viral life cycle comes from the evolution of multiple mechanisms for cell-to-cell movement through PD and in different tissue types, and evidence of the convergent evolution of similar strategies by very different genera/families of viruses, e.g., those with positive-sense ssRNA and double-stranded (ds) DNA genomes.

In this review, we will present the types and properties of major plant virus cell-to-cell movement mechanisms. For each mechanism, we will focus on a representative virus, and provide a brief background, an update of the current understanding of the cell-to-cell movement mechanism, and present a model depicting the viral and host factors involved. The first type includes a large group of positive-sense ssRNA viruses that subtly modify PD and move cell-to-cell as a vRNA-MP and are represented by *Tobacco mosaic virus* (TMV), or as a vRNA-MP-CP, most likely in a non-virion complex, and are represented by *Potato virus X* (PVX). These two viruses use the 'classical' cell-to-cell movement mechanism that occurs in non-phloem (epidermal and mesophyll) cells and that involves a MP that binds ssRNA, targets PD, increases the PD SEL and mediates cell-to-cell movement of a viral replication complex (VRC). We also discuss cell-to-cell movement of potyviruses, another positive-sense ssRNA virus, which also infects non-phloem cells but utilizes a different mechanism, possibly involving virions. The second major type is a diverse group of non-phloem limited viruses that drastically modify PD into MP-lined tubules, through which intact virions move cell to cell. Evidence for convergent evolution of this mechanism in plant viruses comes from the fact that viruses with very different genomes and virion properties share this cell-to-cell movement mechanism. The third major type includes phloem-associated or -limited viruses. They are represented by divergent viruses, including species in three major families: *Closteroviridae*, *Luteoviridae* and *Geminiviridae*. As these viruses possess distinct genomes (positive-sense ssRNA [closteroviruses and luteoviruses] and circular ssDNA [geminiviruses]) and virion properties, it is likely they evolved different cell-to-cell movement mechanisms to infect cells of the phloem compared with those used by non-phloem limited viruses. Moreover, because these viruses are phloem-limited, they may utilize different and perhaps less extensive cell-to-cell movement mechanisms. For example, cell-to-cell movement of phloem-limited viruses involves the specialized plasmodesmata that interconnect cells of the phloem (e.g., sieve elements [SE] and companion cells [CCs]) and different types of MPs. In this review, we will mostly focus on the cell-to-cell movement of phloem-associated (e.g., the bipartite begomovirus *Bean dwarf mosaic virus* [BDMV]) and phloem-limited (e.g., the monopartite begomovirus, *Tomato yellow leaf curl virus* [TYLCV] and the monopartite curtovirus *Beet curly top virus* [BCTV]) geminiviruses.

5.2 DNA Viruses Must Go Through Additional Step in Cell-to-Cell Movement: Passage Across the Nuclear Pore Complex (NPC)

Prior to cell-to-cell movement, DNA viruses must enter the nucleus of the cell for replication and gene expression. Thus, we consider this to be an important step in the cell-to-cell movement of plant-infecting DNA viruses. The nucleus contains the genetic material (DNA) of the cell, and is surrounded by a bilipid membrane, referred to as the nuclear envelope (NE). Trafficking of macromolecules between the nucleus and the cytoplasm is tightly regulated by the nuclear pore complex (NPC), a >60 MDa cylindrical channel composed of multiple copies of ~30 different proteins and a central diameter of ~10 nm (Pemberton and Paschal 2005). The transport of larger proteins across the NPC involves active transport mediated by proteins called karyopherins or importins. Macromolecular trafficking between the nucleus and cytoplasm involves specific amino acid sequences for import (nuclear localization sequences, NLS) and export (nuclear export signals, NES). In most cases, the constraints imposed by the nuclear pore complex do not allow free diffusion of virions across the nuclear pore complex. Thus, viruses have evolved multiple strategies for nucleocytoplasmic transport including (1) transport during mitosis; (2) transport through the NPC; (3) transport after releasing the viral genome at the cytoplasmic side of the NPC; (4) transport of intact virions capsids through the NPC, followed by genome release; and (5) nuclear entry via disruption of the NE.

For this review, we will briefly consider some aspects of the nuclear transport of the two types of DNA viruses for which the cell-to-cell transport mechanism is examined. This includes the family *Caulimoviridae*, genus *Caulimovirus* (pararetroviruses with a circular dsDNA genome) and the family *Geminiviridae*, genera *Begomovirus* and *Curtovirus* (circular ssDNA genome).

5.3 Plasmodesmata

The PD serve as symplasmic channels that mediate intercellular exchange of nutrients and the trafficking of signaling molecules between neighboring cells (Lucas et al. 2009). An individual PD has a diameter of approximately 50–60 nm (Robards 1975; Bell and Oparka 2011; Jahn et al. 2012), and a desmotubule or appressed endoplasmic reticulum (ER) centered within a plasma membrane (PM)-lined cylinder. The space between the ER and PM, termed the cytoplasmic annulus or sleeve, is divided into nano-channels by proteins embedded in the PM and ER, and it is through these channels that nutrients and signaling molecules move cell to cell (Fig. 5.1a). Importantly, most PD nano-channels have average diameters of only 2.5–3.0 nm, which permit intercellular diffusion of small molecules, such as metabolites, ions, and hormones that are less than 1 kDa in size. Thus, the PD SEL

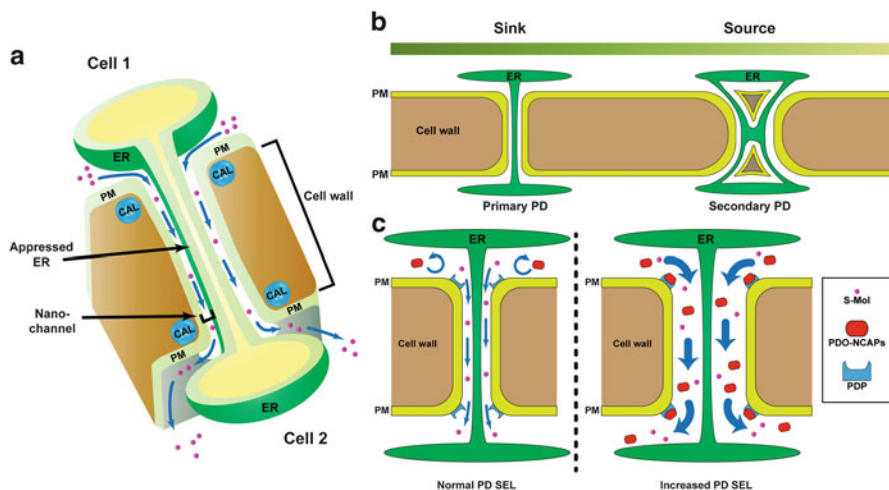


Fig. 5.1 Schematic illustration of the structure and function of a plasmodesma (PD). (a) Small molecules (purple balls; molecular weight <1 kDa) diffuse through PD nano-channels. Callose biosynthesis/degradation at the neck region of PD regulates size exclusion limit (SEL) to control symplasmic diffusion of molecules. CAL, callose; PM, plasma membrane; ER, endoplasmic reticulum. (b) Modification of primary PD to form secondary PD during the sink-to-source transition in developing leaves. (c) Cell-to-cell trafficking of macromolecules involves an increase in PD SEL through the action of PD-opening non-cell-autonomous proteins (PDO-NCAPs). Left image: Nano-channels of PD allow intercellular trafficking of small molecules (S-Mols; <1 kDa). Right image: After PDO-NCAPs, such as viral movement proteins (MPs), there is an interaction with functional PD-proteins (PDP) to increase SEL and permit non-cell-autonomous movement of informative macromolecules through these dilated PD nano-channels. PD SEL of the nano-channels also can be regulated by callose deposition/degradation

is generally far less than the diameter of viral nucleic acids and virions (Balachandran et al. 1997; Ding 1998; Lucas et al. 2009).

According to the developmental stage of the plant, PD are classified as primary or secondary. Primary PD are structurally simple and appear as single PM-lined cylinders (Fig. 5.1b). They are formed during cytokinesis, when the ER becomes trapped and associated with the cell plate during division. Subsequently, in the course of development, the architecture of primary PD can undergo a branching process to form secondary PD, which often have a central cavity (Hepler 1982; Ehlers and Kollmann 2001; Burch-Smith et al. 2011; Fig. 5.1b). The function and structure of PD is also impacted during the sink-to-source transition in leaves, and a model has been proposed where primary PD serve as templates for the formation of secondary PD during the process of cell wall extension (Faulkner et al. 2008). In parallel with this transition, secondary PD that are located in the source region have downregulated SEL, reflecting a difference in function between primary and secondary PD for transport of macromolecules (Oparka et al. 1999).

Diffusion through the PD nano-channels is controlled by callose (β -1,3-glucan) deposition at the neck region of the PD (Vatén et al. 2011; Brunkard et al. 2015;

Fig. 5.1a). This callose deposition is highly coordinated with abiotic and biotic stress, root development, and auxin signaling (Vatén et al. 2011; Lee et al. 2011; Wang et al. 2013; Han et al. 2014). Callose-mediated PD permeability is correlated with the effect of callose binding and synthase activity (Simpson et al. 2009; Benitez-Alfonso et al. 2013; Maule et al. 2013), and can also be influenced in situations where sterol interferes with callose biosynthesis/degradation at the PD (Grison et al. 2015).

In addition, a number of proteins have been identified that can influence PD function. These include PD-localized protein-1, -5 and -6, which are transmembrane proteins with DUF26 domains (cysteine-rich receptor-like kinase domains of unknown function); PD callose binding protein (PDCB1); and Plasmodesmal-localized β -1,3 glucanase1 and 2 (PdBG1 and 2), which influence the processes involved in PD callose deposition/degradation (Levy et al. 2007; Thomas et al. 2008; Simpson et al. 2009; Fernandez-Calvino et al. 2011; Lee et al. 2011; Benitez-Alfonso et al. 2013).

Regarding intercellular trafficking of information macromolecules, such as proteins and RNA, PD often interact with specific classes of proteins that can increase PD SEL to allow non-cell-autonomous movement of these signaling agents through the PD nano-channels (Fig. 5.1c). Several endogenous proteins have been identified that can interact, at the level of PD, to mediate an increase in SEL (Xoconostle-Cázares et al. 1999; Aoki et al. 2002; Yoo et al. 2004; Ham et al. 2009; Lucas et al. 2009). Indeed, one of the key aspects of viral cell-to-cell movement is the evolution and function of MPs, and a wide diversity of MPs have been shown to increase PD SEL and mediate the cell-to-cell movement of viral RNA/DNA through PD (Lucas 2006; Heinlein 2015).

Proteins that function in the regulation of the trafficking of macromolecules through PD have also been identified. For example, remorin (REM), a PM protein detected in PD is involved in controlling cell-to-cell movement of viruses (Raffaele et al. 2009). The PD germin-like protein1 and 2 (PDGLP1 and 2) were shown to regulate primary root growth by controlling resource allocation at the level of the primary and lateral root meristems (Ham et al. 2012). A number of PD-localized receptor kinases, such as lysine motif domain-containing glycosylphosphatidylinositol-anchored protein 2 (LYM2), clavata1 (CLV1), Arabidopsis crinkly4 (ACR4), and strubbelig/scrambled (SUB/SCM) are thought to function as mediators between extracellular signals and their related responses by controlling cell-to-cell communication via PD (Faulkner et al. 2013; Stahl et al. 2013; Vaddepalli et al. 2014).

In the angiosperms, PD also play an important role in providing symplasmic continuity throughout the entire body of the plant, allowing the exchange of information between distantly-located organs. The SEs, which function as the major conductive cells of the phloem in the vascular system, undergo a developmental process in which many organelles, such as vacuoles and nuclei, are degraded and modified. PD establish cytoplasmic continuity between adjacent SEs by forming sieve plate pores during SE maturation. Files of enucleate SEs establish sieve tubes (STs), forming efficient and effective routes for transporting various materials, such as nutrients, ions, proteins and RNAs throughout the plant. To

maintain the functionality of these mature enucleate SEs, the CCs that are located next to SEs exchange macromolecules via the specialized PD that interconnect CCs to SEs. In the phloem ST system, PD located within SE-CC, CC-phloem parenchyma cells (PPC), and SE-PPC serve as regulatory valves for gating translocation of macromolecules along the phloem translocation pathway. Coordination of PD gating, within phloem ST system, in terms of PD opening and closing among SE, CC and PPC, is considered as a pivotal mechanism to efficiently deliver macromolecules into target sink organs (Lucas et al. 2013; Ham and Lucas 2014). Indeed, plant viruses have evolved to effectively utilize this system for cell-to-cell and long-distance transport, with the specialized case of the phloem-limited viruses likely reflecting a situation where all necessary components of the virus life cycle are provided in this tissue (Rojas et al. 2005).

5.4 Cell-to-Cell Movement Mechanisms of Non-Phloem-Limited Viruses

5.4.1 *Subtle Modification of PD That Does Not Involve the CP, Tobacco mosaic virus*

Tobacco mosaic virus (TMV) is a monopartite positive-sense ssRNA virus with a genome size of ~6,400 nts and rigid rod-shaped virions that measure 300×18 nm. The virus is readily mechanically transmitted and insect vectors are not required for plant-to-plant transmission. Once a susceptible cell is inoculated, usually an epidermal or mesophyll cell, the viral RNA is uncoated and replication is initiated in the cytoplasm.

TMV was one of the first viruses used in studies of viral cell-to-cell movement. Early studies established that cell-to-cell movement was CP-independent, and involved a specialized viral protein (MP, referred to as P30 for TMV), which mediates cell-to-cell movement through PD. Key properties of the P30 include non-specific cooperative binding to ssRNA, localization to PD, and a capacity to increase in the SEL, without any striking structural abnormalities (Boevink and Oparka 2005; Lucas 2006; Heinlein 2015). The P30 is localized to ER membranes, and a VRC including the vRNA and the replicase is formed. The next question is how does the VRC get delivered to PD? One possibility is via ER-derived vesicles that traffic, via the cytoskeleton (microfilaments or microtubules), to the cell periphery and PD. Evidence for this came from transient expression studies in which P30-GFP was localized to mobile, microtubule-associated structures, and time-lapse microscopy studies that showed microtubule and ER/actin networks acting together in guiding and controlling the trafficking of ER-associated P30 and possibly VRC to PD. In the same study P30-GFP also mediated transport of its mRNA to PD (Sambade et al. 2008). In a related study, P30-GFP interacted with γ -tubulin *in vitro* (Ferralli et al. 2006; Sambade et al. 2008), and mutation of a tubulin

binding motif in the P30 reduced cell-to-cell movement (Boyko et al. 2000). Recently, a plasmodesmatal localization signal was identified in the N-terminus of the P30 (amino acids val-4 and phe-14) that targeted PD, but that was not involved in the entering or trafficking of P30 through PD (Yuan et al. 2016). Alternatively, this may involve the formation of a different complex, in which the cytoplasmic exposed domain of P30 binds a vRNA-replicase complex that then forms a raft that moves along the ER to the PD, possibly via myosin motor proteins (Heinlein 2015; Fig. 5.2).

Targeting of P30 to ER and PD is facilitated by calreticulin, an ER-lumen-localized chaperone, which regulates Ca^{+2} signaling (Jia et al. 2009; Chen et al. 2005). Interaction of P30 with other cytoskeleton components may be facilitated by microtubule end-binding protein 1 (EB1), a microtubule polymerization factor (Brandner et al. 2008) and actin filaments (McLean et al. 1995). Co-expression of P30-RFP with EB1-GFP revealed evidence of co-localization to microtubules. Interaction of P30 with EB1 could allow the VRC to interact with the cytoskeleton for transport. This was further supported by the finding that a mutant with reduced microtubule polymerization (ATER2) had reduced cell-to-cell movement and reduced symptoms compared with wild-type plants (Ouko et al. 2010).

Once the P30 and, perhaps the entire VRC, is delivered to the PD, gating must occur to allow for cell-to-cell trafficking of the infectious form of the virus. This involves viral- and host-encoded factors. One way this occurs is through the severing of actin microfilaments by the P30 at the PD, resulting in an increase in the SEL and cell-to-cell trafficking (Su et al. 2010). Consistent with this finding, treatment with the F-actin stabilizing agent, phalloidin, inhibited this process. These results also were consistent with studies showing that TMV bound to and trafficked along microfilaments. An important host factor that mediates cell-to-cell movement at the PD or the cell wall is callose, which accumulates in and around the neck region of PD. Callose accumulation is controlled by the counteracting activities of β -1,3-glucanase and β -1,3-glucan synthase, which degrades and synthesizes callose, respectively (Bucher et al. 2001). Evidence for the importance of callose in cell-to-cell movement of TMV came from the observation that callose is down regulated at PD during infection, and it has been suggested that TMV cell-to-cell movement is dependent on β -1,3-glucanases (Epel 2009). The host factor ankyrin repeat-containing (ANK) interacts with P30, and is re-directed to the PD, resulting in a downregulation of callose, thereby increasing the PD SEL and cell-to-cell movement (Ueki et al. 2010). Further evidence for this came from the finding that overexpression of ANK facilitated TMV spread, whereas local infection was slowed in silenced lines. Thus, TMV may recruit specific β -glucanases to PD during infection to induce callose hydrolysis and increase the SEL (Lee and Lu 2011). Finally, there is an association of pectin methyl esterase (PME) with cell walls, including those around PD. PME may help target P30 to PD or by modifying (increasing) the SEL. However, a clear role for PME in cell-to-cell movement has yet to be established (Chen and Citovsky 2003; Faulkner et al. 2008).

Amari et al. (2014) recently found that myosins XI-2, XI-K, VIII-1, VIII-2 and VIII-B play a role in TMV cell-to-cell movement, which is in agreement with

reports for cell-to-cell movement of other types of viruses. The motility and structure of the ER was severely affected by inhibition of myosin XI-2 or XI-K, which resulted in the confinement of P30 to ER aggregates and reduced targeting of P30 to PD. In addition, inhibition of myosins VIII-1, VIII-2 and VIII-B negatively affected accumulation of P30 along the plasma membrane and its association with PD. These results also were consistent with an earlier study in which the cell-to-cell movement of TMV VRCs was blocked by inhibition of myosin and filamentous actin, but not by MT inhibitors (Kawakami et al. 2004).

A role for ER and membrane trafficking in tobamovirus cell-to-cell movement was also revealed based on an interaction of P30 and the host factor synaptotagmin (SYTA). SYTA is a Ca^{+2} /lipid binding protein first identified in animals, and more recently characterized in plants (Chapman 2008). Synaptotagmins tether the cortical ER to the PM to maintain ER morphology and stabilize ER-plasma membrane (ER-PM) contact sites for intracellular lipid and Ca^{+2} signaling (Manford Andrew et al. 2012; Giordano et al. 2013). Evidence that SYTA might have a role in tobamovirus cell-to-cell movement came from findings that SYTA interacted with P30, and reduced endosomes and cell-to-cell movement (Lewis and Lazarowitz 2010). These results suggest a role for the ER and endosomal system in the delivery of the VRC to PD, possibly via vesicles.

In conclusion, the early events of the TMV infection involve an association with ER membranes and formation of punctate P30 bodies, as part of the early VRC formation. The VRCs are then targeted to the PM and PD, possibly via microtubules and myosins (Fig. 5.2). Numerous host factors play a role in this process, including targeting and trafficking to ER and PD, possibly via the VRC. Indeed, the punctate VRCs, which appear at the leading front of the infection, likely play a key role in cell-to-cell spread to uninfected cells. Later in the infection process, when large quantities of CP are synthesized and used to generate virions, the large crystalline inclusion bodies composed of virions are formed. The role of these inclusions is to provide virions for plant-to-plant spread of the virus.

5.4.2 Subtle Modification of PD That Require CP, Potato virus X

The potex- and hordeiviruses are positive-sense single-stranded RNA viruses with flexous rod-shaped virions, which use the triple gene block (TGB) strategy for cell-to-cell movement (Morozov and Solovyev 2003; Verchot-Lubicz et al. 2010). These viruses are typically introduced into epidermal and mesophyll cells via some sort of mechanical damage or, in the case of hordeiviruses, by seed transmission. Here, we will focus on PVX as an example of a non-phloem-limited virus that moves cell-to-cell by subtle modification of PD and requires the CP, though probably not in the form of a virion. There are many similarities of cell-to-cell movement of PVX and hordeiviruses, e.g., *Barley stripe virus*, with the exception that the CP is not required (Jackson et al. 2009).

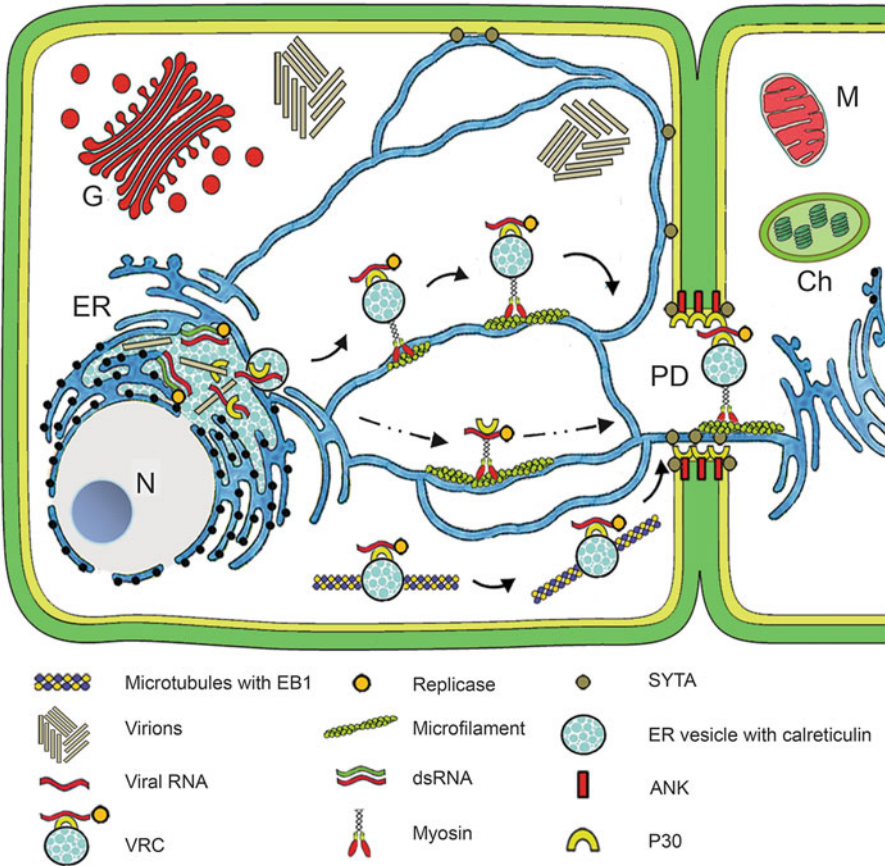


Fig. 5.2 Model for cell-to-cell movement of *Tobacco mosaic virus* (TMV) in non-phloem cells. Initially, the viral replication complex (VRC) is assembled in association with the ER membranes and the interaction of the vRNA, the replicase and P30. Transport of the VRC to the PM and PD occurs via either ER-derived vesicles or a ribonucleoprotein complex (vRNA-P30) that is transported along the cytoskeleton (microtubules or microfilaments), possibly mediated by ATP-dependent motor proteins. PD targeting of the VRC might also be mediated by host factors such as calreticulin, SYTA and the microtubule polymerization protein (EB1). Once at the PD, P30 and possibly ANK, increases the SEL and mediates the passage of a P30-vRNA complex or VRC to an adjacent uninfected cell, where the vRNA replication process begins again. *N* nucleus, *G* Golgi complex, *PD* plasmodesmata, *ER* endoplasmic reticulum, *M* mitochondria, *Ch* chloroplast

PVX has a genome size of 6,435 nts, and flexuous-shaped virions that measure $\sim 500 \times 13$ nm. Once virions are mechanically introduced into a susceptible cell, the viral RNA is uncoated in the cytoplasm. The infection process is sequential and can be divided into at least three phases: (1) the early replication phase and formation of a VRC; (2) an early to middle cell-to-cell movement phase, where an infectious form, most likely a non-virion composed of vRNA and viral proteins,

moves cell to cell through PD; and (3) a late phase where large quantities of vRNA and CP are generated. In this late phase, virions are synthesized and large fibrous inclusions, often in a parallel arrangement, are produced for plant-to-plant spread. Also at this late phase, the proteins involved in cell-to-cell movement are thought to be sequestered to allow for unimpeded formation of virions (Tilsner et al. 2012).

In the initial phase, viral replication and protein expression occurs in ER-associated vesicles and a VRC is produced. During cell-to-cell movement (phase 2), PVX induces a proliferation of the ER, with the ER playing a key role in cell-to-cell movement. The key players in cell-to-cell movement are: (1) the vRNA; (2) the triple gene block (TGB) proteins, TGB1, TGB2 and TGB3; and (3) the CP, most likely in a non-virion form. The TGB1 is an ~25 kDa multifunctional protein. It is expressed at the highest level of the TGB proteins, and it plays an important role in replication, VRC formation and cell-to-cell movement. The TGB1 protein binds ssRNA; has helicase activity; localizes to the PD and increases the SEL, but does not actually target PD; suppresses gene silencing; and activates translation (Howard et al. 2004; Lucas 2006; Verchot-Lubicz et al. 2010; Fig. 5.3). The TGB1 binds with the 5' non-translated portion of the vRNA to form a complex. The CP is also essential for cell-to-cell movement, but this does not involve virions or a CP-mediated increase in the PD SEL. There are three possibilities for how the CP mediates cell-to-cell movement. First, a TGB1-vRNA-CP complex is formed and moves through PD; second, the complex that moves cell to cell is composed of TGB1 and vRNA; and third, the complex that moves is composed of TGB1 and virions. It has been challenging to sort out the distinct roles of the CP in PVX cell-to-cell movement and virion formation. However, there is evidence for an interaction between TGB1, vRNA and a non-virion form of CP. First, deletions in the C-terminus of the CP interfered with cell-to-cell movement, but not virion formation (Verchot-Lubicz et al. 2007; Kumar et al., 2015). Second, the interaction of the NTPase/helicase motif of TGB1 with the C-terminus of the CP supported the formation of a non-virion TGB1-vRNA-CP complex.

The TGB2 (~13 kDa) and TGB3 (~8 kDa) are membrane-associated proteins that induce proliferation of ER vesicles, consistent with TGB2 having two transmembrane domains and TGB3 having one. Mutational analyses revealed that these domains are important for cell-to-cell movement of PVX, and this is likely by targeting TGB1-vRNA-CP (or another cell-to-cell movement complex) to PD (Fig. 5.3). Thus, it is believed that TGB2 and TGB3 serve as accessory factors, which mediate the delivery of the vRNA complex to PD via some type of vesicle (Lucas 2006). The TGB2 and TGB3 vesicles are motile along the ER network, via the actin cytoskeleton and myosin motor proteins (Kumar et al. 2015; Fig. 5.3). At the PD, the TGB2 plays a role in increasing the PD SEL, whereas TGB2 and TGB3 target the infectious form of the virus to PD. Results of microinjection and particle bombardment studies have indicated that the TGB2 and TGB3 do not move through PD (Lough et al. 1998; Serazev et al. 2003). Rather, the infectious form of the virus traffics through PD, whereas TGB2 and TGB3 remain in the infected cell and are recycled via the endosomal system to re-initiate the process (Fig. 5.3). Once the

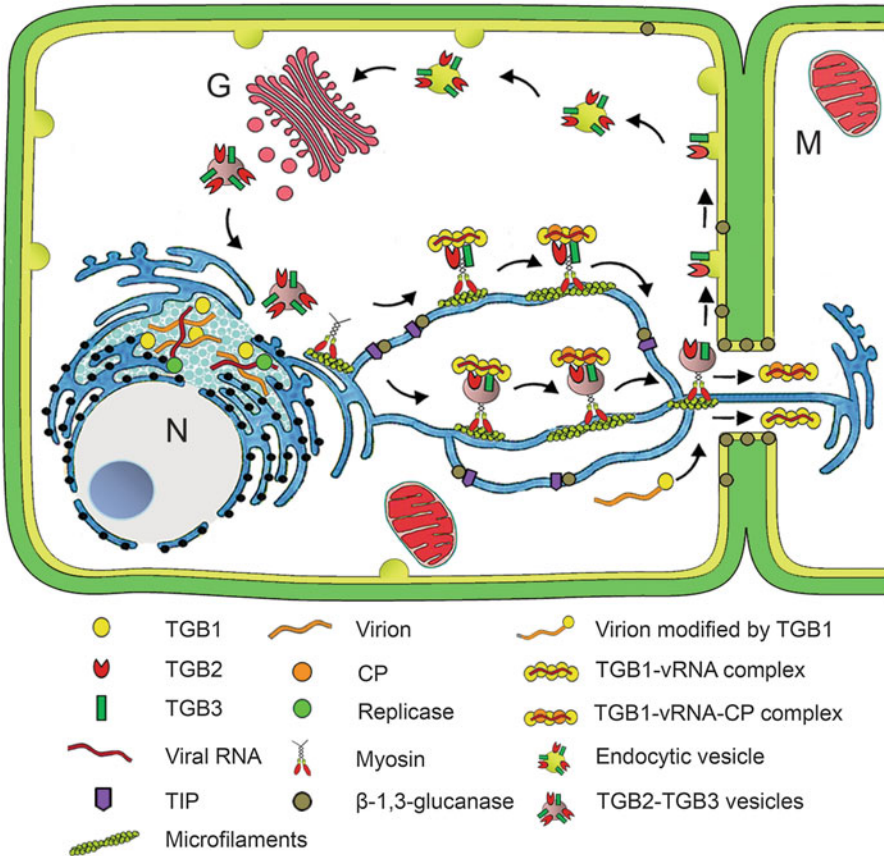


Fig. 5.3 Model for cell-to-cell movement of *Potato virus X* (PVX) in non-phloem cells. Replication of the viral RNA induces remodeling of the ER and formation of the viral replication complex (VRC). At the VRC, TGB1 interacts with the vRNA and possibly CP and forms a RNP complex (TGB1-vRNA or TGB1-vRNA-CP). TGB1 could also interact with the CP to create a modified movement competent virion. The VRC is then transported to the plasma membrane (PM) and PD via TGB2 and TGB3 vesicles that are motile along the ER network by ATP-dependent motor proteins guided by TGB3. The VRC may also be transported to PD by TGB2 and TGB3 without the vesicle. At the PD, the TGB1 and TGB2 increase the SEL, and a complex of TGB1-vRNA or TGB1-vRNA-CP moves, cell-to-cell, to the adjacent cell where vRNA is released and initiates viral gene expression and replication. TGB2 and TGB3 remain in the infected cell and are recycled by the endocytic pathway. The host factor TIP1 interacts with TGB2 and TIP1 is localized with the β -1,3-glucanase at the PM and may increase the SEL. Remorin also interacts with TGB1 and may increase the SEL. *N* nucleus, *G* Golgi complex, *M* mitochondria

complex has passed through the PD, the vRNA is believed to be released for translation of replicase and initiation of replication.

A number of host factors have been identified that play a role in PVX cell-to-cell movement (Fig. 5.3). A yeast-two-hybrid screen using TGB2 as bait identified three interacting host proteins: TIP1, TIP2 and TIP3. Using the same approach, it was

further established that the TIP1 protein interacted with a plasma membrane localized β -1-3 glucanase (Fridborg et al. 2003; Hyun et al. 2011). The associated callose degradation is likely to result in an increase in the PD SEL and cell-to-cell movement. Another host factor that may play a role in PVX cell-to-cell movement is remorin, which was found to interact with TGB1 in a yeast-two-hybrid screen. The remorin protein localized to the plasma membrane and interacted with TGB1. Furthermore, over expression of remorin resulted in reduced cell-to-cell movement, indicating an interference with this process (Raffaele et al. 2009).

5.4.3 *Potyvirus Cell-to-Cell Movement: A Different Mechanism of a Non-Phloem-Limited Movement That may Involve Virions*

The viruses in the genus *Potyvirus*, family *Potyviridae* comprise a large group of positive-sense ssRNA viruses characterized by having a single ~10 kb genomic RNA and long flexuous rod-shaped virions measuring 700–800 nm. In nature, these viruses are transmitted by aphids in a non-persistent manner, but they also can be transmitted mechanically (i.e., via sap) and, in some cases, via seeds. The genome has 5' end-linked terminal protein (VPg) and a polyadenylated sequence at the 3' end. The genome strategy involves expression of a single large open reading frame (ORF), which encodes for a polyprotein that is cleaved into functional proteins by viral-encoded proteinases (reviewed in Revers and García 2015). Potyviruses replicate in the cytoplasm, and produce striking inclusion bodies. It has been suggested that the main site of replication is chloroplast-associated membranes, and that translation occurs in association with the ER (Huang et al. 2010; Wei et al. 2010). In the case of *Turnip mosaic virus* (TuMV), it has been suggested that replication takes place in association with globular structures derived from the ER, and that intracellular transport as well as cell-to-cell movement via PD occurs via actin microfilaments (Grangeon et al. 2013).

Potyvirus cell-to-cell movement involves several viral and host proteins. The cylindrical inclusion (CI) forms the striking structures associated with PD that can be seen by light microscopy and electron microscopy (Rodríguez-Cerezo et al. 1997; Roberts et al. 2003). P3N-PIPO is an ~25 kDa potyvirus protein that has been associated with cell-to-cell movement based on the finding that TuMV mutants replicated but did not move cell to cell (Chung et al. 2008). Similarly, P3N-PIPO mutants of *Wheat streak mosaic virus* (WSMV) showed a phenotype of small clusters of infected cells, consistent with a deficiency in cell-to-cell movement (Choi et al. 2005). It has been proposed that P3N-PIPO plays a role in potyvirus cell-to-cell movement by formation of a complex by interacting with the CI protein to form a conical structure essential for cell-to-cell transport (Wei et al. 2010). This CI and P3N-PIPO complex is initially produced adjacent to the nucleus and in association with ER membranes, but it is then delivered to the PD via the secretory system (Huang et al. 2010).

To localize to PD, the PIPO domain of P3N-PIPO, interacts with the host factor plasma membrane-associated cation binding protein 1 (PCaP1), which has a myristoylation domain for targeting the PM and localization to PD (Vijayapalani et al. 2012). Experimental evidence for P3N-PIPO-PCaP1 interaction and its localization to PD came from yeast-two-hybrid, bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation experiments. A TuMV-GFP reporter was used to reveal that Arabidopsis PCaP1 mutants were replication competent (3 days post inoculation), but had greatly reduced cell-to-cell movement (Vijayapalani et al. 2012). A CI/P3N-PIPO/PCaP1 complex appears to serve as an anchor for accumulation of additional CI proteins for the formation of the conical structure at the PD (López et al. 2001; Vijayapalani et al. 2012).

The potyvirus cell-to-cell movement complex has yet to be identified and could be 1) a type of vesicle, 2) a RNP complex or 3) even modified virions. In the case of *Potato virus Y* (PVY), the movement complex may be a specialized form of the virion, with a modified tip that protrudes from one end of the virion (Puustinen et al. 2002; Torrance et al. 2006; Gabrenaite-Verkhovskaya et al. 2008). This modified end of the virion is most likely at the 5' end/N-terminus, because it interacts with the VPg (Torrance et al. 2006). HC-Pro and CI were also detected at this specialized tip structure, possibly through interacting with the VPg (Torrance et al., 2006). Thus, CI may interact with the virion tip through binding with the HC-Pro/VPg, and targeting the complex to the conical structure for cell-to-cell movement (Revers and Garcia, 2015). Additional ultrastructural evidence, possibly revealed by high pressure freezing and immunolabelling techniques, could reveal if the modified virion is the elusive potyvirus cell-to-cell movement structure.

The CP of potyviruses also plays a role in cell-to-cell movement. It is delivered to the PD through microfilaments, together with vRNA, and this complex binds to the CI conical structures (Wei et al. 2010; Seo et al. 2013). The role of CP and HC-Pro in cell-to-cell movement were investigated using microinjection studies with *Bean common mosaic necrosis virus* and *Lettuce mosaic virus*, where both proteins increased the SEL of the PD, trafficked cell-to-cell through PD and facilitated cell to cell movement of vRNA (Rojas et al. 1997). HC-Pro was particularly effective at increasing the SEL of PD, and the finding that this multifunctional protein also was a suppressor of gene silencing has suggested a possible link between these two functions, e.g., in the establishing of silencing suppression in newly infected cells. Protein-protein interaction experiments also supported the idea that HC-Pro and CP interact to mediate cell-to-cell transport of vRNA (Roudet-Tavert et al. 2002).

Hofius et al. (2007) used yeast-two-hybrid screens to show that the PVY CP interacts with *Nicotiana tabacum* DnaJ-like (HSP40) proteins, and these were named capsid protein interacting proteins (NtCPIPs). DnaJ proteins act as co-chaperones for Hsc70s to mediate a range of functions such as protein folding, trafficking, secretion and stress response signaling (reviewed in Qiu et al. 2006). Many of these functions could play a role in viral cell-to-cell movement. Transgenic tobacco plants expressing dominant negative NtCPIPs mutants were resistant

to PVY infection, suggesting a role for this protein in virus infection. Binding assays confirmed the capacity of the PVY CP and NtCPIP to interact and form a complex *in vitro* and *in vivo* (Hofius et al. 2007). Further evidence that the CP plays an important role in cell-to-cell movement came from results showing that viral replication was not impaired in the NtCPIP mutants (Hofius et al. 2007).

Another viral protein, the VPg, interacts with the host factor potyvirus VPg interacting protein (PVIP), and this may be important for cell-to-cell movement. This was based on findings that mutations that interfered with the interaction of PVIP and VPg caused severe impairment in movement, but did not affect replication. Similar results were obtained with analyses of GFP reporters and hybridization experiments with specific viral probes (Dunoyer et al. 2004). It is also interesting to note that PVIP is normally found in the nucleus (Saiga et al. 2008); therefore, this raises the intriguing possibility that PVIP/VPg interactions can regulate the expression of host genes involved in viral movement, rather than being directly involved (Revers and García 2015).

Numerous questions remain to be answered regarding potyvirus cell-to-cell movement including: (1) do potyviruses move cell-to-cell as vesicles, a non-virion RNP complex or as specialized virions; (2) what is the function(s) of the viral proteins that modify PD SEL; (3) what is the association, if any, of the cell-to-cell movement and gene silencing functions of HC-Pro; and (4) how does the potyvirus movement complex interact with the conical structure for cell-to-cell movement?

5.5 A Cell-to-Cell Movement Mechanism for Non-Phloem-Limited Viruses that Involves Virions and MP-Lined Tubules

A number of types of plant viruses utilize a mechanism of cell-to-cell movement that involves the extensive modification of PD into MP-lined tubules through which virions pass across the cell wall barrier. Compelling evidence for this type of cell-to-cell movement came from the observation of tubules protruding from the surface of protoplasts expressing MPs of these viruses.

Five main genera in four families utilize the tubule-based mechanism of cell-to-cell movement: *Caulimovirus*, *Nepovirus*, *Comovirus*, *Umbravirus* and *Tospovirus*. These genera are represented by viruses having three types of genomes and genome strategies: dsDNA, positive-sense ssRNA and ambisense ssRNA. This indicates that this mechanism of cell-to-cell movement has likely evolved independently on a number of occasions. It also reflects the selection pressure under which plant viruses are placed to evolve mechanisms to pass through the cell wall (PD). In this chapter, we will focus on on viruses in the genera *Caulimovirus*, *Nepovirus* and *Comovirus*.

5.5.1 *Cell-to-Cell Movement Mechanism Involving Virions and MP-Lined Tubules: Cauliflower mosaic virus (CaMV), a dsDNA virusa*

The genus *Caulimovirus* belongs to the family *Caulimoviridae*. It is a pararetrovirus with an ~8 kbp dsDNA genome, and an icosahedral virion of ~50 nm in diameter. The type species is *Cauliflower mosaic virus* (CaMV), and this is the virus for which cell-to-cell movement has been most extensively studied in this genus and family (Schoele et al. 2015). CaMV infects plants in the families Brassicaceae (e.g., *Arabidopsis thaliana*) and Solanaceae (e.g., *Nicotiana benthamiana*), which also has facilitated studies of cell-to-cell movement (Angel et al. 2013; Carluccio et al. 2014; Rodriguez et al. 2014).

The infection cycle of CaMV begins when virions are inoculated into the cell, either by an aphid vector or mechanical inoculation. Because CaMV has a dsDNA genome, it must first gain access to the nucleus for transcription. As soon as the virions enter the cytoplasm they are directed to the periphery of the nucleus (Leclerc et al. 1999). Because the virion is not able to pass through the NPC, there are two possibilities for how the vDNA gains access to the nucleus. First, virions are partially uncoated or physically distorted, allowing for passage of the vDNA through the nuclear pore. In the second, vDNA together with CP in a non-virion form and possibly another protein, such as reverse transcriptase, are transported through the NPC into the nucleus (Karsies et al. 2002). Once inside the nucleus, the minichromosome is formed and transcribed into the 19S and 35S RNAs, which are directed to the cytoplasm for translation.

The 19S RNA is responsible for translation of the 58 kDa P6 protein, which forms inclusion bodies (IBs) in the cytoplasm, and these eventually give rise to the virus factory (VF). The IBs will serve as sites for translation of additional proteins via the 35S RNA to give rise to P1 (MP), P2, P3, P4 (CP), P5 (reverse transcriptase) and P7. Relatively early in the life cycle, the MP is expressed and is localized to the PD (Carluccio et al. 2014). The MP is the only viral protein required for tubule formation, and it is through these tubules that the intact virions move cell to cell (Huang et al. 2000; Fig. 5.4). The transport of MP from the cytoplasm to the PM and PD may be mediated by vesicular transport (Carluccio et al. 2014). Evidence for this came from the finding that a MP mutant, lacking the Tyr sorting signal responsible for the binding of an adaptor complex that promotes the trafficking of small coated vesicles, was not directed to the PD and accumulated into the cytoplasm. Because CaMV requires a large amount of MP for tubule formation, it appears that MP is recycled and re-targeted to the PM and the PD. The three Tyr sorting signals present in the CaMV MP mediate protein recycling by the trans Golgi network (TGN)/early endosome (EE) via PM-derived vesicles. The regulation of the MP and TGN/EE allows for targeting to PD for tubule formation. Excess CaMV MP is degraded via multivesicular bodies, which will release the MP into the vacuole (Carluccio et al. 2014).

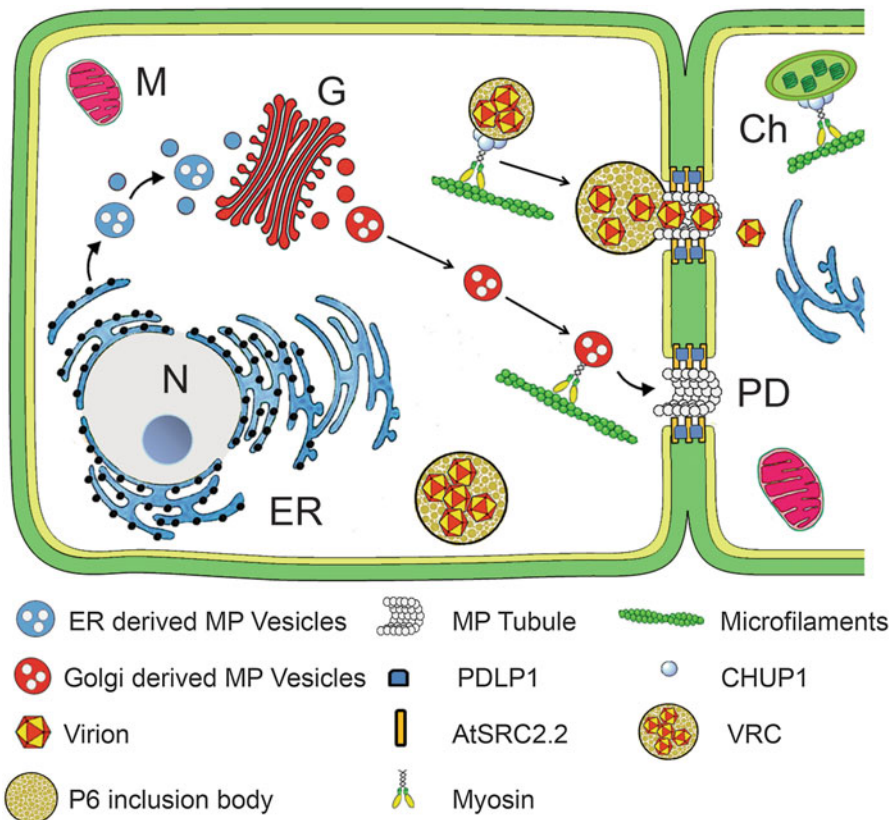


Fig. 5.4 Model for cell-to-cell movement of *Cauliflower mosaic virus* (CaMV) in non-phloem cells. The CaMV particles are inoculated into host cells during the aphid feeding process. Then, the virions are delivered to the nucleus where the DNA discontinuities are repaired and transcription occurs. Translation of the 19S RNA occurs in the cytoplasm, resulting in the production of the P6 protein that condenses into large inclusion bodies surrounded by ribosomes (electron dense inclusion bodies), and trans-activates the expression of P1 (MP), P2, P3, P4, P5 and P7 encoded from the 35S RNA. Newly synthesized 35S RNA is encapsidated and retro transcribed in the inclusion bodies forming a viral factory (VF). Simultaneously, MP is targeted to PD via the secretory pathway. MP transforms PD into a tubule structure that allows cell-to-cell movement of intact virions, possibly via a MP-P3-CP interaction. Interaction of P6 with CHUP1 allows the VF to traffic to the PD along the actin microfilaments. Once the VF is delivered to the PD, the MP-containing tubule structure interacts with the P3-CP complex allowing the virions to move cell-to-cell to the adjacent cell. For virion delivery and transport through the tubules, PDLP1 and AtSRC2.2 help in the targeting and/or in forming a complex with P6 and MP. MP recycling is mediated by TGN/EE via PM-derived vesicles. *N* nucleus, *G* Golgi complex, *PD* plasmodesmata, *ER* endoplasmic reticulum, *M* mitochondria, *Ch* chloroplast

The CaMV MP interacts with proteins in the *Arabidopsis thaliana* PRA1 gene family that are involved in regulation of vesicle trafficking between different cellular compartments, including the ER, endosomes, and Golgi (Alvim Kamei et al. 2008; Amari et al. 2010; Huang et al. 2001). Using the yeast-two hybrid

system, Huang et al. (2001) identified three such proteins, MPI1, MPI2 and MPI7, which interact with CaMV MP. A domain in the MPI7 protein was identified that interacted with MP and abolished tubule formation when deleted (Huang et al. 2001). A bioinformatics analysis of a partial proteome of an Arabidopsis cell wall preparation allowed for the identification of a family of proteins localized to the PD (Thomas et al. 2008). These proteins were named plasmodesmata located proteins (PDLP), and evidence has been presented that these proteins play a role in CaMV cell-to-cell movement (Huang et al. 2001; Thomas et al. 2008). Amari et al. (2010) used fluorescence resonance energy transfer (FRET) studies to show that PDLP1 interacts with the CaMV MP. Moreover, mutations introduced into the *PDLP 1, 2* and *3* drastically reduced CaMV cell-to-cell movement, without altering replication (Amari et al. 2010). This was interpreted to indicate that PDLPs are involved in directing MP to PD, possibly as some sort of receptor. However, it is not clear exactly where the interaction of PDLP1-MP, occurs (Amari et al. 2010).

Later in the virus life cycle, P6 accumulates within the IBs in the cytoplasm, and there is a shift to virion formation in the virion factory (Schoelez et al. 2015). CaMV virions are composed of vDNA and the 56 kDa CP (P4). In addition, the 15 kDa P3 protein is closely associated with CaMV virion, although virion formation and replication does not require P3 (Leh et al. 2001; Stavolone et al. 2005). The P3 protein is required for cell-to-cell movement as well as aphid transmission. The CaMV MP does not interact directly with the CP, this occurs indirectly via the P3 protein (Leh et al. 2001; Stavolone et al. 2005). After there is sufficient virion assembly in the IBs, there is an interaction between P6 and the host factor CHUP1 (Chloroplast unusual positioning protein), which allows virions to move along the actin microfilaments for delivery to the PD (Angel et al. 2013). CHUP1 is an Arabidopsis protein with an actin-binding motif that is located in the chloroplast outer envelope, and its function is relocation in the chloroplast in response to light intensity (Oikawa et al. 2008). Once the virions are delivered to PD, the first contact with the MP and P3-CP-vDNA is thought to occur at the entrance or within the PD (Stavolone et al. 2005). The P6 also interacts with two other host proteins that are located near the PD: PDLP1 and AtSRC2.2 (*Arabidopsis thaliana* soybean response to cold). These are membrane-bound proteins that are localized adjacent to the MP tubule (Rodriguez et al. 2014). Additionally, P6 interacts with MP (Hapiak et al. 2008). It is possible that PDLP1 and AtSRC2.2 form a complex with P6 and MP, which help to deliver virions to the tubule.

What triggers the release of the virions from the P6 IBs to the tubules is still unknown. However, the close proximity of CaMV proteins in the virion factory and PD may facilitate the loading of virions into the tubules. The CaMV CP also binds to the C-terminus of P6 (Himmelbach et al. 1996) and, as mentioned above, host factors may also help deliver virions to tubules. The mechanism by which the virions move inside the tubules is also not known; however, there is the possibility that the association between P3-CP-vDNA with MP, PDLP1 and AtSRC2.2 within the tubule could support a treadmilling mechanism (Schoelez et al. 2015; Fig. 5.4).

The treadmilling mechanism, also called head-to-tail polymerization, is defined as “a unidirectional flux of subunits through the polymer as a result of continuous net assembly at one end of the polymer and continuous net disassembly at the other end”. This treadmilling movement was first described for actin filaments (Wegner 1976). The mechanism least understood is how the appressed ER is removed from the tubules. This may involve a cascade of signaling to contract or destroy the appressed ER.

5.5.2 Cell-to-Cell Movement Mechanism Involving Virions and MP-Lined Tubules: Nepoviruses, Bipartite ssRNA Viruses with a Single Capsid Protein

The genus *Nepovirus* belongs to the family *Secoviridae* in the order *Picornavirales*. The *Nepovirus* genome is composed of a bipartite positive-sense ssRNA, and is encapsidated in icosahedron virions of ~28–30 nm diameter. The type species is *Tobacco ringspot virus* (TRSV), but *Grapevine fanleaf virus* (GFLV) is the species for which cell-to-cell movement has been most extensively studied. The GFLV MP is 38 kDa, and is encoded on the RNA 2. Similar to CaMV, expression of the GFLV MP results in generation of tubules that protrude from the PM of transfected protoplasts (Ritzenthaler et al. 1995a). However, in contrast to CaMV, there is a direct interaction between MP and CP (virions) (Belin et al. 1999; Ritzenthaler et al. 1995a, b).

GFLV RNA 1 can replicate independently of RNA 2; however, RNA 2 encodes proteins responsible for encapsidation and cell-to-cell movement (Ritzenthaler et al. 1995a). After GFLV inoculation by nematode vectors, RNA 1 and RNA 2 are unencapsidated in the cytoplasm, and are translated separately into polyproteins (Gaire et al. 1999). After the RNA1 polyprotein is processed, proteins assemble into a VRC. Subsequently, RNA 2 is associated with the VRC, where it is translated and polyprotein processing occurs (Gaire et al. 1999). This temporal gene expression allows the distinct steps of the viral life cycle to occur.

Ritzenthaler et al. (2002) established the critical role of the ER in the VRC, as well as the redistribution of the ER after virus infection. Furthermore, following infection, *de novo* synthesis of membranes and endomembrane vesicular trafficking, essential for GFLV replication, occurs (Ritzenthaler et al. 2002). The processing of the RNA 2 polyprotein results in the rapid release of the MP protein from the VRC, and delivery to the plasma membrane and the cell periphery (Ritzenthaler et al. 2002). Laporte et al. (2003) proposed that the transport of the MP to the cell periphery was dependent on the microtubular cytoskeleton, because inhibition resulted in accumulation of MP in the cytoplasm. The finding that the GFLV MP interacted with the host factor, KNOLLE, a member of the t-SNARE

family responsible for host vesicle-mediated intracellular trafficking, was consistent with a role for vesicle trafficking in MP synthesis.

Another host factor that plays a role in GFLV cell-to-cell movement is myosin, a family of ATP-dependent motor proteins. It was established that myosin XI, especially class XI-K and XI-2, but not VIII, are important in transport of GFLV MP. Myosin XI-K was important for localization of MP to PD, based on experiments with chemical treatments and results with dominant lethal mutants. The primary contribution of myosin motors was proposed to be transport of MP, via the ER-Golgi-PM network (Amari et al. 2011). This may also require PDLP1 for targeting of PD (Amari et al. 2011).

As with CaMV, GFLV MP interacts with PDLP, and PDLP1 appears to be an important factor in tubule biogenesis and virus movement. This is because inhibition of trafficking of PDLP1 reduced tubule assembly and led to GFLV MP accumulation in the cytoplasm rather than at PD. In addition, the triple mutant *pdlp1/2/3* reduced GFLV local and systemic infection (Amari et al. 2010).

These findings led to development of a microtubule motor driven endomembrane transport pathway model, similar to some animal-infecting viruses, as opposed to diffusion-based models. More information is needed regarding the delivery of virions and movement through the tubules.

5.5.3 Cell-to-Cell Movement Mechanism Involving Virions and MP-Lined Tubules: Comoviruses, Bipartite ssRNA Viruses with Two Capsid Proteins

The genus *Comovirus* belongs to the same order and family as the genus *Nepovirus*, and these viruses have a similar type of nucleic acid and virions shape. The type species of the genus *Comovirus* is *Cowpea mosaic virus* (CPMV). A major difference between CPMV and GFLV is that members of the genus *Comovirus* have two CPs: small CP (CPS)-23 kDa and large CP (CPL)-37 kDa. The MP is 48 kDa, and it interacts with the CPL, but not the CPS (Carvalho et al. 2003). Deletion of the C-terminal 48 amino acids of the MP abolished the interaction between MP and CPL and cell-to-cell movement (Lekkerkerker et al. 1996). Interestingly, protoplasts electroporated with this deletion mutant formed tubules, but these were empty and the virus was unable to move cell to cell (Lekkerkerker et al. 1996). The authors concluded that the interaction between CPL and MP is required for cell-to-cell movement. The mechanism by which the virions move from the replication site to the PD is not known. However, Pouwels et al. (2002) showed that this may not involve the cytoskeleton nor the secretory pathway.

5.6 Phloem-Associated and -Limited Viruses-A Case of Specialized or Degenerated Cell-to-Cell Movement

A number of important families of plant-infecting viruses are limited to infecting cells of the phloem, and these include positive-sense ssRNA viruses such as closteroviruses and luteoviruses and many species of the circular ssDNA viruses in the family *Geminiviridae*. Most of these viruses are not mechanically or seed-transmitted, and are specifically transmitted by phloem-feeding insects, e. g., aphids, leafhoppers and whiteflies, by a persistent mode of transmission. These viruses infect and spread in cells of the phloem, and it has been hypothesized that inability to infect non-phloem cells is not due to inability to replicate in these cell types, but the lack of a MP that allows cell-to-cell movement in non-phloem cells or an inability to suppress gene silencing. It has been further speculated that these viruses may be highly evolved, such that the phloem provides all necessary functions, i.e., a luxury apartment rather than a jail. The big question is what role does cell-to-cell movement play in this process? Here, we can envision at least three options for phloem-limited viruses: (1) cell-to-cell movement through PD mediated by one or more MP, (2) passive cell-to-cell movement mediated via cell division in rapidly dividing progenitor cells (e.g., protophloem cells), and (3) virus-stimulated cell division resulting in increased numbers of cells for infection. Then, the question arises as to whether there is a specific cell-to-cell movement mechanism, e.g., for the movement of infectious nucleic acid from the SE to the CC and PP, and then back again, and the nature of the movement complex. Here, we might be tempted to argue for the existence for a cell-to-cell movement process for phloem-limited viruses because (1) it is unlikely that virions freely move through the PD interconnecting the SE-CC (even with a greater SEL limit); (2) the existence of MPs, presumably for cell-to-cell movement; and (3) the failure to observe virions in the PD interconnecting SE-CC.

In the cases of closteroviruses and luteoviruses it is possible that virions, perhaps modified in some way, may move cell-to-cell along with one or more MP. In the case of closteroviruses, which have large complex positive-sense ssRNA genomes (~15-19 kb) and long flexuous rod-shaped virions (1250–2200 × 10–13 nm), cell-to-cell movement involves five proteins, including a protein with cell-to-cell movement properties (p6) and four structural proteins (Alzhanova et al. 2000; Peremyslov et al. 2004; Avisar et al. 2008). The p6 localizes and interacts with the ER, whereas the HSP70h interacts with the virions, in an asymmetric manner, and targets PD. The HSP70h traffics along the actomyosin, and this requires the VIII myosins. The current model favors the cell-to-cell movement of the virion, mediated by the HSP70 and other proteins. It remains unclear how this cell-to-cell movement mechanism interacts with the PD of the SE-CC, including increasing the SEL of these PD. Luteoviruses are also positive-sense ssRNA viruses, but they possess a genome of ~5.5 kb and have icosahedral virions that are 25–30 nm in diameter. These viruses also encode a cell-to-cell MP, in this case an ~17–21 kDa protein encoded by the ORF4 gene. This MP has nucleic acid binding properties,

and is localized to PD (Link et al. 2011). Mutational analysis of the read through domain of the CP has provided some evidence for a distinct type of movement involved in short-distance movement, but this needs to be further investigated (Mutterer et al. 1999). For closteroviruses and luteoviruses additional studies are needed to elucidate the cell-to-cell movement mechanism, including the form in which the virus moves.

5.6.1 The Geminiviruses Are a Large and Diverse Family of ssDNA Viruses That Utilize Different Mechanisms of Cell-to-Cell Movement

The family *Geminiviridae* is comprised of a large and diverse group of viruses characterized by having a small circular ssDNA genome, either a single genomic DNA of ~2.9 kb (monopartite) or two ~2.6 kb ss DNA components (bipartite), and quasi-twinned icosahedral virions measuring $\sim 18 \times 30$ nm (Hanley-Bowdoin et al. 2013). Geminiviruses possess a wide range of biological properties, including being transmitted by a range of insect vectors (e.g., the whitefly species *Bemisia tabaci*, various leafhopper species and possibly even aphids), infecting dicot and monocot plant species, possessing different tissue tropisms, inducing different symptom types and being mechanically transmitted or not.

A common feature of all geminiviruses is the need to enter the nucleus for transcription of the genome and replication. Geminiviruses encode at least two karyophilic proteins: the CP and the nuclear shuttle (NSP). For the monopartite viruses, which lack the NSP, the CP is multifunctional and plays a role in mediating nuclear import and export as well as encapsidation. In the case of the bipartite begomovirus, e.g., *Bean dwarf mosaic virus* (BDMV), the NSP provides this function, as the CP is dispensable for infection (Sudarshana et al. 1998). NLS and NES domains have been identified within CP and NSP, and the functional nature of these domains have been revealed using mutational analyses and functional analyses (Ward and Lazarowitz 1999; Rhee et al. 2000). It is less clear the nature of the complex involved in nucleocytoplasmic transport, including whether it involves ss- or dsDNA.

In terms of long-distance movement in plants, geminiviruses can be placed in two main groups. The first are those that are non-phloem limited and, in some cases, can be mechanically transmitted. These are represented by a sub-set of species of bipartite whitefly-transmitted geminiviruses (genus *Begomovirus*, e.g., BDMV), and include representatives found in the New and Old World (Rojas et al. 2005). The second is represented by those that are phloem-limited and usually are not mechanically transmitted, and these include begomoviruses with monopartite genomes (e.g., *Tomato yellow leaf curl virus*, TYLCV), some with bipartite genomes (*Squash leaf curl virus*, SLCV, and *Cucurbit leaf crumple virus*, CuLCrV), and the leafhopper-transmitted geminiviruses in the genus *Curtovirus*

(*Beet curly top virus* [BCTV]). Although some early reports indicated that geminiviruses might move via tubules, the current evidence suggests that this is not the case. However, there is strong evidence that these two groups of geminiviruses have evolved to move via distinct mechanisms and forms, and utilize different proteins for cell-to-cell movement. Thus, these two groups will be discussed separately.

5.6.2 Cell-to-Cell Movement of Non-Phloem Limited Geminiviruses (Bipartite Begomoviruses): Viruses That Do Not Require the CP for Movement, Move as a DNA-Protein Complex, and Are Represented by Bean dwarf mosaic virus (BDMV)

The most well studied member of this group is BDMV, a typical bipartite begomovirus that infects common bean (in nature) and *N. benthamiana* (as a laboratory host). In addition to the whitefly vector, BDMV is also transmitted mechanically and via particle bombardment and agroinoculation (Levy and Tzfira 2010). Through the use of infectious cloned DNA-A and DNA-B components and particle bombardment, it was established that the genes encoded by DNA-A were involved in replication and encapsidation, whereas those encoded by DNA-B were involved in movement. Furthermore, genetic analyses established that both of the DNA-B encoded genes were necessary for cell-to-cell movement (Noueiry et al. 1994). Microinjection studies performed with fluorescently-labeled *E. coli*-expressed DNA-B encoded proteins, established that the protein encoded by the *BV1* gene was targeted to the nucleus and mediated the export of fluorescently labelled DNA, whereas the protein encoded by the *BC1* gene targeted the nuclear periphery and cell periphery and mediated the cell-to-cell movement of viral ss- and ds-DNA (Noueiry et al. 1994). Using expression of transiently expressed BV1 and BC1 proteins of SLCV in protoplasts, detected via fluorescently labelled antibodies, it was established that BV1 was re-directed out of the nucleus to the cell periphery in the presence of BC1, consistent with a BV1-BC1 interaction and cell-to-cell movement of ssDNA (Lazarowitz and Beachy 1999). The subcellular localization of the BDMV BV1 and BC1 to the nucleus and the nuclear periphery and the cell periphery, respectively, was further established with transient expression studies and GFP fusion proteins (Rojas et al. 2001). *In vitro* binding studies demonstrated that the BDMV BV1 and BC1 proteins bound ss- and ds-DNA, suggesting a role in transport across nuclear and cell-to-cell (PD) boundaries, respectively. Remarkably, the binding was not sequence specific, but occurred in a size- and form-specific manner (Rojas et al. 1998; Fig. 5.5).

The development of a GFP reporter for BDMV and other bipartite begomoviruses took advantage of: (1) the GFP gene being about the same size as the CP gene, and (2) the CP not being required for cell-to-cell or long-distance

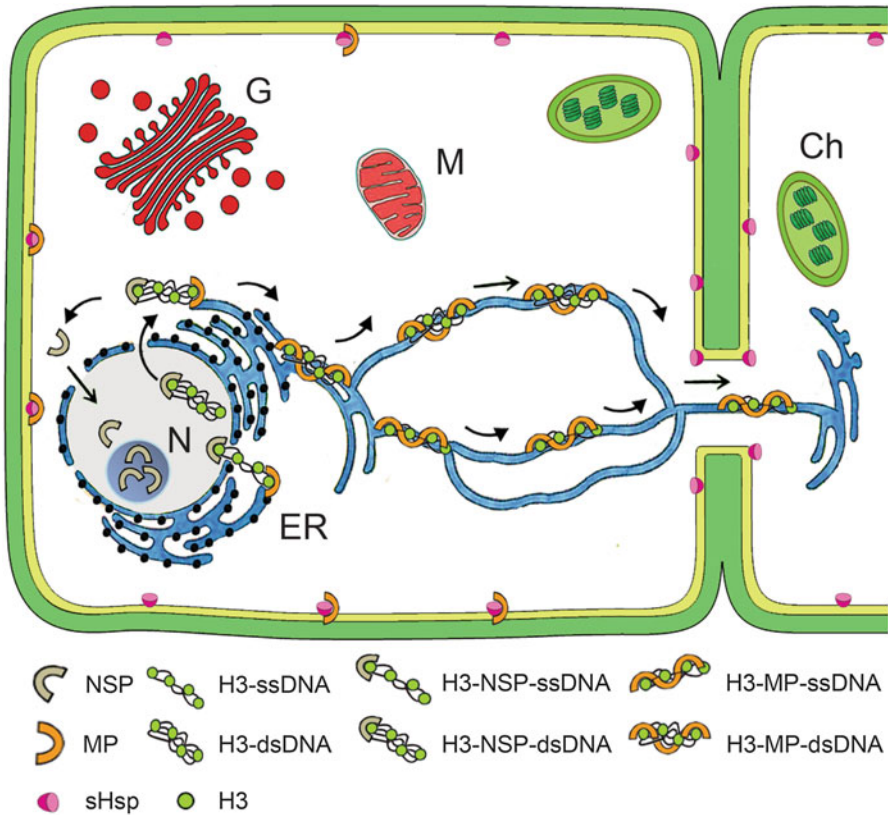


Fig. 5.5 Model for cell-to-cell movement of a non-phloem limited geminivirus that does not require the capsid protein (CP), *Bean dwarf mosaic virus* (BDMV). Viral single-stranded DNA, presumably released from virions in the cytoplasm or at the nuclear pore complex enters the nucleus. The ssDNA is converted to a dsDNA minichromosome for transcription of early genes. This is followed by formation of a movement complex composed of condensed ds (or ss) DNA bound by histones (e.g., H3) and NSP. This complex is exported from the nucleus via the NSP. At the nuclear periphery there is an interaction between NSP and MP in which NSP is displaced and replaced by MP, to form a vDNA-histone-MP movement complex that traffics to PD along the ER membranes. Once delivered to the PD, MP increases the SEL allowing cell-to-cell movement of the MP-vDNA-H3 complex to the adjacent cell. MP may be released to allow the histone-vDNA to enter the nucleus. *N* nucleus, *G* Golgi complex, *ER* endoplasmic reticulum, *M* mitochondria, *Ch* chloroplast)

movement (Sudarshana et al. 1998; Gilbertson et al. 2003). The BDMV-GFP reporter was used to confirm, *in vivo*, that a two component MP system was utilized for cell-to-cell movement and that it did not require the CP. These findings helped to name the BV1 protein as the nuclear shuttle (NSP), and BC1 as the MP. The application of the BDMV-GFP reporter was then used to investigate a number of key questions regarding the cell-to-cell movement of bipartite begomoviruses. One of these concerned the issue of size limitation on the viral genome. Although the CP

was not required for cell-to-cell or long-distance movement, attempts to increase the size of the virus genome, i.e., by inserting genes substantially greater than the CP (717 bp), resulted in reversion back to genome size (~2.6 kb) via recombination. By using a size-increased GFP reporter construct, it was established that size reversion occurred at the level of cell-to-cell movement (Gilbertson et al. 2003). This was further confirmed by microinjection studies, in which the BDMV MP preferentially mediated cell-to-cell movement of DNAs at or near the genome size, i.e., ~2.6 kb (Gilbertson et al. 2003). BDMV disease resistance in common bean was activated during cell-to-cell movement, including a hypersensitive response in some cultivars (Seo et al. 2006). It was further discovered that a plant resistance (R) gene, active against an RNA virus, was also upregulated in the BDMV-common bean defense response (Seo et al. 2006).

It is clear that BDMV and related bipartite begomoviruses use NSP to mediate nuclear export of viral DNA, and MP to mediate cell-to-cell movement of viral DNA through PD, respectively. As both NSP and MP interact with ss- and ds-DNA, both proteins play a role in the cell-to-cell movement of DNA. However, it is not clear if the form in which the viral DNA moves cell to cell is as a ss- or dsDNA, and it should be kept in mind that more than one DNA form could move. In one model, the NSP exports viral DNA from the nucleus and then, at the nuclear periphery, there is an exchange with MP, resulting in a complex of DNA-MP and host factors moving cell to cell through PD (Zhou et al. 2011; Fig. 5.5). This is supported by a lack of evidence that NSP moves cell to cell through PD, and the fact that geminivirus DNA delivered into plant cells in the absence of NSP or other viral proteins is infectious. A second model has viral ssDNA complexed with NSP and MP mediating cell-to-cell movement through PD, followed by NSP delivering the infectious viral DNA into the nucleus adjacent cell (Hehnl et al. 2004). Resolving precisely how NSP and MP mediate cell-to-cell movement of DNA may come from identifying host factors that interact with NSP and MP, and purifying and analyzing the cell-to-cell movement complex (no small task as this has not been done for any plant virus).

Recently, a gel overlay approach with ^{35}S -labelled NSP and MP was used to establish (1) a direct interaction between NSP and MP, (2) an interaction between NSP and MP and histones, specifically a structural form of H3, and (3) a redirection of histone H3 from the nucleus to the cell periphery and PD in BDMV-infected leaves (Zhou et al. 2011). These results suggest that histones are part of the viral cell-to-cell movement complex, possibly functioning by condensing viral DNA to <10 nm in diameter, a phenomenon that occurs in chromatin. Furthermore, after histone-bound viral DNA moves cell to cell through PD, it may be delivered into nuclei of newly infected cells (Fig. 5.5). Using a yeast two hybrid screen with MP, a small heat shock protein (sHsp) with an α -crystallin domain was identified that interacts with MP and is targeted to PD (Park et al. unpublished). Expression of a sHsp-GFP fusion protein in transgenic plants enhanced symptom development, whereas silencing of the gene led to delayed symptoms. These results suggested that sHsp may facilitate the cell-to-cell spread of BDMV DNA through PD, possibly by acting as a co-factor in a chaperone network (Fig. 5.5). Together these findings

are beginning to reveal the host factors involved in bipartite begomovirus movement, but many questions remain, including the precise nature of the viral DNA (s) that move through PD, the interactions between viral proteins and nucleic acids and host factors involved in the formation of the movement-competent complex.

In terms of understanding cell-to-cell movement, it will also be important to resolve what are the initial cells infected in the infection process, i.e., after initial delivery by the whitefly vector. This is likely to occur in the shoot and root apices, where the virus is delivered after long distance spread via the phloem. This may be in cells of the protophloem, where virions or a vDNA-protein complex gain access to nucleate cells, from which active cell-to-cell movement or spread by cell division occurs (Fig. 5.5). Alternatively, this could also occur by unloading of infectious virus along the phloem translocation pathway via the specialized PD connecting the SE and CC. Here, viral replication and then cell-to-cell movement into non-phloem cells may occur following a shift from G phase (terminally differentiated cells) into S phase (synthesis phase) cells (Hanley-Bowdoin et al. 2013).

5.6.3 *Phloem-Limited Geminiviruses: Viruses That Require the CP and Virions for Long-Distance Movement and May also Use a Non-Virion Cell-to-Cell Movement Mechanism: Tomato yellow leaf curl virus and Beet curly top virus*

Phloem-limited whitefly-transmitted geminiviruses differ from their non-phloem limited relative (e.g., BDMV) in genome structure (monopartite vs. bipartite), type of symptoms induced (yellows vs. mosaic/mottle), the lack of mechanical transmission, and the nature and role of the genes involved in cell-to-cell movement (Gafni and Epel 2002; Rojas et al. 2005; Levy and Tzfira 2010). The same whitefly vector (*B. tabaci*) transmits the phloem- and non-phloem-limited begomoviruses, but there may be some differences, e.g., cell types from which the virus is acquired and attractiveness of infected leaves to vectors. The monopartite begomovirus TYLCV has a genome of ~2.9 kb, and genetic studies have suggested that the V1 (CP), V2 and C4 are involved in cell-to-cell movement. In the case of the leafhopper transmitted BCTV, viral proteins possibly involved in cell-to-cell movement include V1 (CP), V2, V3 and C4. Clearly, the large number of geminiviruses that are phloem-limited indicates that this is not disadvantageous for the virus; the phloem is a 'luxury apartment rather than a jail'. It may even be advantageous as it has been shown for the luteovirus, *Potato leaf roll virus* (Peter et al. 2009). In some cases, geminivirus tissue tropism may be a function of the host plant, e.g., the apparent preference of monopartite begomoviruses for tomato. In this part of the review, we will emphasize the monopartite begomovirus TYLCV and the monopartite curtovirus BCTV, as they are model systems for the study of phloem-limited geminiviruses.

TYLCV and BCTV are transmitted by whiteflies and leafhoppers, respectively, via a persistent non-propagative manner (though the possibility of propagative transmission has been raised for TYLCV). Virions are likely introduced into the nucleate SEs during feeding, probably in the same manner as non-phloem-limited geminiviruses, and the initial long-distance spread occurs via virions in a source to sink manner. However, for replication to occur, virus must then gain access to nucleate cells. This can occur in at least two mechanisms: (1) in the protophloem, following long-distance movement via the SE, and (2) by egress from SEs to CCs; in both cases this likely involves a vDNA that has been unencapsidated, either before entering the nucleate cells or before the virus enters the nucleus (Fig. 5.6).

Once the monopartite geminivirus infects a nucleate cell and enters the nucleus, viral replication and protein expression occurs. Cell-to-cell movement occurs in these initially infected cells, and this can occur either as (1) a passive process via cell division, possibly facilitated by viral proteins (e.g., C1 or C4) and/or (2) as an active process, that most likely occurs via a non-virion form and involves MPs, e.g., CP (in a non virion form) and V2 or C4 for TYLCV or CP, V2, V3 and C4 for BCTV. Although this cell-to-cell movement mechanism is limited to the nucleate cells of the phloem, it seems to occur very efficiently and it may be an active process that occurs early in the infection (Rojas et al. 2001; Gafni and Epel 2002). Later in the infection process, there may be a shift to virion formation, which is required for long-distance movement and insect transmission of TYLCV (Noris et al. 1998) and BCTV (Soto et al. 2005). Thus, it is intriguing to speculate that these viruses utilize a distinct non-virion form and mechanism for a specialized form of cell-to-cell movement. In the case of TYLCV, some evidence for this might come from the development of the IL-60 vector system, in which the rolling circle single-stranded DNA replication mechanism of wild-type TYLCV has been replaced by a plasmid-based double-stranded DNA replicon, which has a broad host range and is released from phloem-limitation (Peretz et al. 2007).

Further evidence for the existence of a cell-to-cell movement mechanism for these viruses comes from functional analysis of viral gene products. The CP (V1) of TYLCV and BCTV has been localized to the nucleus, and microinjection studies performed with the TYLCV CP established that it mediates the nuclear import and export of fluorescently labelled ss- and ds-DNA, presumably for cell-to-cell movement (Rojas et al. 2001; Gafni and Epel 2002). Based upon subcellular localization and microinjection studies, the TYLCV V2 protein was localized to around the nucleus, to cytoplasmic strands, the cell periphery and punctate bodies and co-localized with the ER (Hak et al. 2015; Rojas et al. 2001). The C4 localized to the cell periphery and PD, mediated by an N-terminal myristoylation site (Rojas et al. 2001; Hak et al. 2015). Based on these results, Rojas et al. (2001) proposed that V2 and C4 acted together to deliver viral DNA to PD for cell-to-cell movement, perhaps analogous to the MP of the bipartite begomoviruses. In microinjection studies, cell-to-cell movement mediated by V2/C4 was limited to the adjacent injected cells, which was considered to reflect the phloem-limited nature of TYLCV (Fig. 5.6). More recently it has been established that the V2 of TYLCV

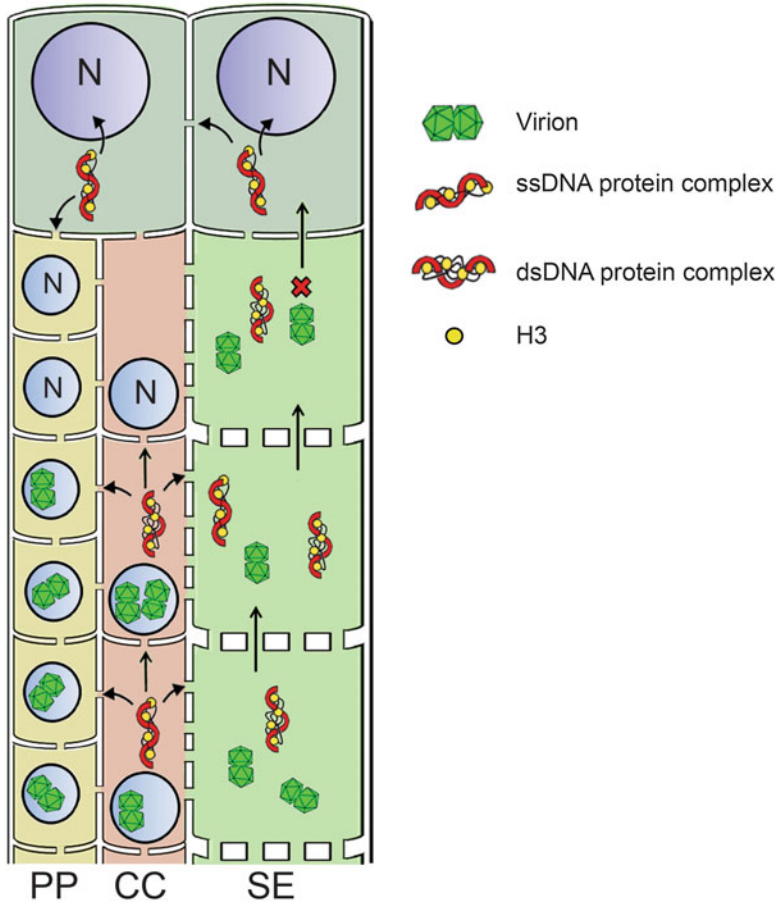


Fig. 5.6 Model for cell-to-cell movement of a phloem limited geminivirus that requires the capsid protein (CP), *Tomato yellow leaf curl virus* (TYLCV). Initially, the virions are inoculated into SEs in the phloem during the feeding of the whitefly vector. The phloem translocation stream carries the virions to the shoot apices and roots, via the source-to-sink translocation pathway. Initial infection occurs in nucleate cells of protophloem, where ss- and dsDNA moves through a PD-like structure, followed by trafficking into the nucleus, via host factors or in association with CP. Viral replication occurs in the nucleus, followed by cell-to-cell spread of ds- or ssDNA forms after nucleus export mediated by CP. Additional cell-to-cell spread occurs via this path or is mediated by cell division. Virions move long distance through SEs to the newly developing protophloem to re-initiate infection (waves of infection model). Alternatively, virions or a vDNA-protein complex moves cell to cell, across the specialized PD interconnecting SE-CC, allowing vDNA to access the nucleus to initiate infection in nucleate phloem cells (CC or PP). Additional cell-to-cell infection can occur as described above, possibly amplified by cell division stimulated by the virus. *N* nucleus, *PP* phloem parenchyma, *CC* companion cell, *SE* sieve element

and other monopartite tomato infecting begomoviruses functions to suppress silencing, either by binding siRNAs or interacting with SGS3, and also possess nuclear export activity (Sharma and Ikegami 2010; Sharma et al. 2011; Hak et al. 2015). Genetic and subcellular localization studies with TYLCV further showed that the phenotypes of V2 mutants, e.g., reduced infectivity, symptomless infections and 10-fold reduced viral titer, were more consistent with a silencing suppressor function, although a role in cell-to-cell movement could not be ruled out. Indeed, it may well be that these results indicated that V2 is a multifunctional protein involved in both functions. Support of this hypothesis and a possible role for V2 in cell-to-cell movement came from the observation of an interaction between the V2-C4 proteins, where co-expression of the proteins in protoplasts resulted in a 'shrunken' phenotype (Rojas et al., unpublished). Further complicating the elucidation of the role of V2 in the biology of monopartite tomato-infecting begomoviruses was the recent discovery of *Tomato leaf deformation virus* (ToLDeV), an indigenous New World monopartite begomovirus, which induces leaf curl disease in tomato and *N. benthamiana* and lacks a V2 gene (Melgarejo et al. 2013). This demonstrated that the V2 is not necessary for pathogenicity in some monopartite begomoviruses, and raises the idea that function of V2 is virus-specific, similar to the situation with gene silencing suppression and the C4.

BCTV is a monopartite geminivirus that is transmitted by the beet leafhopper, *Circulifer tenellus* in a persistent non-propagative manner. The virus is not mechanically transmissible and immunolocalization studies have revealed it is phloem-limited (Latham et al. 1997; Chen et al., unpublished data). A PCR time-course study of BCTV infection in shepherd's purse plants, performed over 1, 3, 5, 7, 9 and 14 days, revealed that the majority of replicating viral DNA was in newly emerged leaves and roots (i.e., moved long distance in a source to sink direction), beginning at 5 dpi and continuing over the course of the experiment. In contrast, considerably less viral DNA was detected in inoculated leaves, and then only at the 9- and 14-day time-points (Chen et al., unpublished). These results indicated that most of the infectious form of the virus (virions) moved out of the inoculated leaves and into the shoot and root apices via SEs and into nucleate-phloem-associated cells, e.g., protophloem cells (e.g., Wang et al. 1996 for BDMV). We speculate that BCTV virions may pass across PD that are transitioning into sieve plate pores (and have larger pore sizes than typical PD) and into nucleate protophloem cells where replication can occur. However, this needs to be experimentally established. Alternatively, a vDNA-protein complex could be generated in the SEs, which can move across the specialized PD connecting SEs and CCs. BCTV then moves cell to cell from these initially infected cells, either by cell-to-cell movement or spread mediated by normal cell division or cell division stimulated by the C4 protein.

Once the infectious form of BCTV is introduced into nucleate cells, the viral DNA is likely unencapsidated and moves across the NPC via host factors. BCTV genes possibly involved in cell-to-cell movement include the CP, V2, V3, and C4 based upon the replication competence of mutants in these genes (Bridson et al., 1989). A functional analysis of these genes performed by transient expression of GFP fusion proteins revealed that the CP localized to the nucleus, V2 localized to

the ER and around the nucleus and V3 to mobile cytoplasmic vesicles that appeared to move on the cytoskeleton (Soto et al., unpublished). Taken together with the lack of evidence of virions outside of the nucleus of infected cells, these data suggest the possibility of a non-virion based cell-to-cell movement mechanism for BCTV. However, additional studies are needed to define whether there exists a bona fide cell-to-cell movement step in the life cycle of BCTV.

A model for cell-to-cell movement of monopartite begomoviruses involves the CP mediating the import and export of viral ss- and/or ds-DNA, in a non-virion form. This is followed by delivery of viral DNA to the PD mediated by the C4, and possibly by V2 (Fig. 5.6; Sharma et al. 2011). It will be important to determine what host factors play a role in this process, and if any of these are specific to phloem-cell and are involved in cell-to-cell movement.

5.7 Summary Points

- Viruses have evolved a diversity of mechanisms for cell-to-cell movement through PD, and this includes viruses that can egress the phloem and those that are phloem-limited. Regardless of their phloem-tropism all viruses encode one or more MP, and these can subtly or drastically modify PD structure and function to mediate the cell-to-cell movement of viral nucleic acids or virions. The diversity of MPs and cell-to-cell movement mechanisms indicates multiple evolutionary events, including convergent evolution of the same mechanism by different viruses and hijacking and modifying host factors involved in macromolecular trafficking.
- There is emerging evidence that VRCs are closely linked to the cell-to-cell movement complex. This can occur through the accumulation of components from the VRC to assemble the cell-to-cell movement complex or the actual transport of the entire VRC across PD.
- Phloem-associated and -limited viruses have effectively evolved to infect plants via multiple mechanisms. These viruses are introduced into SEs and move in a source to sink direction to access nucleate cells for replication and cell-to-cell movement. Access to nucleate cells in the phloem can occur via cell-to-cell movement from SE to CCs, and at the shoot and root apices, e.g., geminiviruses. Some geminiviruses can move cell-to-cell in a non-virion form; however, for bona-fide phloem-limited geminiviruses, closteroviruses and luteoviruses this may require virions. This area needs new research emphasis and experimental approaches.
- With the emergence of new methods in cell biology, spectroscopy, protein-protein interaction, genomics and proteomics more progress in the identification of the host factors involved in virus cell-to-cell movement will be made. Hopefully, this will allow for the identification of the all of the steps involved in viral cell-to-cell movement and additional details of the interaction of viruses and PD. For example,

the application of quantum dots, a more robust and sensitive fluorescent dye, could allow for visualization of virus infection *in vivo*.

- Eventually, it is hoped that this information will reveal new approaches for management of plant diseases caused by viruses, specifically by interfering with cell-to-cell movement of plant viruses.

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Chapter 6

Long-Distance Movement of Viruses in Plants

Jang-Kyun Seo and Kook-Hyung Kim

Abstract Plant viruses are obligate, biotrophic parasites that live in the symplastic space of their hosts. After invading the host plant, viruses begin to multiply in the initially penetrated cells by establishing specific interactions between viral factors and macromolecules, structures, and processes of the host plant. Viruses then spread throughout the plant not only by moving from cell to cell through plasmodesmata, but also by moving long distance through the vascular system, usually through the phloem, to establish systemic infection. The short-distance cell-to-cell movement requires modification of plasmodesmata by viral factors such as movement proteins. Long-distance movement involves passage of viruses through various cellular barriers including the bundle sheath, vascular parenchyma, and companion cells for virus loading into sieve elements. Viruses are then passively transported through sieve elements to distant tissues. While many studies have examined virus cell-to-cell movement, few have focused on the molecular mechanisms regulating virus long-distance movement. Finely characterizing long-distance movement of viruses is challenging because of the inter-dependence of cell-to-cell and long-distance movement and because phloem is located deep within plant tissues. Nevertheless, recent studies have begun to shed light on the molecular mechanisms of long-distance movement by viruses. This chapter discusses some general features, recent progress, and future prospects of long-distance movement of viruses in plants.

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6.1 Introduction

The infection cycle of plant viruses includes invasion of host plants, genome multiplication, encapsidation, short- and long-distance movement, and plant-to-plant transmission by insect vectors, mechanical wounds, or seeds and pollen. Because viruses are obligate parasites that multiply in the host symplasm, a successful infection requires multiple compatible interactions between viral factors (i.e., viral nucleic acids and proteins) and host cellular factors including the host transcriptional, translational, and macromolecular transporting machineries. In this regard, plant viruses have evolved various strategies to interact with pre-existing host cellular factors in order to systemically infect host plants.

Spread of plant viruses from initially infected cells to distal tissues involves four general steps: (1) active replication of viruses in the initially infected cells to produce infectious entities for subsequent movement; (2) cell-to-cell movement of viruses between mesophyll cells through plasmodesmata (PD); (3) virus entry (loading) into sieve elements after passing through several vasculature-associated cell types; and (4) virus unloading from sieve elements into distal sink tissues and reestablishment of virus replication and cell-to-cell movement.

Plant viruses encode functions specifically required for transport of infectious entities from infected cells to uninfected tissues. The first model explaining virus movement was developed from studies of the 30-kD protein encoded by Tobacco mosaic virus (TMV; *Tobamovirus*), which is now known as the movement protein (MP) (Deom et al. 1987; Meshi et al. 1987). The TMV MP modifies the size-exclusion limit (SEL) of PD and binds virus genomic RNA to form a ribonucleo-protein (RNP) complex that is transported through PD for cell-to-cell movement (Wolf et al. 1989). It is now evident that most families of plant viruses encode MPs that facilitate intercellular movement. Nine plant virus genera within the *Alphaflexiviridae*, *Betaflexiviridae*, and *Virgaviridae*, and in the unassigned genus *Benyvirus* encode triple gene block (TGB) proteins that are essential for virus movement (Verchot-Lubicz et al. 2010; Solovyev et al. 2012). Beside MPs, in many cases virus structural proteins or replication-associated proteins also function in cell-to-cell and long-distance movement. The coat protein (CP) is required for long-distance movement of most viruses that move cell-to-cell by a TMV-like mechanism (Vaewhongs and Lommel 1995; Carrington et al. 1996). The potyvirus CP contains discrete domains required for cell-to-cell and long-distance movement: the surface-exposed N- and C-terminal domains are involved in long-distance movement, while the central domain is required for virion assembly and cell-to-cell movement (Dolja et al. 1994; Dolja et al. 1995). The potyvirus-encoded proteins VPg, 6K2, and HC-Pro also contribute to virus movement (Hipper et al. 2013). The tombusvirus-encoded proteins p19 and p22 are critical for systemic infection. p19 is involved in long-distance movement, whereas p22 is required for cell-to-cell movement. In addition, many plant virus-encoded replication proteins also appear to have specific roles in long-distance movement (Scholthof et al. 1995).

The host dependence of virus movement suggests that specific cellular factors play key roles in cell-to-cell and long-distance transport of viruses. A number of

recent studies have focused on identifying the cellular components involved in virus movement with the goal of elucidating the molecular mechanisms of intracellular and intercellular transport and long-distance movement of viruses (Harries and Ding 2011; Solovyev et al. 2012; Hipper et al. 2013; Park et al. 2013). Recent evidence has revealed that viral factors interact with cellular components to modify and utilize existing transport routes in plants, including the intracellular transport system, PD, and the vascular system. The following section review general features of virus movement in plants and recent advances concerning the identification of viral and cellular factors involved in long-distance movement.

6.2 General Features of Virus Movement in Plants

Spread of a systemically infecting virus involves movement through several cell types and tissues (Fig. 6.1). Following entry into a host cell, usually an epidermal or mesophyll cell, a virus must replicate to produce infectious progeny. Viruses then move from the site of replication to the cell periphery in order to transport infectious entities into surrounding cells through the PD (Fig. 6.1). Recent studies have shown that this intracellular transport of viruses is mediated by the cellular cytoskeleton and associated motor proteins and the host endomembrane system (Carrington et al. 1996; Verchot-Lubicz et al. 2010; Harries and Ding 2011; Park et al. 2014). The SEL of PD is a physical barrier that must be overcome for successful cell-to-cell movement, and virus-encoded MPs function here to modify the PD and increase the SEL. Plant viruses can be divided into at least four groups based on the characteristics of cell-to-cell movement. The first group encodes a single, dedicated TMV 30 K-like MP that increases the SEL of PD to allow cell-to-cell movement (Ueki and Citovsky 2011). The second group utilizes both MPs and CPs to form tubule-like structures through the PD. Icosahedral viruses, such as those in the *Secoviridae*, *Bromoviridae*, and *Caulimoviridae*, belong to this group (Laporte et al. 2003). The third group of viruses comprises potex-, carla-, and hordeiviruses and some furo-like viruses, which encode TGB proteins (Verchot-Lubicz et al. 2010). The last group includes the viruses in the family *Potyviridae*, which contains the largest number of positive-stranded RNA viruses. The cell-to-cell movement of potyviruses requires elaborate and complicated interactions among several viral factors including HC-Pro, VPg, cylindrical inclusion (CI) protein, P3N-PIPO protein, and CP (Wei et al. 2010; Vijayapalani et al. 2012).

Virus cell-to-cell movement must be followed by on-going replication in the newly infected cells. Virus cell-to-cell movement continues in phloem tissues via the successive crossing of the bundle sheath, vascular parenchyma cells, and companion cells until sieve elements are reached (Fig. 6.1). Once in sieve elements, the virus is passively transported within the source-to-sink flow of the phloem sap. Invasion of distant cells requires virus unloading from sieve elements into companion cells, followed by cell-to-cell movement into the bundle sheath and mesophyll cells (Fig. 6.1).

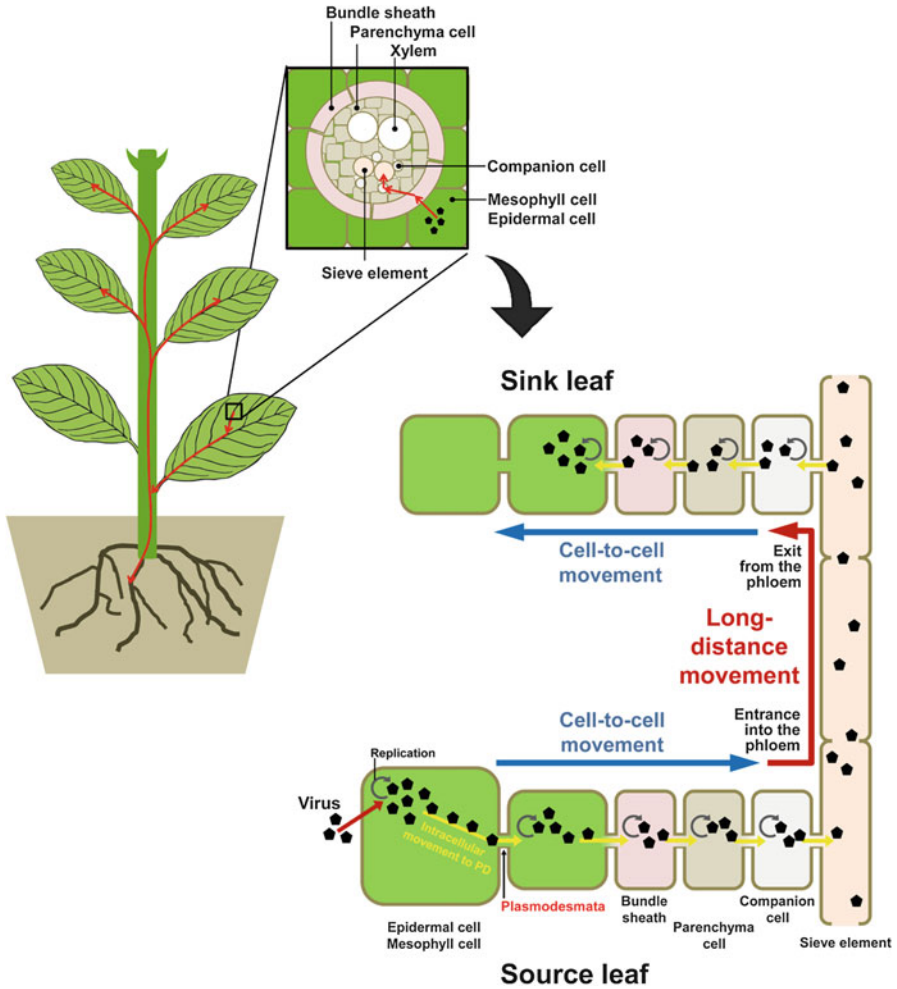


Fig. 6.1 A general view of systemic movement of viruses in plants. Following entry into a host cell, mostly epidermal or mesophyll cell, viruses replicate their genomes. The amplified viral genomes interact with viral proteins and/or host factors to form the movement complexes (virions or RNP complexes). The viral movement complexes are then transported from the site of replication to plasmodesmata by mediation of the host intracellular transport system. Virus replication and cell-to-cell movement continue through the vasculature-associated tissues (bundle sheath, parenchyma cells, and companion cells). The movement complexes are then loaded into sieve elements for long-distance movement and passively transported within the source-to-sink flow of the phloem sap. Invasion of distant systemic cells begins by virus unloading from sieve elements into companion cells, then replication and cell-to-cell movement through bundle sheath and mesophyll cells

Research has established that virus entry into sieve elements occurs in all vein classes of source tissues, while virus exit from sieve elements is limited to major veins of sink tissues, suggesting that virus loading into phloem and virus unloading from phloem involve different mechanisms. Because the entire virus movement pathway is part of an elaborate symplastic network, the virus does not need to cross the plasma membrane after the initial invasion. Each step requires transport through PD, the intercellular channel between adjacent cells. However, virions or infectious RNP complexes are too large to freely move through PD, indicating that viral and cellular factors and their compatible interactions are required to facilitate virus transport through PD.

Understanding how viruses move in plants requires the characterization of the viral complexes transported through PD and phloem tissues. Thus far, researchers have described two forms of these viral complexes: virions and RNP complexes. While a virion is an entire virus particle, consisting of an outer protein shell called a capsid and an inner core of viral genome, an RNP complex consists of the viral RNA genome associated with viral and/or cellular proteins.

Virions are indispensable for the long-distance movement of viruses belonging to the genera *Alfavirus*, *Begomovirus*, *Benyvirus*, *Carmovirus*, *Closterovirus*, *Cucumovirus*, *Dianthovirus*, *Mastrevirus*, *Necrovirus*, *Potexvirus*, *Sobemovirus*, and *Tobamovirus* (Hipper et al. 2013). In Red clover necrotic mosaic virus (RCNMV), which belongs to the genus *Dianthovirus*, transgenically expressed MP was able to complement cell-to-cell movement of RNA1 in the absence of RNA2, indicating that CP and virion formation are dispensable for RCNMV cell-to-cell movement (Vaewhongs and Lommel 1995). This complementation, however, was unable to support long-distance movement of RNA1, and CP accumulation, in the form of virions, was necessary for long-distance movement of RCNMV (Vaewhongs and Lommel 1995). Similarly, while the cell-to-cell movement-defective phenotypes of TMV, Cucumber mosaic virus (CMV; *Cucumovirus*), and Tobacco etch virus (TEV; *Potyvirus*) mutants were complemented in transgenic plants expressing the TMV MP, CMV 3a protein, and TEV CP, respectively, virion formation was required for long-distance movement of these viruses (Holt and Beachy 1991; Dolja et al. 1994; Kaplan et al. 1995).

Some viruses do not require the CP and virion formation for long-distance movement. The CP-defective mutants of Tomato leaf curl virus (ToLCV, *Begomovirus*) and Tomato bush stunt virus (TBSV, *Tombusvirus*) were incapable of virion formation but were capable of long-distance movement and systemic infection of leaves (Padidam et al. 1995; Desvoyes and Scholthof 2002; Qu and Morris 2002). Brome mosaic virus (BMV), the type species of the genus *Bromovirus*, is a well-characterized example for which long-distance movement involves formation of RNP complexes with cellular factors. *Agrobacterium*-mediated independent expression of BMV RNA3 resulted in long-distance movement of the RNA3 without viral replication (Gopinath and Kao 2007). As another example, umbraviruses do not encode their own CP and move naturally in the form of RNP complexes (Ryabov et al. 2001; Taliansky et al. 2003; Kim et al. 2007b).

6.3 Viral Factors Involved in Long-Distance Movement

Involvement of the CP is obviously common in long-distance movement of plant viruses, but other viral proteins including MPs, TGB proteins, and HC-Pro are also required for this process (Waigmann et al. 2004; Hipper et al. 2013). Despite the experimental difficulties, recent studies have focused on the viral factors involved in long-distance movement of viruses with the goal of clarifying underlying mechanisms (Solovveyev et al. 2012; Hipper et al. 2013), and these studies are discussed in the following sections.

6.3.1 Coat Protein

As described above, many virus species move long distances in the form of virions (Waigmann et al. 2004). The requirement of the CP for long-distance movement is often associated with formation of virion particles as described in TMV (Holt and Beachy 1991; Ding et al. 1996), RCNMV (Xiong et al. 1993; Vaewhongs and Lommel 1995), Cucumber green mottle mosaic virus (CGMMV; *Tobamovirus*) (Simon-Buela and Garcia-Arenal 1999), and Rice yellow mottle virus (RYMV; *Sobemovirus*) (Opalka et al. 1998).

For poleroviruses, researchers have proposed that the viruses are transported to non-inoculated distant leaves in the form of virions (Brault et al. 2003; Kaplan et al. 2007). Whether this long-distance transport also require RNA complexes, however, is unclear. A recent study examined this possibility by analysing CP-targeted mutants of Turnip yellows virus (TuYV; *Polerovirus*) that were unable to form virion particles (Hipper et al. 2014). The TuYV CP mutant that could not encapsidate into virions was blocked in their long-distance movement, but long-distance movement was recovered by *in trans* complementation with wild-type CP, confirming that virions are essential for polerovirus systemic movement (Hipper et al. 2014).

For many virus species, the CP contains distinct functional domains specifically required for virus movement and virion formation. The potyvirus CP has three distinct domains. The N- and C-terminal domains exposed on the virion surface are critical for virus long-distance movement, while the conserved central domain forms the core of the virion (Shukla and Ward 1989; Dolja et al. 1994). Recent studies on Wheat streak mosaic virus (WSMV; *Tritimovirus*) have further dissected the requirement of the CP domains for cell-to-cell and long-distance movement, virion assembly, and host specificity (Tatineni et al. 2011; Tatineni and French 2014; Tatineni et al. 2014). Tatineni et al. have examined the effects of a series of deletion and point mutations in the CP cistron on WSMV cell-to-cell and systemic transport and virion assembly (Tatineni and French 2014; Tatineni et al. 2014). They reported that the N-terminal amino acids 6–27 and 85–100 are critical for efficient virion assembly and cell-to-cell movement, while the C-terminal 65 amino

acids are dispensable for virion assembly but are required for cell-to-cell movement, suggesting that the C terminus of CP functions as a dedicated cell-to-cell movement determinant (Tatineni et al. 2014). They also found that WSMV CP C-terminus aspartic acid residues at positions 216, 289, 290, 326, 333, and 334 are not required for virion assembly, but that the mutation of aspartic acid residues at positions 289, 290, and 326 reduced long-distance transport in maize but not in wheat. These findings indicated that the WSMV CP C-terminal amino acids facilitate the expansion of the WSMV host range to include maize by enabling host-specific long-distance movement, and more specifically, by allowing virus ingress into the maize vascular system (Tatineni and French 2014).

The charged residues in the surface-exposed C-terminus of the Soybean mosaic virus (SMV; *Potyvirus*) CP are also critical for virus movement and virion assembly (Seo et al. 2013). Analysis of protein structure of the SMV CP predicted that the C-terminal region contains a short α -helix near the charged amino acid residues (Seo et al. 2013). Interestingly, alanine substitutions of the charged amino acids that disrupted the original α -helix structure in the CP C-terminus resulted in reduced virus movement and defective virion assembly, suggesting that the structural conformation of the C-terminal domain of SMV CP is important for virus movement as well as assembly (Seo et al. 2013). Researchers have suggested that the charged amino acids in the N- and C-terminal regions of potyviral CPs might mediate head-to-tail interactions between CP subunits so that the subunits form ring-like intermediates and thereby virions (Anindya and Savithri 2003). It is likely, however, that the interaction between potyviral CP intersubunit mediated by the C-terminal charged residues are only transiently required for promoting virion assembly because removal of the surface-exposed C-terminal region by limited trypsin treatment does not block virion assembly by potyviruses (Jagadish et al. 1993; Anindya and Savithri 2003).

In a recent study, researchers analyzed the structural motifs of the CMV CP responsible for long-distance movement; they did this by engineering mutations into the CMV CP bearing the corresponding Tomato aspermy virus (TAV; *Cucumovirus*) loops exposed on the surface of the virion (Salanki et al. 2011). This study determined that the surface-exposed β B- β C loop is essential for long-distance movement of TAV, confirming that the domains on the external surface of the virion are important for systemic infection.

6.3.2 Movement Protein

Because PD are too small to allow passive transport of viruses, the transport of many viruses depends on MPs that target and dilate PD. Information on the role of MPs in long-distance movement of viruses is still limited, mainly because of the difficulty in analyzing long-distance movement independent of cell-to-cell movement. Nevertheless, accumulating evidence indicates that MPs perform specific functions in the long-distance movement of some viruses. In RCNMV, point

mutations in the CP do not affect cell-to-cell transport but stop long-distance movement by preventing virus loading into the companion cell/sieve element complex (Wang et al. 1998). In some luteoviruses, such as Barley yellow dwarf virus (BYDV) and Potato leafroll virus (PLRV), MPs associate with the PD of companion cells and sieve elements, and are also required for systemic transport (Chay et al. 1996; Lee et al. 2002). Recent studies have shown that the non-structural protein (NSm) of Tomato spotted wilt virus (TSWV; *Tospovirus*), which is the MP of TSWV, is involved in both cell-to-cell and long-distance movement (Lewandowski and Adkins 2005; Li et al. 2009). The TMV-based expression system showed that the NSm supported long-distance movement and induced TSWV-like symptoms in *Nicotiana benthamiana* (Lewandowski and Adkins 2005), and mutational analyses using deletion alanine-substitution-mutants of TSWV determined that the C-terminal domain of the TSWV NSm is required for tubule formation, long-distance movement, and symptom development (Li et al. 2009). Similarly, another study found that the C-terminal domain of the BMV MP is essential for systemic movement (Takeda et al. 2004). The latter study determined that a BMV mutant with a deletion in the C-terminus of MP could move from cell to cell without CP but could not move systemically, even in the presence of CP, suggesting that the C-terminus of the BMV MP is involved in the requirement for CP in cell-to-cell movement and plays a role in long-distance movement.

Recent advances in understanding of plant virus replication and movement indicate that viruses assemble membrane-associated virus replication complexes (VRCs) in which viral components and host cellular factors required for replication are concentrated. For several RNA viruses, including TMV (Asurmendi et al. 2004; Kawakami et al. 2004), PVX (Bamunusinghe et al. 2009; Tilsner et al. 2013), and RCNMV (Kaido et al. 2009), research has shown that the MP colocalizes with a membrane-associated inclusion that harbors the VRC. Interestingly, the MP facilitates virus transport as part of subcomplexes derived from VRCs, and such subcomplexes are essential for the spread of the virus (Boyko et al. 2007).

6.3.3 Triple Gene Block Proteins

The TGB proteins TGB1, TGB2, and TGB3 are encoded by three partially overlapping open reading frames (ORFs) and are highly conserved among members of the *Potexvirus*, *Hordeivirus*, *Benyvirus*, and *Carlavirus* (Verchot-Lubicz et al. 2010). Extensive studies have examined the molecular mechanisms by which TGB proteins contribute to intracellular and intercellular transport of viruses (Verchot-Lubicz et al. 2010; Solovyev et al. 2012; Park et al. 2013). TGB1 is a multifunctional protein that moves from cell to cell through PD and has RNA-binding, RNA-helicase, and ATPase activities. TGB1 increases the SEL of PD and is also a component of the potexvirus RNP complex (i.e., TGB1-CP-RNA), which is thought to be transported through the PD. TGB1 trafficking to the PD requires TGB2 and TGB3, which are small membrane-associated proteins. Based

on protein sequence analyses, TGB2 is predicted to contain two transmembrane domains, and TGB3 is predicted to have at least one transmembrane domain located at the N-terminus (Krishnamurthy et al. 2003; Wu et al. 2011). Recent studies have shown that TGB3 serves as a driving factor for TGB-mediated cell-to-cell movement (Lee et al. 2010; Wu et al. 2011). TGB2 interacts physically with TGB1, TGB3, and CP in a membrane-associated form, and these interactions are critical for the formation of virus-movement RNP complexes in potexviruses (Lee et al. 2011; Wu et al. 2011).

Genetic analyses of TGB-encoded proteins have revealed two distinct types of TGB, which are termed hordei-like and potex-like TGB (Makarov et al. 2009; Verchot-Lubicz et al. 2010). In contrast to viruses that encode potex-like TGB, viruses that encode hordei-like TGB do not require CP for cell-to-cell and long-distance movement (Makarov et al. 2009; Verchot-Lubicz et al. 2010). Interestingly, hordei-like TGB1 proteins are predicted to contain at least one nucleolar localization signal sequence (NoLS) in the unstructured N-terminal domain (NTD) (Makarov et al. 2009). In support of this prediction, the nuclear/nucleolar localization of hordei-like TGB1 proteins was observed in Potato mop-top virus (PMTV; *Pomovirus*) and Poa semilatifolia virus (PSLV; *Hordeivirus*) (Wright et al. 2010; Semashko et al. 2012a). Mutations of the basic amino acids in the NTD of the hordei-like TGB1 abolished its nucleolar localization, confirming that the NTD is involved in the subcellular localization of TGB1 to the nucleolus (Torrance et al. 2011; Semashko et al. 2012a). Furthermore, research has shown that positively charged motifs in the NTD are not required for cell-to-cell movement but are required for long-distance movement of hordeiviruses and pomoviruses (Wright et al. 2010; Torrance et al. 2011). These findings illustrate possible links between nucleolar localization of viral proteins and virus long-distance movement (See also “The involvement of the nucleus in virus long-distance movement” section).

6.3.4 Potyviral HC-Pro, VPg, and 6K2

HC-Pro is a multi-functional potyviral protein that has papain-like cysteine proteinase activity. HC-Pro is involved in cell-to-cell movement, aphid-transmission, suppression of RNA silencing, and symptom development (Urcuqui-Inchima et al. 2001). HC-Pro also plays a critical role in potyvirus long-distance movement. Mutational analyses showed that the highly conserved central part of HC-Pro is required for systemic spread of TEV (Dolja et al. 1993; Cronin et al. 1995). Specifically, substitution of the conserved protein motif CCE in the central part of TEV HC-Pro with an RPA amino acid sequence dramatically suppresses long-distance movement (Cronin et al. 1995). Additional experiments suggested that HC-Pro is required for both virus loading into and unloading from sieve elements (Cronin et al. 1995; Kasschau et al. 1997).

Interestingly, a transient silencing suppression assay showed that the role of HC-Pro in long-distance movement and genome replication of TEV depends on its

activity in suppressing RNA silencing (Kasschau and Carrington 2001). Similarly, a recent study showed that a Turnip mosaic virus (TuMV; *Potyvirus*) mutant with a defect in the silencing suppression activity of HC-Pro was incapable of systemic infection (Garcia-Ruiz et al. 2010). However, the TuMV mutant regained long-distance movement when the DICER-LIKE proteins DCL4 and DCL2 were knocked out. The TuMV mutant was also able to move systemically in the *rdr1/rdr6*-knock out plant, suggesting that RDR1 and RDR6 act cooperatively to limit systemic movement of the mutant virus (Garcia-Ruiz et al. 2010). Thus, RNA silencing could be a host defence mechanism that reduces virus accumulation and also restricts virus movement.

The viral genome-linked protein VPg of potyviruses is covalently attached to the 5' end of viral genomic RNA. VPg is not only essential for virus replication but is also involved in virus movement (Schaad et al. 1997; Urcuqui-Inchima et al. 2001). In Potato virus A (PVA; *Potyvirus*), a single amino acid mutation in the central domain of the VPg is sufficient to recover long-distance movement, potentially allowing the virus to cross into the phloem (Rajamaki and Valkonen 2002). A histochemical experiment suggested that VPg may act in companion cells to facilitate virus unloading into distant tissues (Rajamaki and Valkonen 2003). VPg is exposed at one end of the virion where it has the potential to interact with other viral and cellular proteins (Puustinen et al. 2002). A mutation in the N-terminal part of the TuMV VPg disrupted its interaction with the cellular protein PVIP and showed long-distance movement (Dunoyer et al. 2004).

The potyviral 6K2 is an integral membrane protein involved in membrane alterations and vesicle production (Beauchemin et al. 2007). 6K2 is targeted to intracellular membranes and recruits VRCs by forming cytoplasmic vesicles. The 6K2-induced vesicles can move intracellularly and from cell to cell (Grangeon et al. 2013). Interestingly, TuMV can be transported systemically through both phloem and xylem in 6K2-induced vesicles (Wan et al. 2015). Electron microscopy revealed 6K2-induced vesicles containing viral RNAs and replication complexes in the phloem sieve elements and in the xylem vessels. Additionally, because the N-terminus of 6K2 is located on the cytoplasmic side of the membrane (Schaad et al. 1997), it can interact with viral and cellular proteins to mediate long-distance virus movement. A recent study showed that the TuMV 6K2 interacts with the COPII coatmer Sec24a for viral systemic infection (Jiang et al. 2015).

6.4 Host Cellular Factors That Regulate Long-Distance Virus Movement

During the last decade, extensive efforts have been aimed at identifying and characterizing the cellular factors involved in virus trafficking (Waigmann et al. 2004; Harries and Ding 2011; Hipper et al. 2013). While some cellular factors can be involved in the formation of virus complexes and can interact with viral

factors to facilitate transport of such complexes to neighboring cells and systemic tissues, other host factors block or restrict virus trafficking, resulting in virus resistance. The following paragraphs describe some early and more recent advances in understanding the roles of cellular factors in regulating long-distance virus movement.

Because systemic trafficking of tobamoviruses including TMV has been extensively studied for more than two decades, the cellular factors involved in systemic transport of tobamoviruses are relatively well described (Waigmann et al. 2004; Hipper et al. 2013). An early study revealed that the *VSM1* gene of *Arabidopsis* can promote systemic movement of Turnip vein-clearing virus (TVCV; *Tobamovirus*) and TMV (Lartey et al. 1998). The *VSM1* protein probably functions at the step of virus entry into phloem in the infected leaf tissue (Lartey et al. 1998). The *DSTM1* gene identified in *Arabidopsis* is also a recessive gene that is required for TMV movement (Pereda et al. 2000). A later study suggested that *DSTM1* is required for correct virion assembly or virus stability (Serrano et al. 2008). Another cellular factor required for systemic movement of tobamoviruses is pectin methylesterase (PME; a cell wall protein) (Chen et al. 2000; Chen and Citovsky 2003). Specific inhibition of PME expression significantly showed of TMV long-distance movement in tobacco plants (Chen and Citovsky 2003). A yeast two-hybrid assay identified another cellular factor, the 16.8 kDa IP-L protein, that binds to the CP of Tomato mosaic virus (ToMV; *Tobamovirus*) (Li et al. 2005). Virus-induced gene silencing (VIGS) of IP-L expression delayed virus accumulation in systemically infected leaves (Li et al. 2005). RPN9, a 26S proteasome subunit, has also been implicated in viral systemic movement (Jin et al. 2006). Knock-down of RPN9 expression inhibited long-distance movement of TMV and TuMV. RPN9 may regulate the auxin transport and brassinosteroid signaling that are critical for vascular development (Jin et al. 2006). A recent study showed that translation elongation factor 1B (eEF1B) is an essential host factor for TMV spread (Hwang et al. 2013). TMV accumulation did not differ in the inoculated leaves of eEF1B-silenced plants and wild-type plants, but systemic accumulation of TMV was dramatically decreased in the eEF1B-silenced plants. The RuBisCO small subunit (RbCS) of *Nicotiana benthamiana* also plays a vital role in ToMV movement because silencing of RbCS enhances local infectivity but delays the systemic spread of ToMV (Zhao et al. 2013). Current studies have emphasized an emerging mechanism that links host transcriptional reprogramming and virus systemic movement (Chen et al. 2013). *Arabidopsis* WRKY DNA-binding protein 8 (WRKY8) was shown to have a role in mediating TMV long-distance movement (Chen et al. 2013). TMV infection resulted in inhibition of WRKY8 expression, and mutation of WRKY8 accelerated the accumulation of TMV in systemically infected leaves. WRKY8 is involved in regulation of plant basal defense responses (Chen et al. 2010). Researchers suggested that WRKY8 mediates the crosstalk between ABA and ethylene signaling during TMV interaction, thereby regulating systemic spread of the virus (Chen et al. 2013).

Arabidopsis genes *RTM1* (Restricted TEV movement1) and *RTM2* were initially determined to restrict long-distance movement of TEV in the vascular system

(Chisholm et al. 2000; Whitham et al. 2000). The *RTM1* and *RTM2* genes are expressed exclusively in the phloem-associated cells, and their protein products localize in sieve elements, which is consistent with the inference that *RTM* genes are involved in the restriction of long-distance rather than cell-to-cell movement (Chisholm et al. 2001). A later study showed that the *RTM* genes also restrict the systemic spread of two other potyvirus species, Lettuce mosaic virus (LMV) and Plum pox virus (PPV) (Decroocq et al. 2006). Some LMV isolates are able to overcome the *RTM*-mediated movement restriction, and a recent study demonstrated that the N-terminal region of the potyvirus CP is responsible for breaking *RTM*-based resistance (Decroocq et al. 2009). A third *RTM* loci (*RTM3*) was recently characterized (Cosson et al. 2010). *RTM3* also restricts long-distance movement of several potyviruses in *Arabidopsis* and interacts with *RTM1* (Cosson et al. 2010). Some cellular factors interacting with the potyvirus VPg protein promote long-distance virus movement. The translation initiation factor eIF4E interacts with VPg, and that interaction is necessary for virus systemic infection (Lellis et al. 2002; Gao et al. 2004). The central region of VPg has been implicated in the interaction with eIF4E (Roudet-Tavert et al. 2007). A later study also emphasized the requirement of eIF4E in potyvirus movement (Contreras-Paredes et al. 2013). Absence or overexpression of eIF(iso)4E does not affect TEV replication but abolishes virus systemic spread in eIF(iso)4E knockout plants. Another cellular protein interacting with VPg is the *Potyvirus* VPg-interacting protein (PVIP) (Dunoyer et al. 2004). The N-terminal 16 amino acids are responsible for the interaction with PVIP. Mutations in the TuMV VPg that disrupt the interaction with PVIP reduce local and systemic movement of TuMV. Silencing of PVIP in transgenic *Arabidopsis* confirmed that PVIP is not required for virus replication but functions as an ancillary factor that supports potyvirus infection and movement (Dunoyer et al. 2004). Recently, a linkage and association mapping study identified a recessive resistance gene, referred to as *sha3*, that restricts PPV long-distance movement (Pagny et al. 2012). The gene was predicted to be positioned in a region at the bottom of chromosome 3, which contains seven *RTM3*-like TRAF domain-containing genes. However, because the *rtm3* knockout plants are susceptible to PPV infection, gene(s) other than *RTM3* in the small identified region may be necessary for PPV long-distance movement (Pagny et al. 2012).

Research has also shed light on the roles of cell wall proteins and polysaccharides in regulating virus movement. One of the well-studied examples is the negative regulation of long-distance movement of tobamoviruses by a cadmium (Cd)-induced, glycine-rich protein (*cdiGRP*) (Ueki and Citovsky 2002). Treatment with low concentrations of cadmium induced the *cdiGRP* gene and inhibited the systemic movement of TMV and TVCV in tobacco plants. The *cdiGRP* protein is localized in the cell wall of sieve elements and companion cells because it contains an amino-terminal secretion signal, an internal glycine-rich domain, and a carboxy-terminal cysteine-rich domain that are responsible for cell wall targeting (Ueki and Citovsky 2002). Silencing of *cdiGRP* facilitates systemic spread of virus, whereas *cdiGRP* over-expression reduces virus movement. The authors suggested that *cdiGRP* may induce callose formation within the cell wall of cells associated

with the vascular system (Ueki and Citovsky 2002). Callose is one of the polysaccharide cell wall components deposited between the plasma membrane and cell wall and in PD (Northcote et al. 1989; Ueki and Citovsky 2002). Thus, callose deposition reduces the SEL of PD and thereby can restrict virus movement through PD. Callose deposition is regulated by signal transduction mediated by the plant hormone abscisic acid (ABA) (Luna et al. 2011). In the *R* gene-mediated extreme resistance against SMV, a subset of the type 2C protein phosphatase (*PP2C*) genes is specifically up-regulated (Seo et al. 2014). The *R* gene-mediated resistance is elicited by the SMV CI protein and is accompanied by ABA accumulation and callose deposition. In this *R* gene-mediated resistance, virus accumulation was detected in the initially inoculated cells, and virus movement was inhibited. Over-expression of *PP2C* activated callose deposition and inhibited virus movement, indicating that *PP2C* is a negative regulator of virus movement and a signaling component that links ABA accumulation to callose deposition (Seo et al. 2014).

6.5 The Involvement of the Nucleus in Long-Distance Virus Movement

There is growing evidence that viral proteins of various virus species localize to the nucleus and that the interactions between viral and cellular proteins in the nucleus are critical for long-distance virus movement (Hipper et al. 2013; Solovyev and Savenkov 2014). As mentioned above, hordei-like TGB1 proteins that contain the NoLS localize to the nucleolus (Semashko et al. 2012a). The hordei-like TGB1 proteins interact with coilin, the major structural component of Cajal bodies, which are the subnuclear structures found in the nuclei of many eukaryotes; mutational analyses showed that this interaction requires the nucleolar localization of the TGB1 proteins (Semashko et al. 2012b). The hordei-like TGB1 proteins are also able to interact with fibrillarin, the major nucleolar protein essential for RNA processing. This suggests that a nucleolar phase is involved in life cycle of viruses that encode hordei-like TGB1 proteins, although whether the interactions between the TGB1 and either coilin or fibrillarin are critical for long-distance movement of these viruses remains unclear. On the other hand, the involvement of fibrillarin in the long-distance movement of umbraviruses has been well-characterized. Silencing of fibrillarin expression in *Arabidopsis* does not affect replication or cell-to-cell movement of umbraviruses but inhibits their systemic movement (Kim et al. 2007b). The ORF3 protein plays an essential role in the long-distance trafficking of Groundnut rosette virus (GRV; *Umbravirus*) (Ryabov et al. 1999). Fibrillarin interacts directly with the GRV ORF3 protein, and this interaction mediates nucleolar localization of the ORF3 protein. The ORF3/fibrillarin complexes are then relocalized from the nucleus to the cytoplasm to form viral RNP complexes, which are competent for long-distance movement (Kim et al. 2007a;

Kim et al. 2007b). Collectively, the findings concerning the nucleolar phase of viral proteins such as hordei-like TGB1 and umbravirus ORF3 proteins provide new models for the formation of 'transport-competent' viral RNP complexes and for the involvement of host factors in long-distance virus movement.

6.6 Conclusions

Recent studies have provided new information about viral factors involved in long-distance transport of viruses in plants and about the involvement of cellular factors in the regulation of virus trafficking. Research has established that, in addition to the CP, many non-structural viral proteins including the MP, TGB proteins, HC-Pro, and VPg are components of viral transport complexes and facilitate the cell-to-cell and long-distance movement of viruses within plants. Plant viruses depend on interactions with cellular factors throughout their life cycle. Because viruses use the host vascular system for long-distance transport, they must overcome various cellular barriers to load into sieve elements. At the same time, plants deploy various mechanisms to prevent systemic movement and infection by viruses. By identifying compatible interactions between viral and cellular factors, researchers are uncovering the molecular mechanisms that regulate virus movement. Extensive studies have provided strong evidence for the role of pre-existing cellular trafficking machineries including the cytoskeleton, membrane systems, and vascular systems in virus movement in plants. Recent findings emphasize the involvement of nuclei and other organelles in the formation of virus movement complexes and in virus transport. In particular, the nuclear localization of viral proteins implicated in virus movement has been observed in many virus species, suggesting that viruses may target the nucleus to support their systemic movement in plants. Comparative transcriptome analyses should be directed towards elucidating whether the nuclear localization of viral proteins changes the cellular transcriptional programme. Systemic spread of viruses in a plant requires the integration of processes affecting intracellular, intercellular, and long-distance movement through symplastic interconnections of plant cells. Thus, in-depth characterization of cellular factors that regulate virus trafficking will not only increase our understanding of virus movement but will also provide insight into basic cellular processes associated with macromolecular trafficking. Finally, a fundamental understanding of virus movement mechanisms and of the regulation of macromolecular trafficking will help in the development of anti-viral tools.

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Chapter 7

ER Stress, UPR and Virus Infections in Plants

Lingrui Zhang and Aiming Wang

Abstract The endoplasmic reticulum (ER) endomembrane is a central site for protein synthesis. Perturbation of ER homeostasis can result in an accumulation of unfolded proteins within the ER lumen, causing ER stress and the unfolded protein response (UPR). In humans, ER stress and UPR are closely associated with a vast number of diseases, including viral diseases. In plants, two arms that govern the UPR signaling network have been described: one that contains two ER membrane-associated transcription factors (bZIP17 and bZIP28) and the other that encompasses a dual protein kinase (RNA-splicing factor IRE1) and its target RNA (*bZIP60*). Although early studies mainly focus on the essential roles of the UPR in abiotic stresses, the significance of UPR in plant diseases caused by virus infections has recently drawn much attention. This chapter summarizes the latest scenario of ER stress and UPR in virus-infected plant cells, highlights the emerging roles of the IRE1 pathway in virus infections, and outlines exciting future directions to spark more research interest in the UPR field in plants.

7.1 Introduction

Protein folding guided by entropic and energetic forces has been one of the most intensely studied topics in biology in the past half century (Hartl and Hayer-Hartl 2009). Although the mechanism(s) by which the final folding is determined by the amino acid sequence still largely remains elusive, some important aspects in this field have become to be understood in the recent years. For instance, it has been known that a large fraction of proteins during synthesis (especially secreted and membrane proteins) are loaded in an unfolded state into the endoplasmic reticulum (ER) lumen (Hartl and Hayer-Hartl 2009). The newly synthesized polypeptides

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trans-located in the ER undergo folding aided by ER-associated chaperons and organelle-specific posttranscriptional modifications to reach their higher-order three dimensional states efficiently on a biologically relevant timescale (Ellgaard and Helenius 2003; Hartl and Hayer-Hartl 2009; He and Klionsky 2009; Marciniak and Ron 2010). For those proteins that fail to fold and modify properly, a surveillance mechanism composed of the ER quality control (ERQC) system and the ER-associated degradation (ERAD) system is assigned to eradicate their deleterious effects in order to maintain cell health (Howell 2013).

However, the load of client proteins may exceed the assigned processing and eliminating capacities of the ER, leading to ER stress, which is a pervasive characteristic of eukaryotic cells (Ron and Walter 2007; Gao et al. 2008; Liu and Howell 2010; Marciniak and Ron 2010; Hetz et al. 2011; Iwata and Koizumi 2012). ER stress can be primed by developmental or physiological fluctuations and genetic mutations that erode the ER protein homeostasis networks (Brewer and Hendershot 2004; Schröder and Kaufman 2005; Balch et al. 2008; Kim et al. 2008; Marciniak and Ron 2010; Hetz et al. 2011). In eukaryotic cells, a substantial body of evidence has also shown that multiple types of environmental stimuli (abiotic and biotic stress), including pathogenic invaders, chemicals, and depletion of energy or nutrients, can exert stress on the ER by disruption of cellular redox equilibrium and calcium (Ca^{2+}) homeostasis, interference of post-translational modifications and assemblies, and demand for an increased protein synthesis capacity (Dimcheff et al. 2004; Gao et al. 2008; Liu and Howell 2010; Ye et al. 2011; Iwata and Koizumi 2012; Zhang and Wang 2012; Zhang et al. 2015). In general, perturbation of ER homeostasis associated with accumulation of unfolded proteins in the lumen of the ER triggers an evolutionarily conserved signaling pathway referred as the unfolded protein response (UPR) (Ron and Walter 2007; Kim et al. 2008).

The primary goal of the UPR is to reestablish cellular homeostasis, to relieve stress imposed on the ER, and to prevent the cytotoxic impact of malformed proteins via inhibition of mRNA translation and activation of adaptive mechanisms (Xu 2005; Kim et al. 2008; Preston et al. 2009; Ye et al. 2011). As a result of this adaptation, particular groups of genes are expressed to enhance the protein folding capacity of the ER and to promote the misfolded protein degradation capacity mediated by ERAD (Kim et al. 2008; Verchot 2014). The signal-transduction events that are commonly associated with innate immunity and host defense, including mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), reactive oxygen species (ROS) networks, Ca^{2+} signaling and autophagy pathways, are also induced to produce a synthetic cellular response to ER stress (Fig. 7.1) (Kaneko et al. 2003; Tardif et al. 2005; Xu 2005; Chen et al. 2008; Kim et al. 2008; Ke and Chen 2011). However, if attempts to restore cellular native settings fail, a final mechanism called programmed cell death (PCD), also called apoptosis in eukaryotes, is triggered. Under this scenario, cell death is presumably useful to protect the organism from the expansion of potentially harmful substances produced by the damaged cells (Fig. 7.1) (Liu et al. 2005; Ron and Walter 2007; Kim et al. 2008).

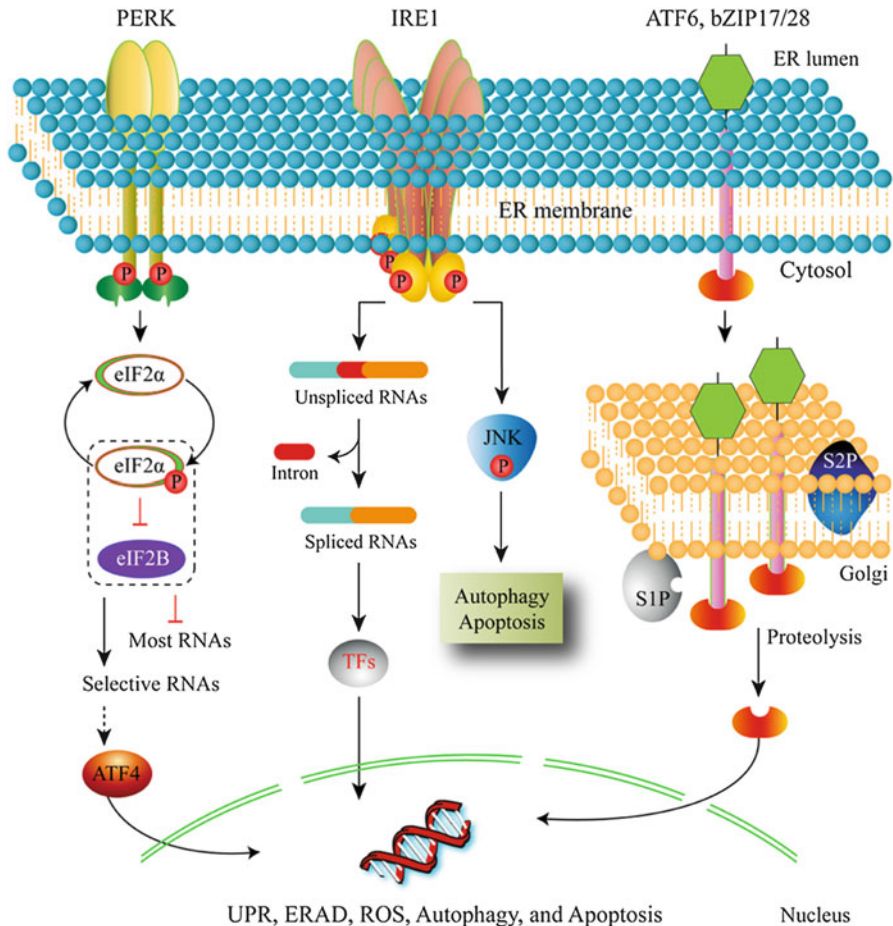


Fig. 7.1 The UPR branches in eukaryotes. The PERK arm is identified only in animals. On ER stress induced by abiotic or biotic cues (such as virus infection), the PERK kinase oligomerizes in the ER membrane and is activated via *trans*-autophosphorylation. The activated PERK phosphorylates initiation factor 2 alpha (eIF2 α), which inhibits the activities of eIF2B and the eIF2 complex. This accounts for all of the important consequences of PERK activity, such as translation inhibition of most mRNAs, which reduces protein synthesis and lowers ER loading. However, some mRNAs such as *ATF4* gains a selective advantage for translation via phosphorylated eIF2 α . ATF4 in turn contributes to the transcriptional activation of *CHOP*, *XBP1*, *GADD34*, and other genes involved in ROS signaling and apoptosis. The IRE1 arm is conserved in eukaryotes. IRE1 unconventionally splices the bZIP transcription factors *XBP1*, *bZIP60* and *HAC1* mRNA in mammals, plants, and yeast, respectively. The spliced bZIP transcription factors enter into the nucleus to regulate UPR, ERAD and autophagy target genes. In mammals, IRE1 arm also activates kinases such as JNK to initiate autophagy and apoptosis. ATF6 in animals and bZIP17/bZIP28 in plants reside in the ER membrane in unstressed cells. Upon ER stress, they are relocated from the ER to the Golgi apparatus, where they are subject to cleavage twice, first by the luminal S1P and then the intra-membrane S2P, to release their cytosolic transcription factor domains. Subsequently, the transcription factor domains then enter into the nucleus and activate a subset of UPR target genes

In mammalian cells, the UPR is mediated by two types of ER transmembrane proteins (ER stress sensors). The type I ER stress sensor is composed of IRE1 (inositol-requiring transmembrane kinase/endonuclease) including two IRE1 isoforms IRE1 α and IRE1 β , and PERK (PKR-like ER kinase), whereas type II includes ATF6 α and ATF6 β (activating transcription factor 6) (Fig. 7.1) (Cox and Walter 1996; Sidrauski and Walter 1997; Oikawa et al. 2010; Hetz et al. 2011). In contrast to that in animals, the UPR in yeast (*Saccharomyces cerevisiae*) is controlled by only one signaling pathway, the type I transmembrane ER protein IRE1p (Fig. 7.1) (Cox and Walter 1996; Sidrauski and Walter 1997; Oikawa et al. 2010). Over the last 10 years, significant advances have been made in understanding the ER stress and UPR signaling pathways in plants (Urade 2007; Vitale and Boston 2008; Deng et al. 2011; Nagashima et al. 2011). Thus far, two UPR pathways have been identified in plants, one mediated by IRE1-*bZIP60* (basic leucine zipper), and the other by site-1/site-2 proteases (S1P/S2P)-*bZIP17/bZIP28* which is analogous to the animal ATF6 pathway (Fig. 7.1) (Koizumi et al. 2001; Liu et al. 2007a, b.; Urade 2007; Vitale and Boston 2008; Liu and Howell 2010; Deng et al. 2011; Nagashima et al. 2011). Most recently, the beta subunit of the heterotrimeric G protein complex AGB1 has also been found to be essential for the plant UPR, adding further complexity to the UPR pathways (Chen and Brandizzi 2012, 2013).

In humans, it has been known that ER stress is implicated in numerous diseases, including cancers, neurodegeneration, diabetes, inflammation, and viral diseases (He 2006; Hetz et al. 2011). Therefore, there is a significant biomedical interest in illustrating the UPR molecular mechanisms and developing procedures to manipulate this pathway (He 2006; Hetz et al. 2011; Zhang and Wang 2012). In plants, much of the work in this field has concentrated on ER stress induced by environmental cues (Irsigler et al. 2007; Liu et al. 2007b; Costa et al. 2008; Gao et al. 2008; Liu and Howell 2010; Deng et al. 2011; Iwata and Koizumi 2012). In contrast to mammalian systems, in which the virus-induced UPR has been extensively studied (He 2006; Zhang and Wang 2012), it has only been recently that the essential role of the UPR in plants in response to viral attack has drawn attention (Ye et al. 2011; Ye and Verchot 2011; Zhang and Wang 2012; Ye et al. 2013; Zhang et al. 2015). This book chapter presents the latest progress in and viewpoints on the research of virus-induced ER stress and UPR in plants, with a focus on a recent discovery that IRE1 and *bZIP60* operate as a conserved pair to regulate virus infections. Through presenting evidence on how the ER transmembrane proteins sense the unfolded settings, we delineate the mechanisms of UPR activation under virus infections in mammals and plants, and discuss the functional implication of the UPR in virus infections and host responses. Finally, the physiological relevance of virus-induced ER stress with other signaling pathways and cellular processes is introduced and discussed, aiming to provide an integrated view of ER stress in multicellular eukaryotes and to suggest possible future directions of research on plant UPR.

7.2 The UPR Sensing Mechanisms during Virus Infections

Currently, there is general acceptance in the scientific community that the UPR signaling is initiated by UPR stress sensors, the ER resident transmembrane proteins (Fig. 7.1). Great efforts have been made in this area during the past decade (Bertolotti et al. 2000; Shen et al. 2002; Kimata et al. 2003, 2004; Credle et al. 2005; Gardner and Walter 2011), and several models have been proposed to depict the intricate mechanisms of how the protein misfolding is detected by UPR stress sensors (Hetz et al. 2011; Zhang and Wang 2012).

7.2.1 The Models of UPR Activation

Initially, the indirect recognition model proposes the binding immunoglobulin protein (BiP) as a repressor of UPR, which is dissociated from PERK, IRE1 α or the yeast homolog IRE1p to bind unfolded proteins upon ER stress, leading to the activation of the ER transducers (Bertolotti et al. 2000; Kimata et al. 2003). This model is also suggested to operate in the control of type-II transmembrane sensor activation (Shen et al. 2002). However, two major observations challenge this model. First, genetic evidence shows that deletion of the BiP-binding site does not cause constitutive activation of IRE1p (Kimata et al. 2003, 2004). Second, the finding of central groove formed by α -helices in the crystal structure of IRE1p suggests that IRE1p itself has the intrinsic ability to sense ER stress (Credle et al. 2005). Thus, the unfolded protein is proposed to play a pivotal role in stabilizing the activated ER stress sensors after the releasing of BiP in order to trigger robust UPR, which is the so-called two-step activation model (also called semi-direct recognition model) (Kimata et al. 2003, 2004; Credle et al. 2005). Subsequently, time resolved analysis of IRE1p signaling and elegant biochemical assays reveal a new quantitative model (also called direct recognition model), in which IRE1p is in a dynamic equilibrium with BiP and unfolded proteins. This model mainly stresses that the unfolded protein binding to IRE1p is sufficient and the only prerequisite for activation of the UPR, ruling out BiP as the principal determinant that governs the state of the UPR, and regarding BiP as a buffer and a timer to fine-tune the sensitivity and dynamics of the UPR (Pincus et al. 2010; Gardner and Walter 2011). Nevertheless, the direct recognition model does not apply to human IRE1 α , due to the facts that the groove in IRE1 α is too narrow for peptide binding, and that the recombinant IRE1 α does not interact with unfolded proteins *in vitro* (Zhou et al. 2006; Oikawa et al. 2009). Therefore, it is self-evident that the complexity of unfolded protein sensing is far beyond our initial expectation, and the task of identifying the fine molecular mechanisms in this field is far from complete. Interested readers are suggested to refer to the reviews for the details of the current recognition models (Hetz et al. 2011; Zhang and Wang 2012). In this section, we will mainly discuss how the UPR is activated upon virus infections.

7.2.2 *The Strategies to Manipulate UPR by Human Viruses*

During the course of millions of years of co-inhabitation with their hosts, viruses have evolved many sophisticated mechanisms of inducing and/or manipulating the UPR to assist in their own infections, which can be summarized into several categories as follows (Fig. 7.2). Viruses employ unfolded proteins encoded by their own genome to bind non-covalently the ER-resident protein BiP, thus leading to the activation of UPR (Fig. 7.2). These viral proteins include glycoprotein G of vesicular stomatitis virus, hemagglutinin-neuraminidase (HN) glycoprotein of paramyxovirus SV5, hemagglutinin of influenza virus, and E1 and E2 proteins of hepatitis C virus (HCV) (Kozutsumi et al. 1988; Hurtley et al. 1989; Ng et al. 1989; Machamer et al. 1990; Choukhi et al. 1998; Liberman et al. 1999). The second strategy is that viruses may exploit their own proteins(s) to directly and specifically modulate the ER stress sensors (Fig. 7.2). For instance, among seven proteins encoded by simian virus 5, only the HN glycoprotein that is inserted into the ER is capable of stimulating the UPR (Watowich et al. 1991). This also holds true for the ER-resident proteins encoded by flaviviruses and retroviruses (Tardif et al. 2004; Tardif et al. 2005). Recent studies with severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) have also revealed that one of the accessory proteins of SARS-CoV, the ER-resident protein 8ab protein, can bind directly to the luminal domain of ATF6, the type II ER stress sensor, to activate the UPR (Sung et al. 2009), whereas the ER-resident protein 3a selectively activates the PERK pathway (Minakshi et al. 2009). In addition, viruses may borrow the interaction of host factors and viral protein(s) in the ER site to induce the UPR (Fig. 7.2). This notion is supported by the finding that human cytomegalovirus protein US11 provokes the UPR in a manner depending on the interaction of US11 with Derlin-1 within the lipid bilayer of the ER (Tirosh et al. 2005). Lastly, but far from over, several other studies have also suggested a connection between the UPR and viral replication (Fig. 7.2). These include herpes simplex virus (HSV) 1, Japanese encephalitis virus (JEV), and HCV (Su et al. 2002; Cheng et al. 2005; Tardif et al. 2005).

7.2.3 *The Possible Mechanisms of UPR Activation during Plant Virus Infections*

In plants, several groups have independently shown through microarray analysis that the chaperon BiP is upregulated in *Arabidopsis* infected by *Turnip mosaic virus* (TuMV) and *Oilseed rape mosaic virus* (ORMV) (Whitham et al. 2003; Yang et al. 2007; García-Marcos et al. 2009). Consistently, other ER-resident chaperones have also been found to be induced in *Arabidopsis*, potato (*Solanum tuberosum*) and *N. benthamiana* (*Nicotiana benthamiana*) infected by *Potato virus X* (PVX) (Whitham et al. 2003; Yang et al. 2007; García-Marcos et al. 2009; Ye et al. 2011;

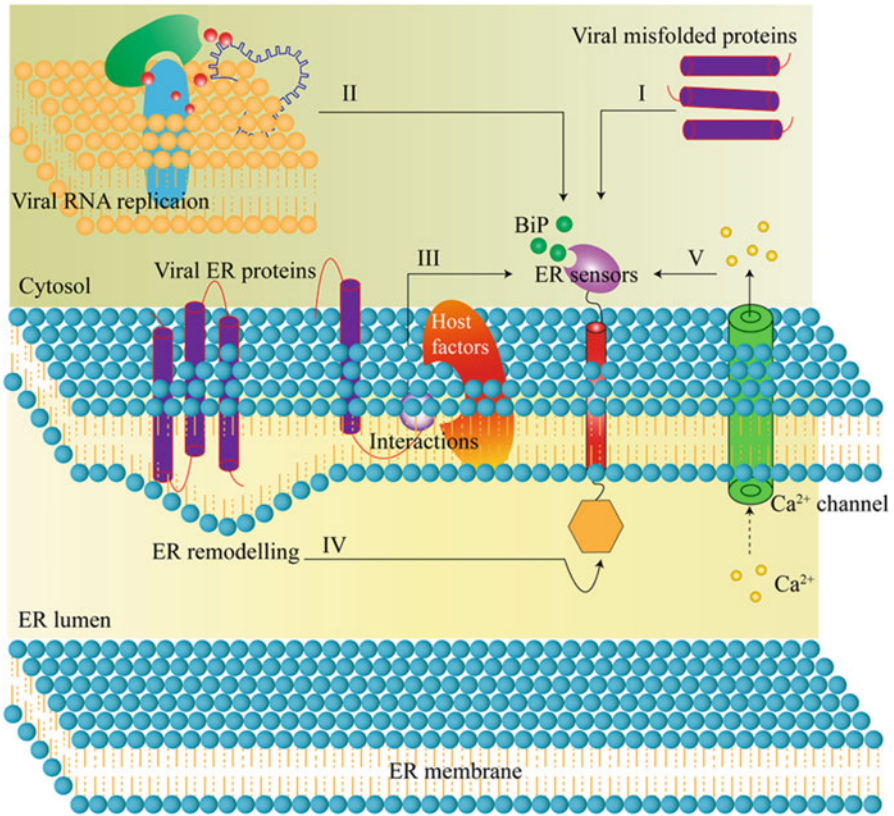


Fig. 7.2 The activation or/and manipulation of the UPR by viruses. The UPR branches are usually activated or manipulated by viral invaders by several ways as follows: (I) viruses such as HCV and influenza employ unfolded proteins encoded by viruses themselves to bind non-covalently BiP, thus leading to the activation of UPR; (II) viral genome replication can also initiate specific UPR arm due to the facts that replication vesicle are originated from ER membrane, and, moreover, unlimited viral RNA replication itself exerts a burden on cellular homeostasis; (III) viruses also borrow the interaction of host factors and viral protein(s) within the lipid bilayer of the ER to induce the UPR; (IV) viruses may exploit their own proteins(s) to directly and specifically modulate the ER stress sensors or to remodel the ER structure to induce the UPR; (V) in animals, virus infection can mobilize the ER calcium stores, resulting the activation of the UPR

Ye and Verchot 2011). However, even though it is clear that the UPR downstream signaling is indeed activated during viral infections, we are only just beginning to understand the molecular mechanisms involved in the activation of virus-induced UPR in plants.

Studies with PVX identified the viral movement protein (MP) TGBp3, which resides in the ER, as an elicitor of the expression of ER-resident chaperones in *Arabidopsis* and *N. benthamiana* (Bamunusinghe et al. 2009; Ye et al. 2011, 2013). It seems that, similar to the ER-resident proteins encoded by human viruses, TGBp3 modulates the level of the ER chaperones as a means to cope with robust viral

protein synthesis (Tardif et al. 2004; Chan and Egan 2005; Sung et al. 2009). Nevertheless, the mechanism(s) by which TGBp3 activates the UPR pathway (s) in PVX infections still remain unknown. Recently, we have shown that, of the 11 viral proteins of TuMV, only 6K2 has the ability to induce the splicing of *NtbZIP60* mRNA in *N. benthamiana* (Zhang et al. 2015). The potyviral 6K2 protein is an integral ER membrane protein and elicits the formation of ER-derived virus replication factories at ER exit sites (Laliberté and Sanfaçon 2010; Wei et al. 2010). Therefore, the finding of 6K2 as an inducer of the UPR pathway in plants is consistent with the well-documented conception in mammalian cells that virus-encoded ER targeting proteins induce the UPR (Fig. 7.2) (see discussion above). Based on all these observations (Bamunusinghe et al. 2009; Ye et al. 2011, 2013; Zhang et al. 2015), it is reasonable to conclude that the virus-encoded ER-targeting proteins may also be the potential inducers of the UPR in plants.

Although the mechanism of 6K2 triggering the UPR has yet not been fully understood, genetic and molecular analyses have revealed that the ER-resident chaperones are not only up-regulated, but the *bZIP60* also undergoes unconventional splicing mediated by IRE1 in *Arabidopsis* and *N. benthamiana* under TuMV infections (Zhang et al. 2015). It was further been proven that the virus infections are suppressed in the *ire1a-3 ire1b-4* double mutant, which can be rescued by complementing the mutant with not only IRE1A or IRE1B but also the spliced *bZIP60* (*bZIP60* S) (Zhang et al. 2015). Moreover, the virus infection suppression phenotype resulting from dysfunction of the IRE1-*bZIP60* branch is independent of the S1P/S2P-*bZIP17/bZIP28* arm (Zhang et al. 2015). These data directly show that the IRE1-*bZIP60* branch, rather than the other arm, is responsible for the virus-induced UPR, and IRE1 and *bZIP60* as a matched enzyme-substrate pair regulate virus infections, providing the first evidence that the IRE1-*bZIP60* arm is preferably manipulated by plant viruses. Considering that no interactions between 6K2 and IRE1 in plants have been experimentally demonstrated (Zhang et al. 2015), it is unclear how the virus-encoded 6K2 manipulates the UPR pathway to benefit viral infection. It is possible that 6K2 induces the UPR through its physical interaction with the ER or subsequent ER remodelling (Fig. 7.2) (Laliberté and Sanfaçon 2010).

7.3 The Roles of UPR in Plant Virus Infections

Virus infections trigger an arm race between virus and the host. On one hand, the host mobilizes the UPR machinery in an attempt to hamper virus infections. On the other hand, viruses exploit or even manipulate the UPR branches for their own benefits (Chan 2014). In mammalian cells, the intimate and complicated relationship between virus and three UPR pathways has been well reviewed (He 2006; Zhang and Wang 2012; Jheng et al. 2014; Verchot 2014). Of them, the IRE1/*bZIP60* branch is the most intensively studied (Jordan et al. 2002; Baltzis et al. 2004; Netherton et al. 2004; Sun et al. 2004; Tardif et al. 2005). Here, we will discuss the crosstalk between the UPR pathway mediated by IRE1 and virus

infections in plants as this is the only pathway found to be implicated in viral infections in plants.

Several lines of evidence suggest that the IRE1-mediated UPR branch promotes virus infections in plants. In the case of PVX, silencing *NtbZIP60* in *N. benthamiana* can suppress the expression of the UPR marker genes and reduce PVX accumulation (Ye et al. 2011, 2013). In virus-infected plants, membrane-associated virus replication or accumulation of large amounts of viral proteins can disrupt the fine equilibrium within cells (Ye et al. 2011, 2013; Smith 2014); therefore, the activation of the UPR mediated by *bZIP60* may serve as a compensatory mechanism required for the host to alleviate cytotoxicity and to restore cellular normal state and functions. This notion is supported by several earlier reports that the infections caused by a set of viruses, including *Cucumber mosaic virus*, *Oil seed rape mosaic virus*, *Turnip vein-clearing virus*, *Potato virus Y* and TuMV, can up-regulate the expression of *bZIP60* and ER marker genes in plants, suggesting a general role of this UPR pathway and ER chaperones in virus infections (Whitham et al. 2003; Yang et al. 2007; García-Marcos et al. 2009).

From the angle of the virus, the increased expression of ER-resident chaperones may facilitate virus infection through assisting in the assembly of replication complexes and the synthesis and folding of viral proteins as well as the assembly of viral particles (Hafrén et al. 2010; Howell 2013). In single-celled yeast, the host Ssa1/2p molecular chaperone (yeast homologue of HSP70) is required for the assembly of the tombusvirus replicase and to enhances viral RNA replication (Serva and Nagy 2006; Pogany et al. 2008). In plants, up-regulation of *HSP70* by potyvirus infection depends on the cytoplasmic UPR pathway (Aparicio et al. 2005; Sugio et al. 2009). Moreover, HSP70 is a component of the membrane-associated viral ribonucleoprotein complex, playing a critical part in viral genome expression and replication (Hafrén et al. 2010; Jungkuntz et al. 2011). In agreement with these findings, we have recently shown that treatment of *N. benthamiana* with pharmacological small molecular chaperones can promote TuMV infection (Zhang et al. 2015).

The IRE1-*bZIP60* pathway is also crucial for TuMV infection. First, in response to TuMV infection, the IRE1-*bZIP60* arm of the UPR is activated in both locally and systemically infected leaves (Zhang et al. 2015). Second, the mutant *bzip60-2* without detectable *bZIP60* S significantly inhibits viral accumulation and remarkably suppresses the development of disease symptoms, which can be rescued by genetic transformation of *bZIP60* S into the mutant (Zhang et al. 2015). Third, two different double mutants of *IRE1A* and *IRE1B*, in which the *bZIP60* splicing is blocked, also display the reduced levels of viral RNA accumulation and suppress viral symptom development. The absence of *bZIP60* S and suppression of virus infection in the double mutants can be rescued by complementation with either *IRE1* or *bZIP60* S (Zhang et al. 2015). Collectively, these data demonstrate that IRE1 and its processed *bZIP60* S function as a projected cognate system to promote viral infection in plants. This result is in accordance with several recent studies in mammalian cells that the UPR can be hijacked by viruses, such as influenza A virus, to favor viral infection, and inhibition of IRE1 activity compromises viral replication (Hassan et al. 2012).

7.4 The Link of UPR with Other Cellular Events during Virus Infections

The UPR has emerged to be more than an independent cellular response to virus infections. It is intimately linked to a variety of signaling networks and cellular responses either by modulating signaling pathways or as part of the cellular responses (Fig. 7.1) (Chan 2014). Here, we outline the physiological relevance of virus-induced ER stress with the ROS signaling network, autophagy and the ER quality control system mediated by ERAD.

7.4.1 Virus-Induced ER Stress and ROS Signaling Network

It has been known that ROS plays a pivotal role in various clinical diseases, including those associated with atherosclerosis and viral infection (Tardif et al. 2005). Many studies in human cell lines have proved that there exists a crosstalk between ER stress and the ROS pathway during virus infection (Tardif et al. 2005). For instance, infection by HCV in liver cells leads to the leakage of Ca^{2+} from ER stores, which is then taken up by mitochondria, inducing a sustainable accumulation of ROS (Fig. 7.3) (Ivanov et al. 2013; Paracha et al. 2013). ROS in turn activates cellular tyrosine and serine/threonine kinases to cause the translocation of NF- κ B and STAT-3 transcription factors into the nucleus in favor of its genome translation and replication (Fig. 7.3) (Gong et al. 2001; Tardif et al. 2005; Ivanov et al. 2013; Paracha et al. 2013).

In plants, ROS function as signaling molecules to control a variety of physiological and pathological processes (Apel and Hirt 2004; Zhang and Xing 2008; Zhang et al. 2009). Early studies with *Tobacco mosaic virus* (TMV) showed that the activity of the gp91^{phox} NADPH oxidase homologs is enhanced in virus-infected leaves, and ROS is induced within seconds after challenging the tobacco epidermal cells with virus (Allan et al. 2001; Sagi and Fluhr 2001). These studies also demonstrate that the rapid induction of ROS bursting can be prevented by specific inhibitors of NADPH oxidase, pointing to the plasma membrane NADPH oxidase, also known as the respiratory burst oxidase (RBO), as a biochemical source of ROS production in virus-infected plants (Allan et al. 2001; Sagi and Fluhr 2001). This notion is further confirmed by the observation that the rapid systemic generation of ROS in response to *Cauliflower mosaic virus* (CaMV) infection is abolished by the double mutation of *AtrbohD* and *AtrbohF* (two *Arabidopsis* genes homologous to gp91^{phox}, *Arabidopsis* RBO homolog) in *Arabidopsis* (Love et al. 2005).

Although virus-induced ROS production is linked to NADPH oxidase, the mechanisms underlying the activation and regulation of plant NADPH oxidase homologs in response to virus infections have yet to be understood. Studies with *Pseudomonas syringae* pv. *tomato* have indicated that calcium is one of the fastest responses upon pathogen infection, and is required for ROS production in plant

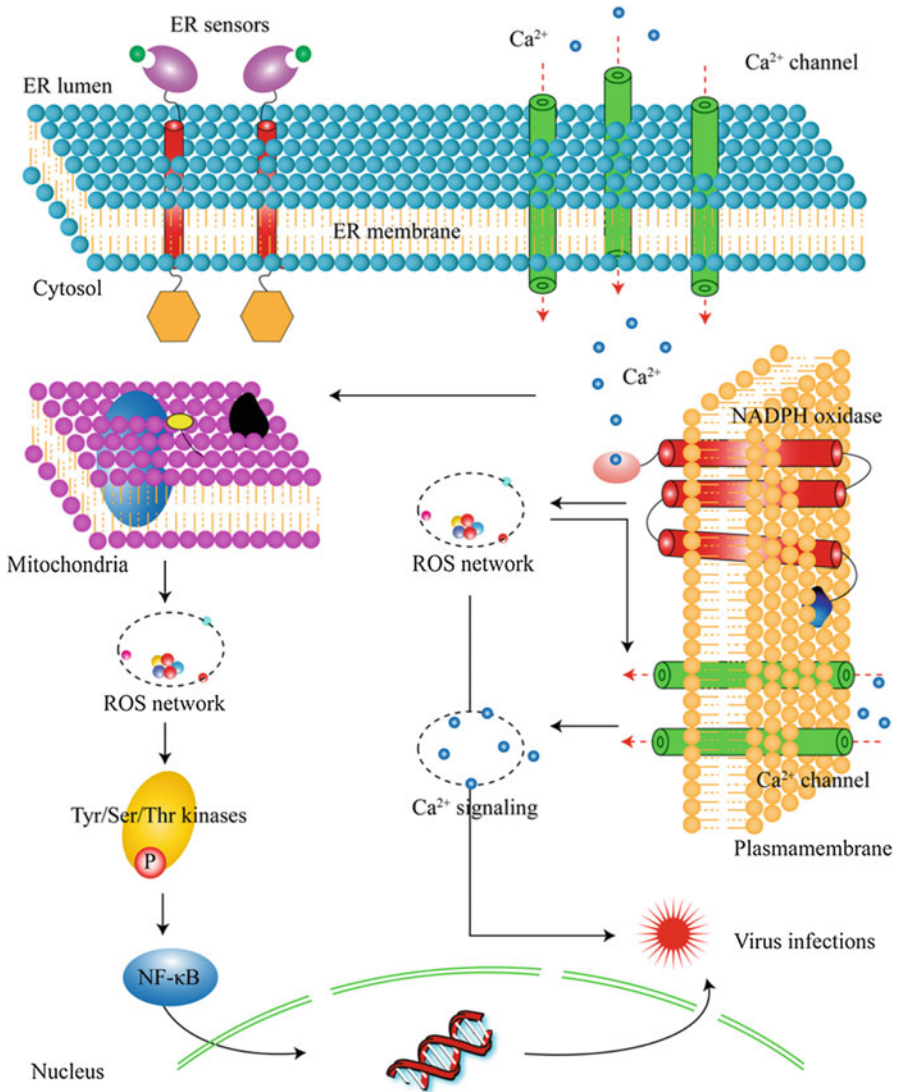


Fig. 7.3 Virus-induced ER stress and ROS signaling. In mammals, upon sensing virus-induced increase in Ca^{2+} , mitochondria initiate ROS signaling. ROS in turn activates tyrosine/serine/threonine kinases-NF- κ B pathways, to assist in viral genome translation and replication. In plants, ER stress also play a role in maintaining persistent virus infection and promoting virus spread through manipulating ROS and Ca^{2+} signaling networks. Here, we propose a ROS signaling cascade in virus infection in plants. Virus-induced ER stress generates a calcium signature during the early stage, which primes the downstream effectors such as NADPH oxidases to initial ROS production. The increased ROS further activate plasma membrane Ca^{2+} channels for eliciting a Ca^{2+} influx. The ROS and Ca^{2+} signaling networks contribute in a synergetic relation to virus infection through alerting host defense systems

defense response (Blume et al. 2000; Grant et al. 2000). Interestingly, in the case of *Pseudomonas syringae* pv. *glycinea*, ROS is also required to prime Ca^{2+} influx to activate a physiological cell death program (Levine et al. 1996), indicating that calcium functions not just upstream but also downstream of ROS production in response to bacterial pathogens (Nürnberg and Scheel 2001; Apel and Hirt 2004). Therefore, all these data indicate a complex and common spatiotemporal connection of ROS and Ca^{2+} signaling networks in plant biotic stress responses (Fig. 7.3). Along with the fact that all plant Rboh proteins contain two EF-hand motifs in their N-terminal region that are regulated by Ca^{2+} (Torres et al. 2006), it is logical to propose a possible signaling cassette for virus infection as follows. Virus-induced ER stress may produce a calcium signature during the early stage, which is sensed by the downstream effectors such as NADPH oxidases to initial ROS production. The elevated ROS may further activate plasma membrane Ca^{2+} channels for eliciting a Ca^{2+} influx to virus' own advantages or to alert host defense system(s) (Fig. 7.3).

It should be noted that the links of ER stress to ROS signaling during plant virus infection may not be limited to just turning on the ROS signaling cascade. During the long-term evolution, ER stress and the ROS signaling network might have developed a sophisticated and coordinated relationship to cope with environmental cues. Recently, it has been shown that the expression of PVX movement protein TGBp3 in *N. benthamiana* leads to ROS accumulation and cell death, which can both be prevented by the co-expression of ER molecular chaperon BiP (Ye et al. 2011, 2013; Ye and Verchot 2011). These data indicate that ER stress may play a contributing role towards maintaining persistent virus infection and/or promoting virus spread through manipulating the ROS signaling pathway. In addition, in *Plum pox virus* (PPV)-infected cells, accompanying with ROS production, the levels of apoplastic antioxidant enzymes are also increased in susceptible peach cultivar to maintain sustainable oxidative stress conditions, creating a co-existence environment for the host and the virus (Díaz-Vivancos et al. 2006; Hernández et al. 2006). In this sense, the role of ER stress-induced manipulation of ROS pathways is associated with the host antioxidant systems and is required for the establishment of persistent virus infection. Further studies are needed to elucidate the molecular link and functional relevance of the UPR branches and the ROS signaling network upon virus infections.

7.4.2 Virus-Induced ER Stress and Autophagy

Autophagy is a double-membrane vesicular process that results in the degradation of the sequestered components (Blázquez et al. 2014; Jheng et al. 2014). In mammalian cells, autophagy executes its cell-context specific functions in a four-step program: (1) autophagy induction mediated by activation of the Unc51-like kinase (ULK1) complex (Inoki et al. 2003; Mizushima 2010; Egan et al. 2011; Kim et al. 2011; Markus et al. 2011; Randhawa et al. 2015); (2) vesicle nucleation regulated by the Beclin1-PI3KC3 complex through the recruitment of PI3P

effectors and lipids required for auto-phagosome construction (Proikas-Cezanne et al. 2004; Axe et al. 2008; Hayashi-Nishino et al. 2009; Matsunaga et al. 2009); (3) vesicle expansion followed by the conjugation of microtubule-associated protein light chain 3 (LC3) with phosphatidylethanolamine (PE) and vesicle completion, represented by the enclosure of cytosolic cargos into double membrane vesicles, leading to the formation of auto-phagosomes (Geng and Klionsky 2008; He and Klionsky 2009; Shpilka et al. 2011); (4) auto-phagosome maturation into autolysosome by sequential fusion with endosomes and lysosomes, which is related to the expression of lysosomal-associated membrane protein 2 (Lamp-2), leading to the degradation of the loaded contents by internal hydrolases (Liang et al. 2008).

In mammalian cells, although the activation of the UPR and the induction of autophagy have been described during infections by a wide variety of viruses, the relationships between these two cellular processes remain controversial. In the case of HCV infection, the down-regulation of a variety of UPR modulators by siRNA has been found to result in a suppression of HCV-induced LC3-PE conjugation and a decrease of HCV RNA replication (Chen et al. 2008; Ke and Chen 2011). It has also been reported that HCV-induced eIF2 α phosphorylation through PERK activates autophagy (Dreux and Chisari 2011), and CHOP activated by the PERK-ATF4 and ATF6 pathways plays a leading role in promoting ATG12 and LC3 protein expression (Ke and Chen 2011; Wang et al. 2014). Moreover, knockdown of IRE1 is found to inhibit the formation of auto-phagosomes as well as the conversion of LC3-I to LC3-II (Joubert et al. 2012). All these data suggest an epistasis role of the UPR in autophagy activation. However, the independence of autophagy induction on the UPR has also been observed. The expression of a sub-genomic replicon of a pegivirus results in an increased LC3-II level, but does not induce the UPR (Howell 2013). Furthermore, the cause-effect relationship between UPR and autophagy is also far from fully understood (Mohl et al. 2012). For instance, WNV triggers the UPR but does not always up-regulate the autophagy pathway (Vandergaast and Fredericksen 2012). All these mixed observations show that further studies are still needed to unravel the connection between the UPR and autophagy pathway during virus infection.

It is well known that the basic process and the essential components of autophagy are highly conserved among eukaryotes from yeast to animals and plants (Liu and Bassham 2012). Unlike animal autophagy, however, the cargo of autophagy is destined to the vacuole in plants (or, in yeast, the analogous vacuole) for degradation (Chen and Klionsky 2011; Liu and Bassham 2012). Like the implication of autophagy in health and disease processes such as cancer, neurodegeneration, aging, and longevity in animals (Yang and Klionsky 2010), autophagy in plants are associated with a variety of stresses, pathogen infections, and senescence (Bassham 2007; Hayward and Dinesh-Kumar 2011). Consistent with the findings from studies using mammalian cells (Chen et al. 2008; Ke and Chen 2011), autophagy genes play a role in host defense against virus infection in plants. For example, when tobacco plants are infected with TMV, autophagy is induced in both the infected and the uninfected area (Liu et al. 2005). Similar to mammalian *BECLIN1*, the plant orthologs of three autophagy genes, *BECLIN1*, *ATG3* and *ATG7*, restrict viral replication (Liu et al. 2005). In contrast to

mammalian *BECLIN1*, which prevents cell death in virus-infected tissues (Orvedahl et al. 2010), plant autophagy genes play a role in preventing cell death in uninfected tissues (Liu et al. 2005). Similar results have also been observed in *Arabidopsis* *ATG6* knockdown plants and *ATG5* knockout mutants (Yoshimoto et al. 2009) challenged with an avirulent bacterial pathogen, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (*avrRPM1*) (Patel and Dinesh-Kumar 2008). These results suggest that autophagy functions to prevent runaway cell death in plants.

In plants, autophagy is known to function during ER stress. When ER stress is induced by tunicamycin (TM) or dithiothreitol (DTT), auto-phagosomes accumulate in *Arabidopsis* root cells; therefore autophagy is activated by ER stress in plants (Liu et al. 2012). Moreover, the activation of autophagy by TM and DTT is mediated by the ER transducer IRE1B, rather than IRE1A (Liu et al. 2012). Although IRE1B is identified as an upstream regulator of autophagy during ER stress in plants, the detailed regulatory mechanism is still unclear (Liu and Bassham 2013). In yeast, ER stress-triggered autophagy relies on the endoribonuclease splicing activity of IRE1 toward its mRNA substrate. In animal cells, ER stress-triggered autophagy is mediated by the kinase activity of IRE1 through the JNK pathway (Fig. 7.1), rather than its splicing activity (Liu and Bassham 2013). In *Arabidopsis*, ER stress-induced autophagy also does not depend on the splicing activity of IRE1B toward *bZIP60* (Liu et al. 2012). Considering that the JNK pathway does not appear to exist in plants, it is possible that either IRE1B has other splicing targets besides *bZIP60*, or IRE1B has unidentified functions in addition to its splicing activity related to induction of autophagy (Liu and Bassham 2013).

7.4.3 Virus-Induced ER Stress and ERAD

During virus infection, viral activities, such as viral genome replication and protein translation, pose an enormous biosynthetic burden on the ER, leading to ER stress (Noueiry and Ahlquist 2003). In this regard, the re-establishment of ER homeostasis by activating UPR pathways to slow down protein synthesis and to upregulate the capacity of the ER to fold client proteins is an adaptive cellular strategy that is beneficial to invaders and hosts (Verchot 2014). To prevent congestion of the ER folding machinery with terminally misfolded proteins, eukaryotic cells have evolved an ERQC surveillance mechanism and an ERAD degradation system for removal of proteins unable to re-fold properly or unable to fold within a reasonable time (Fig. 7.1) (Meusser et al. 2005; Byun et al. 2014; Verchot 2014). It has been shown that ERAD is vital to the maintenance of healthy cells, and its failure to destroy misfolded proteins is associated with a growing number of illnesses, such as Parkinson's, Alzheimer's and Huntington's diseases (Howell 2013; Byun et al. 2014).

Basically, the ERAD system recognizes misfolded proteins to be eliminated, and then extracts them through membrane channels in an energy-dependent manner for poly-ubiquitination, and subsequently degradation in proteasomes (Howell 2013; Byun et al. 2014). The identification of terminally misfolded proteins from the

nascent glycoproteins in the process of being folded is a key step for the ERQC system. This step involves the bipartite recognition of the terminal α -1, 6-linked mannose on the C chain and the misfolded protein moiety, which are the two major features for terminally misfolded proteins (Denic 2011). In yeast, this recognition is performed by the Hrd1 complex, which is composed of E3 ubiquitin ligases Hrd1 and Hrd3 as well as Yos9 lectin. Yos9 recognizes in collaboration with Hrd3 the terminal α -1,6-mannose linkage on the C chain of the N-glycan of a glycoprotein, which is exposed by the action of the mannosidase Htm1 (Gauss et al. 2011). The ERAD substrates recognized by Yos9 and Hrd3 are recruited as a client protein for cytosolic ubiquitination by the E3 ligase Hrd1. Therefore, the Hrd1 complex spares nascent glycoproteins from early degradation, and misfolded proteins bearing modified glycans are consigned to ERAD (Gauss et al. 2011). The misfolded protein recognized and labelled by the Hrd1 complex is extracted from the ER lumen by CELL-DIVISION/CYCLE protein 48 (CDC48, an AAA-ATPase motor) and then targeted to the 26S proteasome for degradation.

In mammalian cells, some viruses, including hepatitis B (HBV) or HCV, exploit the ERAD to reduce the amounts of glycoproteins and particles produced to avoid the innate and adaptive immune systems, leading to chronic infections (Byun et al. 2014). Interestingly, both viruses can induce the UPR, which in turn increases the levels of certain ERAD components (Byun et al. 2014). Therefore, virus-induced UPR and ERAD benefit virus infections. The 69K MP of the plant virus *Turnip yellow mosaic virus* (TYMV) is poly-ubiquitinated for subsequent rapid and selective proteolysis by the proteasome in the *in vitro* reticulocyte lysate translation system (Drugeon and Jupin 2002). In plants, *Potato leaf roll virus* (PLRV) MP and the PVX protein TGBp3 are the targets of the proteasome (Vogel et al. 2007; Ju et al. 2008). Trans-locating TGBp3 from the ER to the cytoplasm for degradation depends on the ERAD pathway (Ju et al. 2008).

As a conserved chaperone controlling protein fate in yeast and animal cells by extracting protein substrates from membranes or complexes (Meyer et al. 2012), CDC48 has been recently identified as a cellular factor regulating viral MP accumulation patterns in plant cells. *Arabidopsis CDC48* is upregulated upon TMV infection, and the encoded protein interacts with viral MP in ER-associated inclusions, the viral factories (Niehl et al. 2012). Later in the infection cycle, more misfolded MPs further accumulate in the ER-inclusions, and then are extracted by the CDC48 complex to the cytosol for poly-ubiquitination and subsequent degradation in an ERAD-dependent pathway (Reichel and Beachy 2000; Niehl et al. 2012). As a result, the 26S proteasome becomes saturated, and increased amounts of MP stabilize microtubules to hinder the transport of viral complexes along the ER into the neighboring cells (Niehl et al. 2012). In this regard, CDC48 function may represent a host defense mechanism by which the viral protein is removed to ensure membrane maintenance and to control viral movement (Niehl et al. 2012). However, this system may be exploited by the virus to increase replication efficiency since the extraction of MP from ER-inclusions by CDC48 may render the RNA translatable and consequently assist further replication of the virus. It will be interesting to test the importance of CDC48 in regulation of the cell-to-cell transport and replication of the virus during viral infection (Niehl et al. 2013).

7.5 Conclusions

The UPR was originally thought to maintain and re-establish cellular homeostasis upon ER stress. Now it is clear that the signaling of the ER stress-induced UPR pathways has broad associations with other signaling networks and cellular events, bringing important consequences for diverse physiological and pathological processes. Moreover, the elements of the UPR systems can be exploited or/and manipulated by pathogenic viruses in favor of genome translation and replication, viral particle assembly and persistent infections.

Despite the recent advances made in plants towards understanding the UPR implicated in abiotic and biotic stress, including viral infection, we are still far away from fully dissecting the UPR. The molecular and structural basis for recognition of the unfolding settings by the ER stress sensors are still missing in plants. The understanding of virus-induced UPR will be very rudimentary without a thorough elucidation of the structural basis of ER stress sensors. As discussed above, in mammalian cells, a variety of approaches can be used by viruses to activate and/or to block specific UPR pathway(s). However, in plants, viral proteins are the only known inducer of the UPR, and just one pathway is identified in virus infections thus far. Therefore, extensive studies are needed in the future to disclose the relationship between virus infections and the host UPR.

In addition, although ER stress and UPR are closely associated with other signaling networks and cellular processes, how those processes are coordinated to function is still unclear. A comprehensive study on these questions will certainly shed new light in the UPR pathways, lead to a better understanding of host-virus interactions, unlock novel antiviral mechanisms and targets and, in the long run, assist in developing novel effective antiviral strategies.

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Chapter 8

Plant Virus Diversity and Evolution

Anthony Stobbe and Marilyn J. Roossinck

Abstract Historically, the majority of plant virology focused on agricultural systems. Recent efforts have expanded our knowledge of the true diversity of plant viruses by studying those viruses that infect wild, undomesticated plants. Those efforts have provided answers to basic ecological questions regarding viruses in the wild, and insights into evolutionary questions, regarding the origins of viruses. While much work has been done, we have merely scratched the surface of the diversity that is estimated to exist. In this chapter we discuss the state of our knowledge of virus diversity, both in agricultural systems as well as in native wild systems, the border between these two systems and how viruses adapt and move across this border into an artificial, domesticated environment. We look at how this diversity has affected our outlook on viruses as a whole, shifting our past view of viruses as purely antagonistic entities of destruction to one where viruses are in a mutually beneficial relationship with their hosts. Additionally, we discuss the current work that plant virology has put forth regarding the evolutionary mechanisms, the life histories, and the deep evolution of viruses.

8.1 Introduction

Until recent years, our knowledge of the breadth of plant virus diversity was limited. The field of plant virology traditionally has focused on agricultural systems, with little study of viruses found in wild plants. In the past decade, several efforts have begun to fill these gaps in the form of biodiversity surveys. These surveys have given us a new view into the true diversity of plant viruses, as well as their distribution.

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One of the most powerful advances in microbe discovery has been massively parallel sequencing, or Next Generation Sequencing (NGS). Previously the most common methods for virus detection were protein based immunological tests such as ELISA, or nucleotide specific PCR assays. Neither of these are sensitive enough to detect low titers of virus in wild plants or general enough to detect novel viruses, or even related strains or species. NGS technology has boosted our ability to fully sequence whole genomes, and advanced the field of metagenomics, the study of all the genetic information from a given environment. When the requirement for culture is removed, the ability to sequence and identify fastidious or unculturable microbes becomes possible.

NGS has become the gold standard for metagenomics. Metagenomics can be used to identify novel virus species, using various techniques to enrich viral nucleic acids such as isolating specific forms of RNA (dsRNA, siRNA, ssRNA) or virus particle isolation. Each method has positive and negative aspects (Stobbe and Roossinck 2014; Roossinck et al. 2015). While NGS can be used for virus discovery, it also has been applied to plant virus diagnostics (Stobbe et al. 2013; Massart et al. 2014). NGS also has been used to look further into the population diversity of individual virus strains. Using this deep sequencing, one is able to determine all of the minor variants found in a given infection (Simmons et al. 2012). NGS has applications in many evolutionary questions regarding systemic movement, vectoring and epidemiology.

In this chapter we look into the recent work looking into the diversity of plant viruses, not only in species diversity but also diversity within the species or quasispecies. This variation comes from many sources, including high mutation rates of RNA viruses, recombination and reassortment. The variation we see within a single plant host has profound effects on the how the virus responds to selective pressures associated with new hosts, and factors such as the bottleneck events associated with cell-to-cell movement or vectoring. Additionally, with our ever increasing knowledge of the breadth of virus diversity, as well as advances in technology, questions of the deep evolutionary history of viruses and their relationship to their hosts are beginning to be answered. While there has been a large body of work on algae-infecting viruses (VanEtten and Dunigan 2012), here we only consider the viruses of vascular plants.

8.2 Viruses Within Agricultural Systems

Agriculture has been an important aspect of virology from the beginning of the field (Beijerinck 1898), and has been the focus of most work in plant virology throughout its 120 year history. Much of the early work characterizing and describing viruses was done with viruses of crop plants. While it is understandable that so much work has been put into a few specific plant species, this has left out a lot of information

about viruses in natural settings. In modern agriculture the use of vast areas of monoculture, extended growing seasons, irrigation, and artificial soil amendments have each impacted plant pathogen prevalence, including viruses.

8.2.1 Human Effect on Virus Diversity

Agriculture is a human invention, and the cultivation of crops has propelled the human race to increasing cultural and technological advances, but with this advancement we have disturbed many natural systems throughout our history. Domestication of the earliest crops probably began about 10,000 to 12,000 years ago (Balter 2007), presumably with their viruses experiencing a shift in selective pressure as well (Stukenbrock and McDonald 2008). Densely spaced, monoculture crops have been increasingly favored due to the ease of production, but these conditions also are excellent for the spread and infection of viruses and other deleterious microbes within the crop (Thresh 1982; Power and Mitchell 2004). To combat the yield loss associated with virus-induced disease, breeders have focused efforts on engineering disease resistant cultivars of crops. However, several forms of virus variation, such as the high mutation rates of RNA and some DNA viruses, recombination, and reassortment lead to resistance breaking (Duffy and Holmes 2008; McDonald and Linde 2002; Harrison 2002). Although breeding of resistant cultivars has had some success, other methods such as increasing the plant species diversity in a given area, breaking the spatial and temporal components of the disease cycle, have been suggested (Ratnadass et al. 2012). For example, genetic diversity (heterosis) induced tolerance to *Turnip mosaic virus* in wild cress (*Lepidium* sp.) hybrids, while plants that were selfed were more susceptible to disease, suggesting that small populations with low genetic diversity could lead to increased disease symptoms, and infection rates (Houliston et al. 2015). Intercropping cowpea with cassava or plantains has reduced the incidence of viruses in Central America (Valverde et al. 1982). These practices suggest that increases in plant diversity, either within a species or with diverse species, could lower the incidence or pathology of viruses (see Sect. 8.2.3).

With the globalization of today's society, it is not surprising to find that humans are playing a role in the movement of plant viruses. Human movement of both the plants and vectors associated with pathogens has facilitated the spread of viruses. The effects of climate change in the form of CO₂ and ozone may change the impacts of viruses on their plant hosts (Trebecki et al. 2015). While many pathogens move closer to the poles as climate change occurs, there is some evidence that viruses and nematodes are moving closer to the equator (Bebber et al. 2013). This may be an analytical artifact as viral symptoms are often misdiagnosed. Increases in the range of insect vectors of viruses due to numerous factors, including climate change, predicts increased virus spread (Feres 2015).

8.2.2 Vectors

A majority of crop viruses are insect vectored, and relationships between plants, viruses and insects are complex (Roossinck 2015b). Using insect vectors as targets for virus discovery is an attractive opportunity. Vector-enabled metagenomics is a recent method for virus discovery that allows one to discover and characterize viruses that are in the area that the vector occupies, including both cultivated and wild plants. In a recent study using vector-enabled metagenomics 79 % of the sequences obtained were related to known viruses, suggesting that many vector transmitted viruses are known (Ng et al. 2011). This number was much higher than the number of identifiable virus sequences found in wild plants, where as many as 60 % of sequences from virus-enriched pools have no similarity to sequences in GenBank (Roossinck et al. 2015).

The “Viral Manipulation Hypothesis” states that by modifying the production of certain volatiles, the plant host will be more attractive to the virus’ vectors (Gutiérrez et al. 2013). Vector transmission mechanisms of plant viruses influence the effects the virus has on the plant host, with persistently transmitted viruses tending to either improve the host quality for the vector or mimic high quality, and nonpersistently transmitted viruses lowering quality to facilitate the rapid dispersal of the viruliferous insect to neighboring plants (Mauck et al. 2012). Host manipulation is seen in unrelated families of plant viruses, implying convergent evolution (Wu et al. 2014). *Barley yellow mosaic virus* (BYDV) is persistently transmitted by the aphid *Rhopalosiphum padi*. Virus-free aphids have a feeding preference for BYDV-infected plants, while the reverse is true for BYDV-carrying aphids (Ingwell et al. 2012). *Cucumber mosaic virus* (CMV), a member of the *Bromoviridae* family, increases the release of volatiles that mimic healthy plants, attracting vectors despite the low quality of the plant for the aphids (Mauck et al. 2015b). Additionally, CMV effects other non-vectoring insects, repelling some while attracting others, in the absence of aphids (Mauck et al. 2015a). In mixed infections, competition can favor one microbe over another, as seen with the potyvirus, *Zucchini yellow mosaic virus* (ZYMV) out-competing another potyvirus, *Watermelon mosaic virus* (WMV). ZYMV and WMV are very similar viruses, in terms of genetics, hosts, and vectors. These similarities places them in direct competition with each other. One important difference is in vector manipulation; ZYMV manipulates the host-aphid relationship, while WMV does not. When co-infecting a plant, ZYMV will maintain its typical level of replication, while the replication of WMV is reduced. Despite this, WMV is still transmitted from a mixed infection, taking advantage of ZYMV host manipulation that attracts the aphid vectors (Salvaudon et al. 2013). In an analysis of genetic turnover during transmission, several clones containing the same mutation leading to a premature stop codon was found within a plant. Further transmissions using this experimental isolate lost this mutation, but this suggests that ZYMV has the ability to complement defective ZYMV genomes in the aphid vector (Simmons et al. 2011).

Viruses not only manipulate their hosts, they also respond to the presence of a vector feeding. *Cauliflower mosaic virus* (CaMV), a double-stranded DNA virus in

the *Caulimoviridae* family, is acquired up by its aphid vector packaged into transmission bodies. The transmission bodies change their morphology in different contexts, such as in response to CO₂ levels or host wounding. In addition, the transmission bodies change to a morphology that favors transmission when in proximity to the saliva from aphid feeding (Martinière et al. 2013).

8.2.3 The Agro-Eco Border: Spill Over and Movement

Obviously there are many differences between agricultural systems, and ecosystems of wild undisturbed plants. We have already touched on the use of monoculture, and the effect of plant biodiversity on the diversity of viruses, in this section we look into how nearby systems can influence viruses. The intersection of wild and agricultural systems has been described as the agro-eco border (Roossinck and García-Arenal 2015). This border may be the source of new pathogenic plant viruses that can impact crops in spillover events (Fig. 8.1).

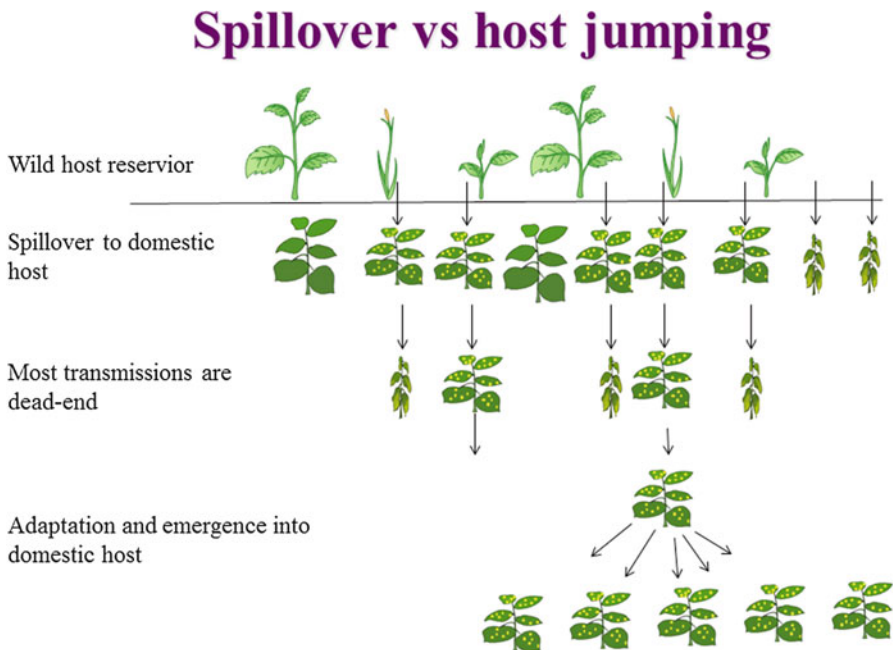


Fig. 8.1 Spillover of viruses from wild plants. Viruses are abundant and often inapparent in wild plants. At the agro-eco boundary viruses may move into crop plants from nearby wild plants. In most cases these infections will be dead-end: either the virus is not competent for further transmission in the new host, it may not establish sufficient virus titer to allow transmission, or it may rapidly kill the host. Rarely, a spillover virus may develop the ability to be further transmitted to more similar hosts, resulting in an emerging virus infection

Due to the relatively recent introduction of modern agriculture to Australia (in the past 200 years), considerable work has been done on this continent to look at the effect of agriculture across this border. Many of the native viruses in Australia have not been influenced by agriculture. Three different new encounter events of legume-infecting potyviruses have been described in Australia. The interspecies genetic diversity of each virus differed, with the native viruses having greater diversity than the exotic viruses (Webster et al. 2007).

Viruses that infect crop plants often find reservoirs within nearby wild plants or in volunteer plants from the previous crop. These viruses, that cause disease in crops, may be asymptomatic in other hosts. For example, *Peanut stunt virus* causes disease in peanuts, but is asymptomatic in clover (Sherwood 1997). The presence of highly susceptible hosts in a plant community can increase the incidence of the virus across all susceptible species in the community (Power 2008). Several scenarios can be seen where the spread of virus moves between an asymptomatic native host to cultivated plants (Jones 2014). Emergent viral diseases may come from “silent” infections within a nearby wild population, and are driven by anthropogenic factors, such as food production or the introduction of vectors (Anderson et al. 2004). In Africa, there have been several emergent viruses in agricultural systems. While many changes in the pathogens themselves have promoted the emergence of disease, changes in agricultural practices have also promoted the introductions. *Rice yellow mottle virus* (RYMV, in the genus *Sobemovirus*) infects *Oryza sp.* in both wild and agricultural systems. RYMV was first described in 1966 in Kenya and is currently an economically important plant virus. The rise of rice production in Kenya is thought to be the main driver of RYMV spread, as epidemics of the virus were not seen until after the intensification of rice production in the 1960s (Fargette et al. 2006). Despite many examples of host jumps leading to disease, there have been examples of viruses that have switched hosts several times with no apparent increase in pathology (Thresh 2006).

The level of biodiversity on the wild side of the agro-eco border affects the emergence of viral movement across the border. A lowered incidence of two begomoviruses was seen in wild peppers with decreasing levels of cultivation or management, suggesting a dilution effect with higher levels of biodiversity (Pagán et al. 2012). This correlation with biodiversity appears to hold true with CMV in wild plants, but not with CMV in crops (Sacristán et al. 2004). The loss of biodiversity appears to increase the movement of a virus across the agro-eco border, but a high degree of biodiversity can lead to a large number of viral species, which may serve as a reservoir for new infections (Keesing et al. 2010). Opportunistic viruses quickly move into susceptible crops, decrease, then recover in the susceptible population, causing a rapid cycle of epidemics and decline (Harrison 1981; Thresh 1981). In France, where ZYMV and WMV are both present, only WMV has significant natural reservoirs, which explains the fragmented nature of ZYMV incidence across France (Lecoq et al. 2014). In Spain, two strains of the potyvirus *Pepino mosaic virus* (PepMV-EU and PepMV-CH2) co-circulate among tomato crops, with the CH2 strain being the dominant strain. PepMV-EU primarily exists in coinfections with PepMV-CH2, and these coinfections allow for an extended host

range of PepMV, thus extending the potential number of reservoirs. This has implications for coinfection effecting the emergence of PepMV in tomato plants (Gómez et al. 2009).

8.3 Viruses Within Natural Systems

Early attempts to explore virus prevalence within wild plants was hampered by a lack of sensitive detection methods (Cooper and Jones 2006). In recent years, there have been a number of plant virus diversity surveys, in which plant tissue was sampled without targeting symptomatic plants (Wren et al. 2006; Roossinck 2013). These tissue samples were then enriched for viral nucleic acid, and sequenced using NGS. The prevalence and distribution of viruses in these studies varies, but inevitably evidence for many novel virus is found, and the variation of viruses in wild systems is much greater than what is seen in crops (Roossinck et al. 2015).

8.3.1 Impacts of Viruses on Wild Plants

The enemy-release hypothesis states that plants invading a new territory may have an advantage because they have left behind their pathogens (Power 2008; Rúa et al. 2011). However, in the invasive grasses of the Pacific coastal region of North America, a non-native plant uses its own adapted *Barley/Cereal yellow dwarf virus* (B/CYDV) to gain an advantage over the native grasses (Malmstrom et al. 2005). The reverse was seen in another related system; *Venetanata dubai* (African wiregrass), an invasive non-native grass that is not adapted to B/CYDV was slowed in its movement across the northwest grasslands of America (Ingwell and Bosque-Pérez 2014).

The extended phenotype of viruses can change based on many contexts, including the genotype of the host (vanMölken and Stuefer 2011), and biotic and abiotic conditions. These phenotypes vary from the classic disease symptoms to host benefitting-qualities such as drought or cold tolerance (Roossinck 2015b). The context of the plant hosts can effect the spread and diversity of plant viruses more than the composition of the plant host species. Competition between BYDV and CYDV was altered by changing the nutrition resources in the form of nitrogen and phosphorous for their hosts (Lacroix et al. 2014). In a B/CYDV survey in North American Pacific coast grasslands targeting three different host species (*Avena fatua*, *Elymus glaucus*, and *Bromus hordeaceus*) virus prevalence was determined not only by host species identity, with *A. fatua* having the highest prevalence and *B. hordeaceus* having the lowest, but also by biotic and abiotic factors, including an increase of virus prevalence with a decrease in precipitation and increase in soil nitrogen (Seabloom et al. 2010).

Many viruses that are found in wild plants have either mild symptoms or are completely asymptomatic (Prendeville et al. 2012; Jones 2014; Davis et al. 2015).

Many wild plants host multiple viruses, in some cases up to seven different viruses were found co-existing in a single plant (Roossinck et al. 2010). In two studies in the United States and Costa Rica, over 50 % of the virus sequences found in wild plants belonged to three virus families: *Partitiviridae*, *Chrysoviridae*, and *Totiviridae* (Roossinck 2012b). These virus families, along with the *Endornaviridae*, have been described as persistent plant viruses (Roossinck 2010, 2015a). Most of the persistent plant viruses are double stranded RNA viruses, although the *Endornaviridae* are likely single-stranded RNA viruses that are isolated as replicative intermediates (Roossinck et al. 2011). Most interestingly, the persistent viruses are wholly transmitted vertically, with no known form of horizontal transmission. In fact, there is no cell-to-cell movement of persistent viruses, spreading throughout the host via cell division (Roossinck 2010, 2012a). The *Partitiviridae*, *Endornaviridae* and *Chrysoviridae* infect both plants and fungi, while the *Totiviridae* also infect fungi and protozoa. While there is no observable effect of these viruses on their hosts, there have been multiple instances of integration of some persistent viral genomes into plant and fungal genomes (Liu et al. 2010; Chiba et al. 2011). This should not be surprising given the intimate symbiotic nature of the relationship. Currently the persistent viruses are understudied and many aspects of their nature are unknown (Roossinck 2015a).

In a study looking at the effect of both CMV and ZYMV in wild populations of *Cucurbita pepo*, the context of the host population, either adjacent to a road, within a managed peanut field, or within an unmanaged pasture, seemed to be the dominate factor in whether ZYMV was detrimental, beneficial or neutral, respectively (Prendeville et al. 2014). While latent viruses are common in wild plants, there are of course pathogenic viruses found in the wild as well (Cooper and Jones 2006).

8.3.2 Difficulties of Virus Discovery

It is not surprising that with an in depth look into the viral biodiversity of plants one would find novel viruses related to known viruses, nor is it surprising that sequences with little or no similarity to anything in a curated database would be found. Often, even when there are related viruses within a database, the curation is not in a state to be useful. There are few centralized databanks to store metadata collected during large biodiversity surveys, though attempts have been made. Metavir, a website service offering basic analysis of viromes, allows for viromes to be made public, and at the time of writing houses 368 different viromes from 67 different projects (Roux et al. 2014). It is unclear how these potential viruses should be treated. In a recent virus survey in Costa Rica, 60 % of the sequence reads received no hit when searched against the GenBank database (Roossinck et al. 2010). For viruses with no known relative, a cluster analysis can give structure to a population of unknown microbes, including viruses (Labonté and Suttle 2013). While having a sequence identity for these viruses can offer some answers, the viruses ultimately need to be characterized experimentally.

8.4 Variation Within Virus Isolates

Evidence for genetic variation of plant viruses was reported as early as 1926 (Kunkel 1947). Numerous studies have looked at variation both within individual virus isolates and among isolates of the same virus species. Variation provides the basis for evolution of traits through natural selection, and has resulted in adaptation of plant viruses to new hosts, to new vectors, and to overcoming host resistance, including natural resistance, resistance introgressed through breeding, or genetically engineered resistance.

8.4.1 Quasispecies

The high levels of mutation generated by viral polymerases leads to high levels of variation within a single infection, known as a quasispecies. The term quasispecies refers to a single replicating population, and is an “individual” that selection acts upon (Holland and Domingo 1998). Do to the many different selective pressures an RNA virus experiences (different hosts, cell tropisms, vectors, etc.), a population that is more genetically robust, having a high degree of evolvability may have a selective advantage. This means in a fitness landscape, quasispecies that have a narrow fitness peak (less robust) experience a sharp decrease in fitness due to a single mutation, and are less likely to adapt rapidly to a new environment. Conversely, those with a wide fitness peak (more robust) will experience a small change in fitness, allowing for multiple mutations to accumulate for selection to act upon. This is known as the survival of the flattest (Wilke 2005).

It was been thought for some time that the lack of error correction within the RNA dependent RNA polymerase (RdRp) of RNA viruses is responsible for the size of the quasispecies (Steinhauer et al. 1992). However, in coronaviruses it is clear that error correction can occur (Denison et al. 2011). The quasispecies is effected by not only the mutation rate of the RdRp, but also by the mode of replication, logrithmic or stamping machine (Safari and Roossinck 2014). While double-stranded RNA viruses replicate predominantly by the stamping machine method, the mode of replication of other RNA viruses is not clear. Although the mutation rate of RNA viruses are high, the level of variation within the quasispecies may be lower than expected (García-Arenal et al. 2003). This is due to both positive and negative selection; however, defective genomes are often carried in the population and can provide extended function in some cases. While there are significant genetic bottleneck during systemic infection (Li and Roossinck 2004), as well as vector transmission (Ali and Roossinck 2010), viruses probably recover their diversity rapidly.

The size of the quasispecies, or level of variation of a virus within a host, is dependent on factors in both the virus and the host. When comparing three related Sindbis-like viruses, CMV, *Tobacco mosaic virus* (TMV), and *Cowpea chlorotic*

mottle virus each had significantly different levels of variation within the same host background (Schneider and Roossinck 2000). In both TMV and CMV, the level of variation changed based on the host background (Schneider and Roossinck 2001). Different strains of CMV, Fny and LS, display different levels of diversity in tobacco and pepper plants, which maps to both the 1a and 2a proteins (Pita and Roossinck 2013b). By using a non-coding satellite RNA the indel fidelity of the CMV RdRp was analyzed *in planta*. While insertion mutations were rare, deletion mutations were more abundant and their rates differed based on the host background and the sequence context (Pita et al. 2007).

NGS is being used to identify minor variants within quasispecies. With a level of coverage of 2500x, the full range of variation can be uncovered (Simmons et al. 2012). This type of analysis can lead to answers to previously difficult questions of quasispecies dynamics in nature. Mutations within the ZYMV quasispecies were maintained through the aphid vector transmission, as well as seen throughout the plant, suggesting that the bottleneck of vector transmission and movement throughout the plant may be lower than previously thought (Simmons et al. 2015). There is evidence that some variants within a quasispecies are necessary for specific functions. Several ZYMV variants were found in different seed transmitted lines, suggesting that these variants have a role to play in seed transmission (Simmons et al. 2015).

Randomly generated point mutations in *Tobacco etch virus* (TEV) were used to determine the effect the mutations had on the virulence and fitness of TEV. The majority of the mutations were lethal, with the majority of non-lethal mutations leading to a significant reduction of fitness. (Carrasco et al. 2007). The lab strain of TEV is adapted to tobacco, but when TEV was adapted to pepper, virulence increased, but was found to decrease in the tobacco host, suggesting a tradeoff in becoming more specialized. No tradeoff was found for becoming more of a generalist (Bedhomme et al. 2012; Elena et al. 2008). Furthermore, pepper-adapted TEV acquires mutations that have a wide range of effects both positive and negative, implying pleiotropic effects. Interestingly, the fitness of mutants in the tobacco host does not predict the fitness in other non-native hosts (Lalic et al. 2011).

By passaging *Plum pox virus* (PPV; M strain) through several different host species for six years and analyzing the fixed mutations after host adaptation, it was found that peach yielded the lowest number of fixed mutations (two fold lower than other hosts). This suggests that PPV-M is highly adapted to peach (Vozárová et al. 2013). Passaging *Pepino mosaic virus* (*Alphaflexiviridae*) through several tomato cultivars with varying degrees of tolerance, convergently leads to isolates with higher pathogenicity. These passages also have an increase in the genetic diversity, with genetic diversity being a good indicator of pathogenicity (Minicka et al. 2015).

Previously it was thought that the high levels of variation within the begomoviruses (circular ssDNA) was due to high levels of recombination (Lima et al. 2013), but begomoviruses have substitution rates much higher than other DNA viruses, on the order of 10^{-4} substitutions/site/year, in line with rates seen in RNA viruses (Duffy and Holmes 2008). *Macrotidium yellow spot virus* (MaYSV) and *Tomato severe rugose virus* (ToSRV), both begomoviruses, were analyzed for their

variability. Interestingly, MaYSV, which primarily infects wild weeds, but does occasionally infect *Phaseolus vulgaris* (the common bean), had a greater diversity than ToSRV, which primarily infects tomato, and has a low incidence in wild plants. Several recombination events were detected for MaYSV, which drove the majority of the variability in the species.

8.4.2 Recombination

Recombination is not only an important part of population variation, but also can be used as a repair mechanism, balancing the high mutation rate of RNA viruses (Nagy and Simon 1997). Recombination is a frequent occurrence in CaMV, with over 50% of isolates being recombinants after only 21 days of infection (Froissart et al. 2005). RNA 3 of bromoviruses may contain recombination hotspots (Bruyere et al. 2000). While recombination is an important part of increasing the variation of a species, recombination events that lead to hybrid proteins are most likely less fit than recombinants between whole genes or protein domains (Bonnet et al. 2005). Recombination offers a path for the adaptation of viruses to a new environment as seen with the introduction of TYLCV into Spain (García-Andrés et al. 2007). Interestingly, it appears the eukaryotic hosts may have adapted a method for modulating or regulating the degree of recombination of their infecting viruses. In a yeast model system modified to allow infection by *Tomato bushy stunt virus*, XRN1, a host exoribonuclease, was found to degrade 5' truncated viral RNA. These truncated RNAs are substrates for recombination (Cheng et al. 2006). Recombination is commonly found in the ssDNA geminiviruses, both within and between different species (Padidam et al. 1999; Pagán and Holmes 2010). *Citrus tristeza virus* (CTV, *Closteroviridae*) is interesting in that many strains of the virus are commonly found within a single host plant. These strains have been phylogenetically analyzed to elucidate their evolutionary history, and it can be inferred that the current diversity of CTV was influenced by the original ancestral diversification, selection pressure of genes between and within strains, and significant recombination among strains (Harper 2013).

A number of studies have looked at recombination frequencies in experimental systems (Bujarski 2013; Sztuba-Solinska et al. 2011), especially in the *Bromoviridae*. In general, recombination frequencies are high in RNA viruses, and hot spots for recombination have been identified that result in exchanges between related RNA molecules, or in deletions leading to defective RNAs. In the cucumoviruses experimental infections with interspecific reassortants have frequently led to recombinants in RNA 3, where the 3' end is exchanged with that of RNA 2, presumably to establish a minus strand promoter that is cognizant with the replicase (Aaziz and Tepfer 1999; deWispelaer et al. 2005; Pita and Roossinck 2013a). In a recent study different strains of CMV had very different frequencies of recombination. Interestingly, the high recombination strain was the same strain that had low mutation frequency, and the 2a protein that encodes the RdRp was responsible for both phenotypes (Pita et al. 2015).

8.5 Plant Virus Deep Evolution

The deep evolutionary history of viruses is a matter for considerable speculation. Since no virus fossils are available, studies have relied on comparisons of extant sequences; however, the recent explosion of complete genome information for plants and many other hosts has led to the development of a new field of virology known as paleovirology, which considers the viral sequences integrated into genomes as molecular fossils (Katzourakis 2012).

8.5.1 Origins of Plant Viruses

The majority of known plant viruses are RNA viruses. The origins of RNA viruses are thought come directly from the ancient RNA world, a time before cellular life, where RNA replicated itself without a DNA phase (Bernhardt 2012). It was proposed three decades ago that animal and plant viruses have a common ancestor, likely an insect virus (Goldbach 1986). Specific motifs in virus hallmark genes (genes which are unique to viruses and are found across many families of viruses) such as the RdRp were analyzed for similarity across a wide range of animal and plant RNA viruses, with positive, negative, and double-stranded genomes. Motifs in both the positive and double-stranded RNA viruses suggests that these groups are monophyletic, with the negative strand RNA viruses less likely to be monophyletic (Koonin et al. 2015). Virus hallmark genes are shared with other selfish genetic elements, such as plasmids and transposons, suggesting that viruses have a deep lineage, which some suggest dates to a pre-cellular time (Koonin and Dolja 2014). One can think of viruses as both an organism and a mobile genetic element, much as light can be thought of as both a particle and a wave. These entities evolve within their environment, and then move as genetic elements through higher organisms (Forterre and Prangishvili 2013). Indeed examples of viral elements being incorporated into their host's genome can be found across all of the tree of life (Katzourakis 2012).

A high degree of recombination and/or reassortment of genetic material allows for modular evolution, where genes, protein motifs, or separate RNA molecules will evolve independently from each other. Within the luteoviruses, recombination is often seen near the gene borders of the RdRp and the coat protein (CP) genes but rarely within genes. This suggests that the genetic histories of these genes are independent of each other (Pagán and Holmes 2010). Phylogenetic analysis of CMV genes implies that each of the viruses three RNA segments have unique histories (Roossinck 2002). The extent of movement of genetic material across viruses can be seen with the recently named *Amalgaviridae*, a double-stranded RNA monopartite virus with 2 open reading frames, an RdRp and another gene. The RdRp most closely resembles that of another double-stranded RNA virus family, the *Partitiviridae*, while the other gene resembles that of the nucleoprotein of negative-stranded RNA viruses of the *Bunyaviridae* family (Krupovic

et al. 2015). The iconic movement protein common and unique to plant viruses, may have been originally derived from the structural proteins used in the formation of the plasmodesmata (Lucas and Wolf 1993).

While there is a lot of movement of genetic material between viruses and their eukaryotic hosts, this movement is vastly biased towards viral genes being moved to their hosts; hence viruses have a major role in the evolutionary history of higher organisms (Forterre and Prangishvili 2013). It is extremely difficult to extract preserved viral nucleic acid from more than a few decades ago, though it is possible (Roossinck, unpublished results). Because of this difficulty, these rare integration events can be used to elucidate the life histories of the virus. Long before so many virus sequences were found in genomes, geminivirus sequences were found within the *Nicotiana* genome (Bejarano et al. 1996). Begomoviruses have been described as being Old world or New world, with several distinct qualities associated with each group. Most notably, the New world begomoviruses are monopartite while the Old world are bipartite. Using the intergration events within *Nicotiana*, an estimate of 20–30 MYA was found for the Old/New world split, suggesting that this virus crossed the Beringian land bridge (Lefeuvre et al. 2011). Cooperation of viruses in mixed infections may be the initial step towards multipartite viruses. Two different monopartite viruses are known to cooperate, using each other's proteins for their own function. If the relationship of the two viruses becomes too dependent, essential gene loss can occur in one or both viruses that may remain viable due to the complementary gene of the other virus. This will eventually lead to merging the two species into a single species (Shirogane et al. 2013).

8.5.2 Early Speciation

A recent phylogenetic analysis of the potyvirus genus suggests that the genus diverged approximately 7250 years ago, in monocots from the Southern Eurasia or Northern African regions (Gibbs et al. 2010). Some ZYMV lineages have been shown to be no older than 800 years old, suggesting that humans had a role to play in their movement and diversification (Simmons et al. 2008). Luteoviridae diversification happened in three stages; The luteo/polerovirus split estimated at 2000 years ago, diversification of each genus estimated at 1000–5000 years ago, and the diversification of the species within the past 300 years (Pagán and Holmes 2010). The knowledge gained from extensive sampling including wild samples give us more insight into the life histories of viruses (Wylie et al. 2008). Potyviruses have a large amount of diversity within and between their species. *Yam mosaic virus* (YMV) is thought to have been originated in Africa. High levels of recombination are found within natural populations of YMV (Bousalem et al. 2000). The endogenization of Caulimoviruses distantly related to *Rice tungro bacilliform virus* into the rice genome have given us insight into the family's evolutionary history. The endogenization events occurred before the divergence of the domestic rice progenitor *Oryza rufipogon*, placing this event at about 160,000 years ago (Chen et al. 2014).

8.6 Conclusions

The diversity of plant viruses is still largely unknown, but what we have learned is that the virus diversity of agriculture systems is vastly different than that of natural ecosystems. While the majority of viruses infecting crops cause disease, it appears that viruses in natural areas are neutral or may provide some small benefit to their hosts. This paradigm shift opens the door to many future applications, as well as exciting implications to the field of virology as a whole. Understanding the mechanisms and consequences of movement across the agro-eco boundary, as well as an increased understanding of the mechanisms underlying virus evolution, may provide us with methods of predicting future epidemics, or attenuating the outbreak of new crop pathogens. The modern era of genomics is revealing new and exciting areas of research into virus evolution, and studies on the origins of viruses will likely lead to an understanding of the very origins of life on earth.

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Chapter 9

Plant Virus-Vector Interactions: More Than Just for Virus Transmission

Clare L. Casteel and Bryce W. Falk

Abstract Viruses dominate the biota on our planet, infecting animals, plants, humans, and even other microbes. Accumulating evidence has demonstrated that the relationships viruses share with other organisms are quite diverse, ranging from detrimental to even mutualistic. The nature of these relationships is particularly important for viruses that depend on other organisms for dissemination in the environment, as is the case for most plant-infecting viruses. Increased interest in vector-virus interactions has determined that some relationships may be synergistic, where the vector and virus proliferate to a greater extent together versus alone, and should be considered a mutualism. However, despite more than 100 years of research on plant viruses, the mechanisms that mediate synergistic relationships with vectors are still poorly understood. Here we review modes of vector transmission by plant infecting viruses and highlight recent evidence on mutually beneficial virus-vector relationships and the mechanisms that contribute to them. Finally we discuss the future directions that will need to be addressed in the field of virus ecology and virus-vector interactions.

9.1 Introduction

Viruses are the most abundant microbes on our planet. Viruses are found infecting animals, bacteria, archaea and plants, and in some cases even viruses have viruses. Viruses are intracellular, molecular obligate parasites, and for survival they must have the means to efficiently encounter and then infect a susceptible host, from which they must again be dispersed. Many virus hosts, such as animals, and even some prokaryotes, have motility. They can move, and by doing so they can encounter, or even spread viruses. By contrast, plants are not motile. Thus, plant-infecting viruses have evolved to have an efficient means to spread among their

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plant hosts, and most plant-infecting viruses use specific vectors to accomplish this. A vector is defined as “the regular and specific agent which transmits the pathogen”. This definition is critical, for in all cases vector-mediated plant virus transmission results from specific interactions between the virus and vector. A number of different types of vectors are known including some fungi, nematodes and insects, but plant-feeding insects are by far the most common.

There are more than 2000 species of plant viruses recognized now and these represent several distinct taxa (Hull 2014), and insects from several different taxa are recognized as plant virus vectors (Table 9.1). Most plant viruses are transmitted by phloem-feeding hemipterans including aphids, leafhoppers, planthoppers and whiteflies (Fig. 9.1), although many different types of insects can transmit viruses to their plant hosts (Granier et al. 1993; Huet et al. 1994). Evidence for virus:vector specificity was shown clearly even in very early studies when it was determined that particular plant viruses were only transmitted by some, but not all similar insects (Ando 1910; Pirone and Blanc 1996; Nault 1997; Gray and Gildow 2003; Ng and Falk 2006; Hogenhout et al. 2008; Blanc et al. 2014; Gray et al. 2014). Further, evidence has clearly demonstrated virus-encoded “transmission proteins” are critical and serve to facilitate the specificity of the process. Successful virus-vector interactions are thus determined by the genetic determinants of both players. Many recent articles have described different aspects of virus-insect-vector interactions (Ng and Falk 2006; Hogenhout et al. 2008; el Ammar et al. 2009; Blanc et al. 2011, 2014; Gray et al. 2014; Whitfield et al. 2015), and those who are interested are encouraged to examine these for information that cannot be presented here.

It is obvious that plant viruses benefit from these interactions, transmission to new host plants ensures virus survival, and viruses encode for proteins that ensure transmission by specific vectors. However, new accumulating information shows that this is not a one way street. Not only does the virus gain the ability to be transmitted to new host plants, but in many cases, the interaction can also benefit the vector. In this chapter we will (1) present some basic background information on virus-insect vector interactions, (2) discuss newly accumulating evidence supporting virus-vector mutualisms, and (3) discuss gaps in knowledge that must be addressed in future research.

9.2 Virus-Vector Transmission Relationships

The most common vectors of plant viruses are phloem-feeding hemipterans including aphids, whiteflies, leafhoppers, and planthoppers (Fig. 9.1) (Nault 1997). The two primary descriptors used to refer to how plant viruses operationally interact with their specific insect vectors are “noncirculative” and “circulative”, but each of these categories is divided further. Noncirculative refers to relationships where the virus does not enter the insect vector body as part of the transmission process, but the virus particles (virions) are retained in the stylet or foregut region. Those that are retained briefly, perhaps only for minutes, are referred to as being transmitted in

Table 9.1 Transmission relationships and representative examples of several Hemipteran-transmitted plant viruses

Transmission relationship	Virus Taxa ^a	Species example ^b	Vector example	Transmission protein(s)
Noncirculative: nonpersistent	<i>Potyvirus</i> ^b	<i>Potato Y potyvirus</i>	<i>Myzus persicae</i>	Capsid protein and HC-Pro
	<i>Cucumovirus</i>			
	<i>Alfamovirus</i>			
	<i>Caulimovirus</i>			
Noncirculative: semipersistent	<i>Crinivirus</i> ^b	<i>Lettuce infectious yellows crinivirus</i>	<i>Bemisia tabaci</i>	Minor capsid protein, CPm
	<i>Closterovirus</i>			
	<i>Waikavirus</i> ^c			
	<i>Badnavirus</i> ^c			
Circulative: nonpropagative	<i>Polerovirus</i> ^b	<i>Potato leafroll polerovirus</i>	<i>Myzus persicae</i>	Capsid protein and capsid protein read through
	<i>Luteovirus</i>			
	<i>Enamovirus</i>			
	<i>Umbravirus</i> ^d			
	<i>Begomovirus</i>			
	<i>Mastrevirus</i>			
	<i>Cutrovirus</i>			
	<i>Betacurtovirus</i>			
	<i>Eragrovirus</i>			
	<i>Turncurtovirus</i>			
	<i>Nanovirus</i>			
	<i>Babuvirus</i>			
Circulative: propagative	<i>Phytoreovirus</i> ^b	<i>Rice dwarf phytoreovirus</i>	<i>Nephotettix cincticeps</i>	Virion protein P2
	<i>Fijivirus</i>			
	<i>Oryzavirus</i>			
	<i>Tenuivirus</i>			
	<i>Tospovirus</i> ^e			
	<i>Nucleorhabdovirus</i>			
	<i>Cytorhabdovirus</i>			

^aShows the majority of plant virus taxa (genera) showing the specific transmission relationship indicated in the first column. Some taxa are not listed because data are not clear regarding their transmission relationship. Others may simply be missed

^bIndicates genus of example in next three columns

^c*Rice tungro badnavirus* and *Rice tungro spherical virus* are transmitted together by their leafhopper vector, but *Rice tungro spherical virus* is a helper virus for vector transmission of *Rice tungro badnavirus*

^dUmbraviruses are only transmitted by aphid vectors when they co-infect plants and interact with co-infecting poleroviruses

^eTospoviruses are not transmitted by hemipteran vectors, but by specific thrips vectors (Whitfield et al. 2005)

a noncirculative:nonpersistent manner, and aphids are the only known vectors of viruses transmitted in this fashion. Those viruses that are noncirculative, but retained by their respective vectors for days (generally 2–7 days) are referred to as being transmitted in a noncirculative:semipersistent manner, and different

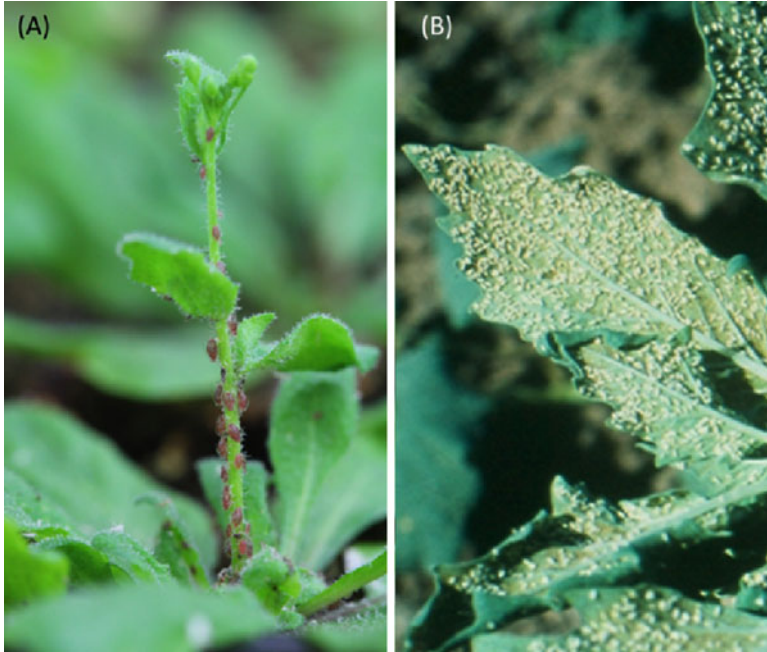


Fig. 9.1 Major vectors of plant viruses. (a) Aphid (*Myzus persicae*). (b) Whitefly (*Bemisia tabaci*)

viruses fitting this descriptor have different types of hemipteran vectors. In contrast, viruses that are transmitted in a circulative manner, pass beyond the foregut into the insect intestine and enter the body as part of the transmission process. Viruses can either only circulate (circulative:nonpropagative) or multiply (circulative:propagative) within the insect vector body, and many can be retained for the life of the insect vector. These types of transmission relationships are found for different viruses with different hemipteran (and in some cases thrips) vectors.

9.2.1 *Noncirculative Transmission of Plant Viruses*

9.2.1.1 *Noncirculative:Nonpersistent Virus Transmission*

Aphids are currently the only recognized vectors that transmit plant viruses in a nonpersistent manner, and both virus acquisition and inoculation are determined in part by aphid behavior. Viruses are acquired and then subsequently inoculated to plants when the aphid vectors first land and then “sample” the plant by probing; inserting their stylets into leaf epidermal and/or mesophyll cells. Probing and virus acquisition or inoculation can occur in less than a minute. Furthermore, retention of the virus is transient; a viruliferous aphid can lose its ability to transmit the specific virus after subsequent probing on only one or two plants. These properties led to

some referring to viruses that are transmitted in a nonpersistent manner as being “stylet borne”. We now know that in fact, at least some are specifically retained within the aphid stylet. The aphid vector behavioral activities that result in spread of viruses that are transmitted in a nonpersistent manner also have important epidemiological implications. Spread is generally relatively local, only a short distance from inoculum sources, and aphid vectors do not have to colonize and feed on plants to acquire or inoculate viruses. Thus, virus spread can be very rapid within susceptible crop plants.

A great amount of research has led to the identification of many of the virus-encoded transmission proteins that facilitate the acquisition and retention of plant viruses that are transmitted by aphids in a nonpersistent manner. It is not surprising that plant viruses have evolved different mechanisms to specifically interact with their vectors and thereby ensure their transmission to plant hosts. For both *Cucumber mosaic virus* (CMV) and *Alfalfa mosaic virus* (AMV) the virus particle (virion) can be purified from plants and mixed with artificial diets, and aphids can acquire the virus from these solutions and subsequently transmit it to host plants. By contrast, for the potyviruses (genus *Potyvirus*, Family: *Potyviridae*) and *Cauliflower mosaic virus* (CaMV) even though purified virions are shown to be intact and highly infectious, virions alone are not sufficient for aphid vectors to acquire and subsequently transmit these viruses to plants. Thus, viruses such as CMV and AMV use a “capsid strategy” for their aphid transmission, but the potyviruses and CaMV require infectious virions plus additional proteins. These are non-virion, virus-encoded proteins called “helper components” (Pirone and Blanc 1996), and serve to help bind the virions to the receptors in the aphid stylet.

9.2.1.1.1 The Capsid Strategy – CMV

CMV is a very widespread and economically important plant virus. Purified CMV virions can be mixed in an artificial diet solution, fed to aphid vectors and subsequently transmitted to plants. The CMV virions most likely bind to receptors in the aphid stylet (see Fig. 9.2) and are subsequently released during probing and inoculation to plant hosts. Perry and colleagues have examined in much more detail CMV coat protein amino acids that affect aphid vector transmissibility (Perry et al. 1994; Perry et al. 1998; Ng et al. 2000, 2005). By making specific recombinant mutants, they identified several capsid protein amino acids which affected aphid transmissibility. Some mutations within the CMV coat protein affected transmission by *Aphis gossypii*, and others affected transmissibility by *Myzus persicae* (Perry et al. 1994). These results suggest that specific amino acid sequences have greater effects on CMV transmissibility by individual aphid species and that corresponding aphid genetic determinants must exist. Mutations that affected virion capsid stability also affected vector transmissibility, including amino acids not even exposed on the virion surface (Ng et al. 2000, 2005). These interactions have recently been reviewed and the reader is encouraged to look there for more specific information (Ng and Falk 2006).

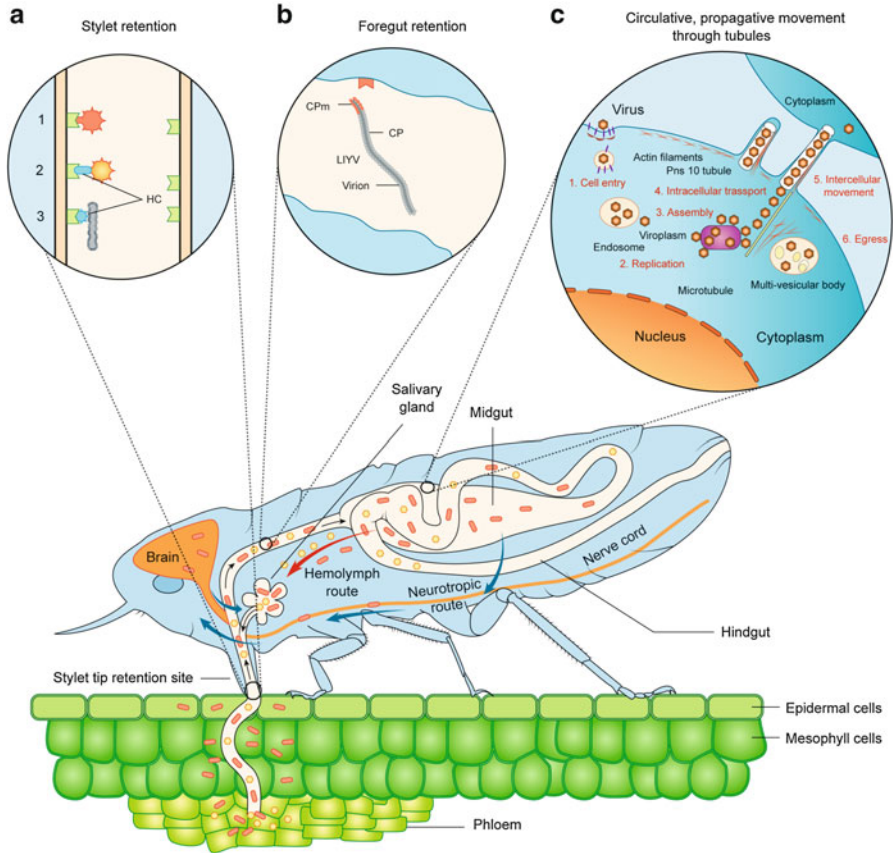


Fig. 9.2 Viruses localize to different sites in their respective insect vector depending on their modes of transmission. Non-circulative viruses bind to the insect stylet (a) or foregut (b). Non-propagative circulative (yellow circles) viruses are generally phloem-limited and move through the insect body via the midgut or hindgut. Circulative viruses use a hemolymph route to reach the salivary glands. In contrast, circulative propagative viruses (red ovals) enter the insect at the anterior region of the midgut and/or filter chamber region. Propagative viruses use a hemolymph route and others such as the Rhabdoviruses use a neurotropic route to reach the salivary glands. Propagative viruses replicate in the midgut cells and other insect tissues. Some propagative viruses are phloem-limited while others are widely distributed in plant tissues. Reoviruses use tubules to move cell to cell in the midgut and another uses the tubular structure to traverse the basal lamina (c). The salivary glands are the final destination for circulative transmission from where they can be inoculated to plant hosts: Magnification of an insect stylet showing the proposed site of virion attachment at the tip of the stylet in the common duct region (a). Numbers designate the different strategies for virion binding and retention in the stylet: capsid strategy, direct binding of coat protein to the aphid stylet as for *Cucumber mosaic virus* (1), helper component strategy for *Cauliflower mosaic virus* (CaMV), the CaMV-encoded P2 proteins serves as a “bridge” between the virion and the aphid stylet (2) and potyviruses, the HC-Pro binds to the aphid stylet and to the virion (3). *Inset b*: Magnification of the foregut retention site and proposed capsid binding strategy used by Criniviruses. The minor coat protein (CPm) is the viral attachment protein (VAP) serving to bind virions to the whitefly vector foregut. *Inset c*: The steps in the reovirus infection cycle and spread to adjacent cells modeled on *Rice dwarf virus*. *Rice dwarf virus* enters cells using the endocytic pathway and after virion release from the vesicle the replication cycle begins (Taken from Whitfield et al. 2015 with permission)

9.2.1.1.2 The Helper Strategy – Potyviruses

Potyviruses and CaMV are examples of viruses that use a helper strategy for their aphid transmissibility, although mechanistically the strategies used by potyviruses and CaMV are quite different. Potyviruses are very widespread and economically important plant viruses. For potyviruses (genus *Potyvirus*) the virus-encoded HC-Pro is a multifunctional protein; it is a proteinase that cleaves itself from the polyprotein, it is a robust suppressor of the plants anti-viral RNA interference defense, and it is the helper component protein that binds potyvirus virions to aphid stylets and facilitates their aphid transmission. Aphids must acquire the helper component before or simultaneously with acquisition of virus particles. This suggests that the helper component most likely binds to receptors in both the aphid stylet, and to potyvirus virions. This has led to use of the term “bridge hypothesis” (Pirone and Blanc 1996) describing the helper component as the “bridge” between the aphid stylet and the potyvirus virion (see Fig. 9.2). Interestingly, the helper components from some potyviruses are more “permissive” than others. That is, these helper components can serve as helpers for the aphid transmission of other potyviruses (Pirone and Blanc 1996). This has important ecological implications as it might serve to increase virus transmission efficiency, or allow for transmission by additional species of aphid vectors.

The potyvirus-encoded molecular determinants of the stylet:HC-Pro:virion capsid interactions are very well studied (Pirone and Blanc 1996). It was first noticed by comparison of potyvirus capsid protein sequences, that the majority of those capsid proteins studied contained a DAG amino acid sequence relatively close (~10–15 amino acids) to the amino terminus if they were from aphid transmissible potyvirus isolates. Subsequent specific mutational analysis showed that these amino acids were critical for successful potyvirus transmission by aphid vectors (Harrison and Robinson 1988; Atreya et al. 1990, 1991). However, some aphid non-transmissible isolates also contained the specific DAG sequence positioned in the proper location, but were not aphid transmissible. Further analyses showed that most of these had mutations in the other virus-encoded participant of the transmission process, the HC-Pro (see below).

The HC-Pro has been shown to contain two motifs, or short stretches of amino acids that are important for interaction with the potyvirus virion CP and with the aphid stylet (Atreya et al. 1992; Atreya and Pirone 1993; Granier et al. 1993; Huet et al. 1994). These sequences are the KITC sequence, located at approximately amino acids 65–68 within ca. 460 amino acids of HC-Pro. The second is the sequence PTK located at ca. amino acids 313–315 in the HC-Pro. *In vitro* binding assays have been used to show that potyvirus CP and HC-Pro bind, and even a minimal amino acid sequence of DTV DAGK is sufficient to bind to HC-Pro. Thus, two potyvirus-encoded proteins required for virus transmission by aphid vectors have been identified (Pirone and Blanc 1996; Ng and Falk 2006). However, we still lack information for which aphid receptors are involved in the transmission process, and regarding how the virions are released from viruliferous aphids in order to infect plant hosts.

9.2.1.1.3 The Helper Strategy – CaMV

The helper strategy for CaMV aphid transmission is more complex than that for potyviruses, as three CaMV-encoded proteins are required. CaMV virions are structurally complex T=7 icosahedra with a capsid composed primarily of the P4 protein, but within the capsid there are also molecules of the CaMV-encoded P3 protein. However, virions alone are insufficient for CaMV transmission by aphid vectors (Lung and Pirone 1974), the CaMV-encoded P2 is also required. P2 was identified early on as the helper component for CaMV aphid transmission (Woolston et al. 1987). Deletions of the region encoding P2 resulted in CaMV mutants that still made virions and were able to infect plants, but were non-aphid transmissible. Like the potyvirus HC-Pro, CaMV P2 serves to bind virions to aphid stylets. P2 interacts with the P3 protein, which is embedded within the capsid, however the capsid is primarily composed of the P4 protein (Plisson et al. 2005; Hoh et al. 2010). Thus, like for potyviruses and HC-Pro, P2 is the “bridge” for CaMV virions to be retained in aphid stylets.

Recent work has also shown that the acquisition of CaMV by aphid vectors is a very dynamic process. Within CaMV- infected plants almost all of the CaMV-encoded P2 protein accumulates in the cell cytoplasm in “transmission bodies”, or TBs (Bak et al. 2013; Martinière et al. 2013). When aphids probe CaMV-infected plant cells, the TBs “sense” aphid activity and P2 traffics on cell microtubules towards the wound site (Bak et al. 2013; Martinière et al. 2013). Sometimes virions are carried along with P2, or they can be separately acquired, but it is suggested that CaMV perceives its vector presence and takes action to ensure its acquisition by that vector. Although biological data and electron microscopic analyses suggest that potyviruses and CaMV are “stylet-borne” by their aphid vectors, the exact receptors are not known. However for CaMV, it has been demonstrated that the P2 protein binds specifically to glycosylated proteinaceous receptors located in the stylet tip, a region named the acrostyle (Uzest et al. 2010).

9.2.1.2 Noncirculative:Semipersistent Virus Transmission

Plant viruses that have semipersistent transmission relationships with their insect vectors are found among many different taxa, and various insects including aphids, whiteflies, or leafhoppers depending on the virus serve as vectors. Like the viruses transmitted by aphids in a nonpersistent manner, some of the semipersistent transmitted viruses use a helper strategy, and some use a capsid strategy. Unlike the viruses which are transmitted in a nonpersistent manner and are acquired and inoculated by their vectors during probing, the great majority of viruses that are transmitted in a semipersistent manner require vector feeding for acquisition. Once acquired, the virus also can be retained for up to several days, but there is no evidence that viruses which are transmitted in a semipersistent manner can be retained if the vector undergoes a molt.

Although there are many plant viruses that are transmitted in a semipersistent manner, so far only for *Lettuce infectious yellows virus* (LIYV) is there an understanding of how the virus interacts with its vector. LIYV is transmitted by the sweet potato whitefly, *Bemisia tabaci* biotype A, in a semipersistent manner, and viruliferous whiteflies can retain the ability to transmit LIYV to plants for up to 3 days (Duffus et al. 1986). LIYV uses a capsid strategy for its transmission by *B. tabaci*. However, the LIYV virions are structurally complex polar filamentous particles, containing four LIYV-encoded proteins. One of the virion capsid proteins, the CPM or minor capsid protein is localized only on one end of the virus particle. Several lines of evidence have shown the CPM is the LIYV-encoded whitefly vector transmission determinant (Tian et al. 1999; Chen et al. 2011).

Purified LIYV virions in artificial diets can be fed to *B. tabaci* vectors and then subsequently transmitted by them to plants. However, when purified LIYV virions are pre-treated with antisera to each of the individual capsid proteins separately, only the CPM antiserum blocked LIYV transmission by the *B. tabaci* vector (Tian et al. 1999). Further, when CPM production was prevented using mutations introduced into the LIYV genome, virion-like particles still formed (lacking CPM) and the LIYV infection still spread systemically in *N. benthamiana* plants (Medina et al. 2005; Stewart et al. 2010), but the resulting LIYV virion-like particles were not transmissible by *B. tabaci*. Chen and colleagues (Chen et al. 2011) then performed detailed studies on LIYV accumulation/localization within vector whiteflies. They showed that LIYV virions, and the CPM alone, bound specifically in the vector *B. tabaci* and not in non-vector *B. tabaci* biotypes. This binding was in a specific region of the anterior foregut (See Fig. 9.2). Although for many plant viruses we know some of the determinants involved in virus-vector interactions and where the virus particles may bind, we do not know how viruses are released and transmitted to plants. The localization of LIYV in the whitefly anterior foregut has implications as to how LIYV may be transmitted to plants by *B. tabaci*. Initial salivation by *B. tabaci* upon first encountering a plant would not allow for LIYV to be inoculated into the plants as the salivary canal is distinct from the foregut region of the food canal where LIYV virions are retained. Thus, it is more likely that LIYV is transmitted to plants when the viruliferous vector *B. tabaci* egests or regurgitates after encountering the phloem.

In contrast to what is seen for LIYV, some other semipersistent-transmitted viruses are believed to use a helper component to facilitate their vector transmission. One of these is *Maize chlorotic dwarf virus* (MCDV), genus *Waikavirus*. MCDV is transmitted predominantly by the leafhopper, *Graminella nigrifrons*, but the molecular determinants of its transmission are not yet identified (Stewart 2011). MCDV virions accumulate in the foregut after acquisition but exactly how is currently unknown. Evidence for the necessity of a helper component is that purified virions alone are non-transmissible if fed to leafhoppers via an artificial diet. However, if leafhoppers first feed on a MCDV-infected plant, they then can acquire and transmit the purified MCDV virions to plants. The MCDV genome has been sequenced and several encoded proteins identified. So far, none have been conclusively identified as the putative helper component (Stewart 2011) although efforts are underway.

9.2.2 *Circulative Transmission of Plant Viruses*

In contrast to viruses transmitted in a noncirculative manner, the plant viruses with circulative transmission relationships must enter the vector body via the gut, pass through specific tissues and ultimately reach the salivary glands from which they can be egested back into host plants for inoculation. Both virus acquisition and inoculation occur mostly during vector feeding, and mostly from the phloem. Plant viruses with circulative transmission relationships have a finite time period between when the vector acquires the virus and when it can subsequently inoculate it to plants. All of the viruses with this type of transmission relationship utilize a capsid strategy (or at least virion structural proteins) for interacting with and thus being acquired by vectors, and receptors for virion interactions are in the insect vector gut. Once acquired, viruses that are transmitted in a circulative manner can be retained often for the life of the insect vector. Epidemiologically this suggests that the potential for long distance virus spread is great. Some circulative-transmitted plant viruses circulate within the insect body, they do not infect and replicate within the insect vector, but some others do infect and replicate within the vector tissues. Thus, these transmission relationships are divided into circulative:nonpropagative and circulative:propagative, respectively.

9.2.2.1 *Circulative:Nonpropagative Virus Transmission*

The most well studied plant viruses with this type of vector transmission relationship are the aphid-transmitted luteoviruses (Family: *Luteoviridae*) and the ssDNA geminiviruses (Family *Geminiviridae*), and of the latter, the whitefly-transmitted begomoviruses (genus *Begomovirus*) are the best known. An excellent, in depth review of the intricacies determining successful circulative:nonpropagative virus transmission by aphid and whitefly vectors is available (Gray et al. 2014). The authors discuss in detail interactions for both of the above systems, here we will only refer to luteovirus transmission by aphid vectors.

Luteovirid (the name used here to refer to all viruses in the family *Luteoviridae*) transmission by aphid vectors is relatively specific. In some cases only one or a very few aphid species are known to be vectors of a given luteovirid (Gray et al. 2014). Successful acquisition of luteoviruses by aphid vectors requires feeding on phloem tissues. After ingestion, the luteovirid virions first interact with specific receptors in the aphid hindgut or midgut, depending on the virus and/or vector. Electron microscopic studies suggest that virions endocytose across the gut membrane and are transported and released into the aphid hemolymph (Gildow 1993). Once in the hemolymph the virions must circulate and make their way to the accessory salivary glands, where again receptor-mediated endocytosis likely transports virions and allows for their release into the salivary duct where they can then be inoculated into plants when the feeding aphid salivates in phloem tissues. Exactly how this circulation occurs is not known, and there is some evidence suggesting that a

protein (called symbionin) secreted by aphid endosymbiotic bacteria of the genus *Buchnera*, binds to virions while in the hemolymph and plays a role in the circulation process [discussed in (Gray et al. 2014)], although this is still questionable. What is known is that there is a finite time between when aphid vectors acquire the luteovirid by feeding on the virus-infected source plant and when they can subsequently transmit the virus into a healthy plant. This time period, often measured in hours, is the latent period.

Luteovirid virions also can readily be acquired by aphids feeding on an artificial diet containing purified virions (Duffus and Gold 1965), thus suggesting that the virion capsid contains the determinants for interacting with the aphid vector and subsequent transmissibility. The luteovirid virion capsid is a $T=3$ icosahedral structure, but is composed of two related species of capsid protein. One of these is the predominant form in the capsid and is referred to as the CP. However the second protein, which occurs in lesser amounts, contains the full CP amino acids at its N terminus, but also an extended sequence of amino acids, and this protein is referred to as the CP-RT (coat protein-readthrough). Both of these proteins appear to play roles in the aphid vector:luteovirid interactions that result in virus transmission to plants. Mutational analyses have shown that luteovirid virions composed completely of CP can be generated (Tian et al. 1995), and some studies show that virions containing only CP can cross the “gut barrier” and enter the hemolymph, but the CP-RT is required for luteovirids to be efficiently transmitted to plants by their vector aphids (Brault et al. 1995; Wang et al. 1995; Chay et al. 1996; Gray et al. 2014; Whitfield et al. 2015).

Most plant-virus-vector transmission studies are done using pure virus and insect vector cultures. However, in nature, plants are often infected by more than one virus and many types of vectors may visit a given plant. In some instances, the co-infecting viruses interact within the plant and one or both of the co-infecting viruses can show altered, or even gained, aphid transmissibility. This is particularly true for mixed infections of luteovirids, and in some cases, unrelated viruses that co-infect plants with specific luteovirids.

Yellow dwarf disease of cereals is caused by several related luteovirids. William Rochow showed in 1970 that yellow dwarf disease mixed infections can result in altered aphid transmissibility of one of the co-infecting viruses (Rochow 1970). In that study Rochow showed that when plants were doubly infected with *Barley yellow dwarf virus* – MAV (BYDV-MAV, genus *Luteovirus*), which is efficiently transmitted by the aphid *Sitobion avenae*, and *Cereal yellow dwarf virus* – RPV (CYDV-RPV, genus *Polerovirus*), which is efficiently transmitted by *Rhopalosiphon padi*, some of the resulting BYDV-MAV progeny became transmissible by both aphids (Fig. 9.3a). The BYDV-MAV that was transmissible by *S. avenae* was of the BYDV-MAV serotype, the virion capsid was composed of BYDV-MAV capsid proteins (Fig. 9.3a). By contrast, the BYDV-MAV that was transmissible by *R. padi* was of the CYDV-RPV serotype, having capsids of CYDV-RPV capsid proteins (Rochow 1970). The interpretation from these studies is that in the doubly infected plants some of the progeny BYDV-MAV RNA became encapsidated by CYDV-RPV capsids and thus gained transmissibility by

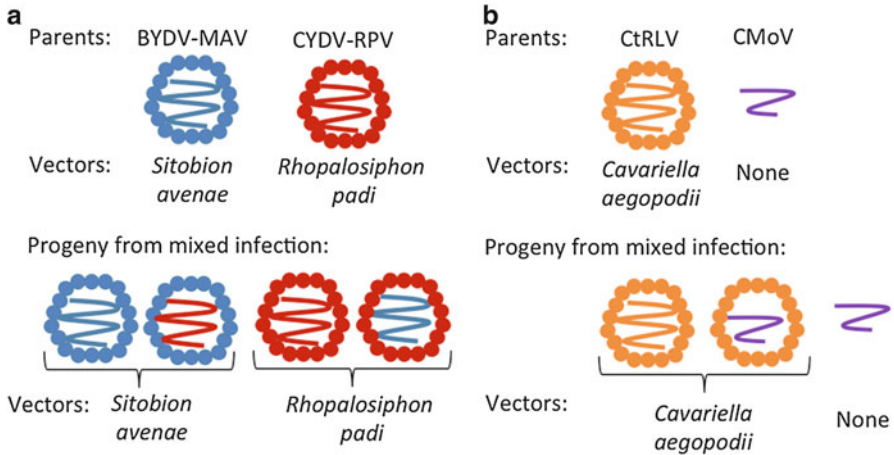


Fig. 9.3 Transcapsidation: mixed virus infections, structural interactions and altered aphid vector transmissibility. (a) depicts possible transcapsidation interactions between related luteovirids (BYDV-MAV in blue and CYDV-RPV in red) and the resulting effects on aphid transmission of progeny virions. Virions are indicated by the outer ring of capsid proteins (*small circles*) and the corresponding genomic RNA, represented by the *line* within the *ring*. BYDV-MAV is transmissible by *Sitobion avenae*, and CYDV-RPV is transmissible by *Rhopalosiphon padi*. When these co-infect the same plant cell it is possible to generate progeny virions where the RNA of one co-infecting parent becomes encapsidated in coat proteins from the other (the virions that are red and blue). When the BYDV-MAV is encapsidated with capsids derived from CYDV-RPV, the chimeric virions are transmitted by *R. padi*. (b) depicts mixed infections between a polerovirus (*Carrot red leaf virus*, CtRLV) and an umbravirus (*Carrot mottle virus*, CMoV). Umbraviruses such as CMoV do not encode a capsid protein. In mixed infections with a polerovirus (e.g. CtRLV), some of the progeny CMoV RNAs become encapsidated within capsids composed of CtRLV coat proteins, and as a result gain transmissibility by the CtRLV aphid vector, *Cavariella aegopodii*

R. padi. This was termed genomic masking or transcapsidation and subsequent studies confirmed these results (Creamer and Falk 1990). These types of interactions are not found only in laboratory studies, but also in nature, and have very important epidemiological implications. These allow for luteovirus transmission by the “wrong” vector, which in turn could allow for virus spread to new plants that are not normally visited by the “right” vector.

This gain of aphid transmissibility is not limited to co-infecting luteovirids. In fact, some viruses of other taxa also are able to take advantage of transcapsidation. These viruses lack the ability to be aphid transmitted when they infect plants alone, but by being in mixed infections with specific luteovirids, they gain the respective luteovirus capsid and aphid vector transmissibility (Fig. 9.3b). Two types of plant viruses are known to do this: viruses of the genus *Umbravirus*, and specific sub-viral RNA replicons which for lack of better nomenclature have been referred to as “luteovirus-associated RNAs” (Murant et al. 1969; Falk et al. 1979; Falk and Duffus 1981, 1984; Chin et al. 1993; Passmore et al. 1993; Watson et al. 1998). Neither of these types of viruses encode for a capsid protein. For aphid transmission

to new plants they rely exclusively on being in plants also infected by specific luteovirids. They gain the luteovirid capsid via transcapsidation and thereby gain aphid transmissibility (Fig. 9.3b).

9.2.2.2 Circulative: Propagative Virus Transmission

As this descriptor suggests, some plant viruses propagate within their insect vectors. Thus, the vector is also a virus host. Plant viruses of three recognized families, plus some from other currently non-affiliated genera, show this type of transmission relationship (Table 9.1). The three families, *Rhabdoviridae*, *Reoviridae* and *Bunyaviridae* also contain members that are transmitted to vertebrate hosts by blood-feeding insects and these viruses infect both their vertebrate and invertebrate hosts, the latter also being the vector. Typically the vector acquires the plant virus by feeding on the virus-infected plants and the virus then enters the vector body, usually via virion interactions with gut receptors. The virus must then multiply and spread within the vector body tissues and the infection must reach the salivary glands before the vector can subsequently transmit the virus to new plant hosts. This time period between acquisition and the ability to transmit the virus is the incubation period and is measured in days (Ng and Falk 2006). There is a great deal known concerning the specific interactions for a few viruses that are transmitted in this manner. A number of different reviews describe these interactions and aspects of how plant-infecting viruses that are transmitted in a circulative-propagative manner interact with their insect vectors (hosts) (Whitfield et al. 2005, 2015; Hogenhout et al. 2008; el Ammar et al. 2009) and readers are encouraged to go to these for more specific information.

9.3 Virus-Vector Interactions Benefit Both Partners

For most plant viruses, transmission by insect vectors is essential, the virus has to move to a new host in order to survive (Ng and Perry 2004). Thus, one could argue that there is likely strong evolutionary pressure for viruses to manipulate plant-insect-vector interactions so as to optimize their own transmission (Sisterson 2008; Ingwell et al. 2012). In support of this, various studies suggest that vectors are more attracted to plants infected with some viruses as compared to healthy plants (Mauck et al. 2012). Increased attractiveness means that virus-infected plants are more likely to be chosen by a vector when settling. In this way, plant viruses are more likely to be acquired and then transmitted and thus the virus benefits by influencing plant chemistry and vector behavior.

Recent studies have also shown that virus infections in plants can also benefit the insect vector (Mauck et al. 2012; Casteel et al. 2014). *Turnip mosaic virus* (TuMV, genus *Potyvirus*) is transmitted in a noncirculative:nonpersistent manner using the helper strategy (Table 9.1). TuMV infection suppresses plant defenses and increases the free amino acid nutrients in virus-infected host plants, increasing

insect vector development rate and reproduction when feeding on these plants (Table 9.2) (Casteel et al. 2014). Furthermore, aphid vectors prefer TuMV- infected plants and rapidly reproduce until crowded, promoting dispersal and then transport of TuMV from these plants. Consequently, insect fecundity is improved on TuMV-infected hosts and there are greater numbers of viruliferous aphid vectors to spread TuMV to new host plants. The vector and virus are thus able to proliferate to a greater extent together as opposed to alone, and this relationship represents a critical and mutually beneficial interaction. Despite the ecological and agricultural importance of interactions between viruses, vectors, and hosts, few studies have addressed the molecular mechanisms, which mediate virus-vector mutualisms.

Just as viruses utilize diverse vector transmission mechanisms, strategies to promote transmission will also differ across virus-vector interactions. Thus, not all virus-vector-plant relationships will be mutualistic. For example, CMV is transmitted in a noncirculative: nonpersistent manner using a capsid strategy (Table 9.1). Like for TuMV, aphids are attracted to CMV-infected squash plants (*Cucurbita pepo*), however, CMV infection reduces quality of this host plant for the aphid vector and aphids disperse rapidly after contacting the plant (Mauck et al. 2012). However, as CMV aphid transmission is nonpersistent, increased aphid contact, probing, and dispersal will benefit CMV through virus spread, but it will not benefit the aphid vector. Thus, this CMV-aphid relationship would not be considered a virus-vector mutualism. For a review on the diversity of impacts of virus infection on vector-plant interactions see (Mauck et al. 2012). In the following sections we will review current knowledge of the mechanisms mediating virus-vector mutualistic relationships.

9.3.1 *Compromising Plant Defenses as a Mutual Goal*

When a viruliferous vector encounters a plant, the plant must recognize, prioritize, and mount the most appropriate response to both the vector and the virus. Plants prosper despite these multiple challenges, largely due to the arsenal of defenses they possess and the sophisticated surveillance systems they utilize to coordinate responses (Jones and Dangl 2006; Howe and Jander 2008). However, viruses and insects share the common goal of colonizing the plant and they have evolved methods to actively compromise plant perception and/or defense responses. Recent studies suggest that both the virus and vector benefit during dual attack through combined efforts resulting in compromised plant defenses (Eigenbrode et al. 2002; Belliure et al. 2005; Mauck et al. 2010, 2012; Casteel and Jander 2013; Casteel et al. 2014b; Li et al. 2014).

While defenses vary widely across plant species, the phytohormones that regulate their production are relatively conserved and thus represent excellent targets for compromising plant defenses. Numerous studies have demonstrated that at least three phytohormones – jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) – have major roles in orchestrating plant defense responses to insects and viruses

Table 9.2 Examples of Potential Virus-Vector Mutualism across Transmission Relationships

Transmission relationship	Virus	Vector	Benefit for vector	Viral protein	Host target (s)	Citations
Noncirculative; nonpersistent	<i>Turnip mosaic potyvirus</i>	<i>Myzus persicae</i>	Increased fecundity	Nla-Pro	?	Casteel et al. (2014) and Casteel et al. (2015)
Noncirculative; semipersistent	<i>Maize chlorotic dwarf virus</i>	<i>Graminella nigrifrons</i>	Increased survival and weight gain	?	?	Hunt and Nault (1990)
Circulative; nonpropagative	<i>Tomato yellow leaf curl begomovirus</i>	<i>Bemisia tabaci</i>	Increased growth rate	βC1	MYC2, ASI	Zhang et al. (2012), Li et al. (2014), Shi et al. (2014), and Song et al. (2014)
Circulative; propagative	<i>Tomato spotted wilt virus</i>	<i>Frankliniella occidentalis</i>	Increased fecundity	?	?	Maris et al. (2004), Stafford et al. (2011), and Abe et al. (2012)

(Bari and Jones 2009; Erb et al. 2012; Pieterse et al. 2012). In general, SA signaling is critical for defense responses against a wide range of pathogens, including viruses (Glazebrook 2005; Carr et al. 2010), while JA and ET signaling have been implemented in defense responses to numerous insect herbivores, including vectors of plant viruses (Howe and Jander 2008; Mauck et al. 2014).

Modulation of hormone composition, timing, and concentration directs the plants' responses to each attacker (Mur et al. 2006; Verhage et al. 2010). Hormones can have antagonistic or synergistic impacts on one another. For example, induction of JA-signaling pathways often negatively influences SA-signaling (Doares et al. 1995; Mur et al. 2006; Leon-Reyes et al. 2010a, b). However, if SA is induced prior to JA, JA's ability to inhibit SA is prevented (Koornneef et al. 2008). Viruses exploit JA-SA antagonisms for their own benefit and these antagonisms benefit the insect vectors that transmit them. *Tomato spotted wilt virus* (TSWV) is transmitted in a circulative:propagative manner (Table 9.1). TSWV induces SA accumulation and SA-regulated defenses, leading to suppression of JA-related defenses during infection. JA signaling mediates defense responses to the western flower thrip (*Frankliniella occidentalis*), an important vector of TSWV. Consequently this insect prefers to feed on TSWV-infected plants and produce larger populations when feeding on infected plants (Table 9.2) (Abe et al. 2012). The specific molecular mechanisms mediating TSWV-thrips vector mutualisms are largely unknown, though evidence is accumulating that manipulation of phytohormones by viruses and vectors may mediate these (Li et al. 2014; Mauck et al. 2014; Song et al. 2014; Casteel et al. 2015).

9.3.2 *Viral Encoded Proteins*

How can a virus manipulate plant signaling and defenses so as to benefit both itself and its insect vector? Viruses encode a limited number of proteins that must interact with the host plant directly to fulfill their infection cycles and likely also alter plant signaling and defense in the process. Despite recent technological advancements and the seemingly simple genomes of plant viruses, few viral proteins have been identified as mediating vector-virus dynamics (Maule et al. 2002; Whitham and Wang 2004; Nelson and Citovsky 2005). This may be due to the multi-functional nature of viral proteins, where secondary functions in vector-virus interactions have been previously overlooked (Urcuqui-Inchima et al. 2001). For example, viruses encode for silencing suppressor proteins that function to inhibit plant RNA interference defenses and promote successful virus infection (Lewsey et al. 2010; Ziebell et al. 2011). These silencing suppressors or other unidentified viral proteins may also mediate virus-vector mutualisms.

Recent studies have identified several viral proteins that alter phytohormones in a way that may contribute to virus-vector mutualisms. For example, *Cucumber mosaic virus* (CMV), increases vector reproduction on CMV-infected tobacco (*Nicotiana tabacum*) as compared to healthy control plants. Lewsey

et al. expressed the CMV-encoded silencing suppressor, 2b, in plants and determined that it disrupts JA-mediated defense responses in *Arabidopsis thaliana* (Lewsey et al. 2010). In addition, plants infected with a CMV 2b deletion mutant were more aphid-resistant, suggesting that 2b is required for enhanced aphid performance (Ziebell et al. 2011) and implicating it in the mutualism.

In another mutually beneficial interaction, the aphid vector, *M. persicae*, is more fecund on TuMV-infected plants compared to uninfected control plants (*A. thaliana* and *N. benthamiana*) (Table 9.2) (Casteel et al. 2014). TuMV infection suppressed aphid-induced callose deposition, an important plant defense response to aphid feeding, in host plants. Using transient expression of individual viral proteins, it was determined production of the TuMV protein, NIa-Pro (Nuclear Inclusion a – Protease domain), was responsible for inhibition of host plant defenses and increased *M. persicae* reproduction. In contrast to what was seen for CMV and tobacco plants, JA signaling does not appear to be the main target of NIa-Pro. Experiments inhibiting ET perception and biosynthesis prevented NIa-Pro from suppressing plant defenses and increasing insect performance on host plants. These findings suggest that NIa-Pro disrupts ET signaling during TuMV infection, thereby enhancing aphid performance (Casteel et al. 2015). Although virus infection has long been known to increase ET production, no generalized role of ET in plant-virus interactions has been established (van Loon et al. 2006; Love et al. 2007; Endres et al. 2010; Mauck et al. 2014).

9.3.3 Host Targets of Viral Proteins

Correct functioning of a plant cell requires orchestration of thousands of genes to direct the most appropriate response to the numerous external and internal signals received. Plant viruses as obligate parasites carry out major disturbances to the cellular balance of the host by interacting directly with plant targets, such as specific proteins. Many examples of interactions between viral proteins and plant proteins have been reported (Ishibashi et al. 2010). Some have even attempted to take a systems level approach to understanding virus-plant interactions, constructing networks of every plant protein-viral protein interaction for a plant-virus system (Elena and Rodrigo 2012). However, mechanistic and systems level approaches have largely ignored insect vectors in plant-virus interactions and important protein interactions may have been overlooked.

In an elegant series of experiments, two host proteins have been identified which mediate plant interactions with *Tomato yellow leaf curl China virus* (TYLCCNV) and its whitefly vector (*Bemisia tabaci*). Whiteflies have increased population growth on TYLCCNV-infected plants as compared to healthy controls, suggesting that TYLCCNV-infected plants have reduced defense responses. TYLCCNV is transmitted with a betasatellite pathogenicity factor, β C1. Expression of β C1 in host plants suppresses plant signaling and related defense responses. Additionally, whiteflies show higher growth on plants expressing the β C1 encoded protein as

compared to control plants (Table 9.2) (Yang et al. 2008; Zhang et al. 2012). Host proteins were identified that directly interact with the β C1 protein and mediate suppression of plant signaling and defense responses. The β C1 protein interacts with the ASYMMETRIC LEAVES1 (Yang et al. 2008), which suppress JA signaling, and with the transcription factor *MYC2*, compromising activation of additional plant defense responses (Yang et al. 2008; Li et al. 2014).

9.4 Conclusions and Future Directions

Insect vector-mediated transmission of plant viruses is not only critical for virus dispersal, it also benefits the insect vectors resulting in mutualistic interactions (Eigenbrode et al. 2002; Belliure et al. 2005; Mauck et al. 2010, 2012). Here we have reviewed aspects of plant virus-vector interactions, highlighted recent evidence on virus-vector mutualisms, and discussed the mechanisms that contribute to them, however, many unanswered questions persist. The majority of plant-infecting viruses have been studied in relation to their ability to cause disease in agriculturally important crops, which often represent recent plant-virus associations. Viruses also exist in natural ecosystems, where viruses have likely co-evolved with plant partners before agricultural associations. Vectors represent the means of transport for viruses between natural and agricultural systems, and additional studies dissecting these interactions in both settings are needed.

Despite the fact that insect vectors transmit most plant infecting viruses, viruses and the functions of the proteins they encode have largely been studied in isolation of their insect vectors. Yet, numerous studies have focused on virus-plant interactions. In this way, key functions and biology may have been overlooked. Viral-encoded proteins that mediate changes in plant chemistry and changes in insect biology and behavior will likely be identified for many systems in the future. A challenge that remains is characterizing the corresponding host and vector targets of these viral proteins. For example the role of CP and HC-Pro in virus-vector transmission is known (Table 9.1), yet little is known about the aphid's genes or proteins that mediate the viral adhesion site on the vector or subsequent release of the virus. Recent innovations and reduced costs in genomics, transcriptomics, and proteomics applied in combination with conventional molecular technologies will facilitate rapid advances in this area.

While many studies have observed changes in insect performance on virus-infected plants and changes in plant chemistry during infection, few studies have actually dissected the mechanisms mediating these changes. Furthermore, viruses can also directly influence insect behavior and biology (Stafford et al. 2011; Rajabaskar et al. 2013; He et al. 2015). Viruses transmitted in a circulative manner, have to pass through various membranes in the insect body. Some studies have demonstrated that viruliferous vectors probe and bite more, which may increase virus transmission and nutrient acquisition for the insect vector (Stafford et al. 2011; He et al. 2015). The molecular basis for these changes in insect behavior

has remained elusive and various vector-encoded targets mediating changes in vector biology and behavior need to be identified.

Increased interest in virus-vector mutualisms is evident in the recent literature (Ingwell et al. 2012; Mauck et al. 2012, 2014; Stafford et al. 2012; Martinière et al. 2013; Li et al. 2014; Musser et al. 2014; Shi et al. 2014; Westwood et al. 2014; Casteel et al. 2015; He et al. 2015) and this trend is likely to continue. As evident in Table 9.2 virus-vector mutualisms exist independent of transmission modes and may be common. Yet, very few systems have been investigated, making generalizations difficult. For example *Thrips palmi*, the melon thrips, transmits the tospovirus *Watermelon silver mottle virus* (WSMV) in a circulative:propagative manner (Table 9.1) and like TSWV, melon thrips are more attracted to WSMV-infected plants and develop faster when reared on infected plants (Abe et al. 2012; Chen et al. 2014). Thrips–Tospovirus mutualisms may be widespread, however currently there are limited data from other systems and the mechanisms mediating these interactions remain unknown. As greater numbers of virus-vector interactions are examined and research is aided by increased use of molecular genetics and genomics, identifying potential mechanisms and defining ecologically important relationships will continue to constitute an important area of research. However, many unanswered questions and knowledge gaps remain.

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Chapter 10

Cross Protection of Plant Viruses: Recent Developments and Mechanistic Implications

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Abstract Cross protection is a well-known phenomenon occurring in virus-infected plants. It is traditionally defined as the protection gained by the plant hosts against infections by more severe virus isolates, as a result of pre-infection with a mild isolate/variant/strain of the same virus. New research during the last 15 years revealed that cross protection completely blocks the multiplication of the secondary viruses in the cells pre-occupied by the mild variant through a novel mechanism that is unrelated to RNA silencing. Reviewing reports of both plant and animal viruses unearths striking similarities between cross protection and superinfection resistance, the latter being a conserved process shared among diverse viruses infecting plants, animals, and humans. These studies further suggest that cross protection/superinfection resistance is a virus-encoded function conferred by one or a few virus-encoded proteins, acting on a step after the translation of viral gene products from the secondary viral genome. A better understanding of the underlying mechanism is expected to lead to improved control of viral diseases through targeted manipulation of viruses.

10.1 Introduction

The term cross protection is used by both animal and plant virologists, but with somewhat different meanings. For animal virologists as well as immunologists, cross protection refers to the fact that immune responses induced by one vaccine or

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virus (or other pathogens) can sometimes protect the immunized (or infected) individuals against other related pathogens. A good example of poor cross protection in that sense is vaccines against influenza viruses, which typically target only a few of the many serotypes of a given influenza virus, making it difficult to prevent flu through vaccination alone.

By contrast, we all know plants have neither adaptive immunity, nor circulating immune cells such as macrophages, T cells, or B cells. Therefore, the first obvious difference would be that cross protection in plants does not involve adaptive immunity. Although there has been some disputes as to whether innate immunity (e.g. RNA silencing) plays a role in cross protection occurring in plants, more recent data do not support a major contribution of RNA silencing to this process (see later).

This article focuses solely on the cross protection occurring in plants infected by plant viruses. Historically, plant viral cross protection has been defined as the protection afforded to host plants by the infection of a mild isolate of a virus (frequently described as “pre-inoculum” or “protector”). As you may have guessed, the protection specifically prevents subsequent infections (known as “secondary infections” or “challengers”) by a more severe isolate of the *same* virus. Cross protection was first described by McKinney in 1929, but was soon confirmed by many other authors working with multiple plant viruses (McKinney 1929). Readers are referred to the excellent review by Ziebell and Carr (2010) for a more detailed historical recount. Our article shall instead dissect the new developments during the last 15 years. We hope that by interrogating a number of key new discoveries and their implications, we can shed some lights on the underlying mechanisms of cross protection, and recommend a few approaches to test these possible mechanisms.

10.2 Recent Developments

10.2.1 *In Cross-Protected Plants, the Secondary Virus Does Not Accumulate*

Earlier reports of cross protection relied almost entirely on the observation of symptoms to distinguish between the mild isolate used as the protector and the more severe field challengers, as the determination of genetic identities of these isolates were not possible until late 1980s. This symptom-centric approach has many drawbacks. For example, one is not entirely clear whether the protector itself consists multiple genetic variants, some of which might cause more severe symptoms under different field conditions. Indeed some of the earlier cross protection-inducing strains are known to cause diseases themselves in different plants or under different temperature conditions.

More seriously, different viruses often induce similar symptoms in plants. Therefore, if a given protector isolate fails to protect the plants from subsequent, more severe virus infections, there could be at least three possibilities. (i) The

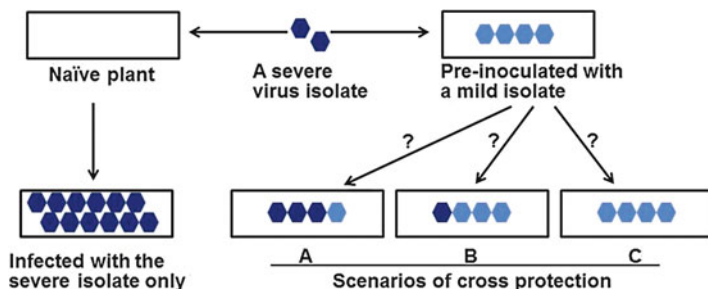


Fig. 10.1 Potential outcomes of cross protection. On the *left*, plants not pre-inoculated with a mild protector viral isolate (naïve plant) permit high level accumulation of a severe virus isolate, accompanied by serious symptoms. On the *right*, plants pre-inoculated with a mild protector isolate develop mild symptoms upon infections by a severe viral isolate. However, until recently it was unclear whether both mild and severe isolates co-exist in cross-protected plants. Recent reports identify “C” as the most frequent outcome

pre-inoculation did not succeed in all plants, which could happen with large plants like papaya plants or citrus trees. (ii) The protector strain could have evolved into, or out-competed by a more severe variant. (iii) The plants became infected by a different virus or even multiple viruses that cause similar symptoms. The third possibility can become even more damaging if synergy occurs between the new virus(es) and the protector virus, leading to symptoms that are more severe than either virus alone, often resulting in the death of the infected plants.

Finally, in plants where cross protection did occur as expected, it was unclear until recently whether the protector virus acts to keep the multiplication of the challenger virus at lower levels insufficient to cause severe symptoms, or it completely excludes the challenger virus (see Fig. 10.1). With the availability of viral genome sequences and infectious viral cDNA clones containing singular, clearly defined sequences, researchers have now resolved this last question with several virus models. The cereal-infecting wheat streak mosaic virus (WSMV) has two closely related isolates, referred to as Type and Sidney 81, for which infectious cDNA clones are available, and their sequences can be distinguished with restriction enzyme digestions of their respective cDNAs. Taking advantage of these WSMV isolates, Hall and colleagues (2001) established that pre-inoculation of either Type or Sidney 81 isolate completely abolished the subsequent infection of Sidney 81 or Type, respectively, in most plants. This study provides the first molecular evidence of complete exclusion of challenger virus by protector virus.

Separately, using variants of cucumber mosaic virus (CMV) that can be easily differentiated by sizes of RT-PCR amplified fragments, Ziebell and colleagues also showed that most of the cross-protected plants contained only the protector variants (Ziebell et al. 2007; Ziebell and Carr 2009). Furthermore, they found that in the few plants in which the challenger variant was detected, the protector and challenger were spatially separated from each other in the co-existing leaves. These results lend further support to the idea that the protector virus cross-protect host plants against challengers by denying the challengers the chance to multiply in the plants.

Similar conclusion was also reached for citrus tristeza virus (CTV) with a CTV variant tagged with a green fluorescent protein (GFP; Folimonova et al. 2010; Folimonova 2012). Here the authors established that incorporation of the GFP cDNA into that of a CTV isolate (T36) resulted in an infectious cDNA clone that could cause systemic infections in susceptible varieties of citrus trees, lighting up the vascular bundles with evenly distributed green foci. However, pre-inoculation of the trees with untagged T36, or other closely related CTV isolates, completely abolished the subsequent infection of the GFP tagged CTV. More recently, complete exclusion of secondary viruses has also been observed as the consequence of cross protection in two more examples, namely pepino mosaic virus (PepMV) and turnip crinkle virus (TCV) (Chewachong et al. 2015; Zhang et al. 2015). Together these results indicate that in cross protection, the protector protects the infected plants from secondary infections by completely blocking the multiplication of the challenger viruses, as depicted in Fig. 10.1, Scenario C. This conclusion has implications in fathoming the underlying mechanism of cross protection.

10.2.2 Cross Protection Is Unrelated to RNA Silencing

RNA silencing is an RNA-targeting defense system conserved in most eukaryotic organisms. RNA silencing-based defense in plants enlists a complex set of proteins to combat intracellular parasites including viruses, retrotransposons, and other highly repetitive genome elements (reviewed by Ding and Voinnet 2007). RNA silencing is commonly triggered by intracellular occurrence of double-stranded RNA (dsRNA) or partially double stranded stem-loop RNA, which are processed by Dicer-like (DCL) nucleases into small RNAs of discrete sizes (21–25 nucleotides [nt]) referred to as small interfering RNAs (siRNAs). siRNAs then serve as sequence-specificity determinants of RNA-induced silencing complexes (RISCs), directing Argonaute (AGO) proteins to complementary RNA or DNA, silencing corresponding viral or plant genes.

In theory RNA silencing could be the ideal mechanism for cross protection. As we discussed earlier, cross protection is most effective when the protector and the challenger are closely related. Indeed, earlier experiments appear to support this assertion (Ratcliff et al. 1997; Baulcombe 2005). However, several lines of new evidence dispute an active role of RNA silencing in cross protection. First, if RNA silencing is at work, then siRNAs produced from the protector genome would be able to target both protector itself as well as the challenger, leading to reduction of genomic RNA levels for both. As a result, one should be able detect both genomes, with the protector genome decreased to lower levels upon challenger invasion (depicted in Fig. 10.1, Scenarios A and B). However, in cross protection typically only the protector genome is detected (see above).

The most definitive decoupling of cross protection from RNA silencing came from use of mutant *Arabidopsis* plants defective in RNA silencing as a result of knocking out multiple DCL genes implicated in antiviral silencing (e.g. *dcl2 dcl4* double knockouts, or *dcl2 dcl3 dcl4* triple knockouts). RNA silencing-mediated defense against CMV in *Arabidopsis* is strongly suppressed by the CMV-encoded

2b protein. As a result, a mutant CMV in which 2b is inactivated (CMV Δ 2b) becomes a milder pathogen than wildtype (wt) CMV. Nevertheless, CMV Δ 2b could infect *Arabidopsis* plants systemically and exerts limited protection against subsequent invasion of wt CMV (Ziebell and Carr 2009). Significantly, this cross protection between CMV Δ 2b and wt CMV persisted or even strengthened in *dcl2 dcl3 dcl4* triple knockouts, thus clearly ruling out a prominent role of RNA silencing in cross protection. Note that while CMV Δ 2b caused mild disease in wt *Arabidopsis* plants, in *dcl2 dcl3 dcl4* triple knockouts it caused more severe symptoms indistinguishable from that of wt CMV, thus confirming the loss of RNA silencing-mediated defense in these plants.

Importantly, Zhang and colleagues (2015) recently confirmed the persistence of cross protection between TCV variants in *dcl2 dcl4* plants, thus strengthening the conclusion that, even though both cross protection and RNA silencing appear to depend on high levels of sequence similarity between the trigger and the challenger, cross protection manifests an independent novel mechanism that is distinct from RNA silencing.

10.2.3 Cross “Protection” Can Occur between Virus Isolates with Similar Pathogenicity

With the availability of tools that can distinguish the protector and challenger genomes at the molecular level, it becomes clear that cross protection occurs not only between a mild and a severe isolate of the same virus, but also between different viral isolates that cause symptoms of similar severity. This was made clear by the report of Ziebell and Carr (2009) showing that CMV Δ 2b and wt CMV caused similarly severe symptoms in *dcl2 dcl3 dcl4* triple knockout plants, yet still cross “protected” against each other. Similarly, the TCV variants used in the study by Zhang and colleagues (2015) were likewise similarly pathogenic. Importantly, similar mutual exclusion also occurs in field isolates with similar pathogenicity levels. For example, the Sidney 81 and Type isolates of WSMV are both field isolates that cause similar diseases in field grown wheat (Hall et al. 2001). Likewise, many of the CTV isolates tested by Folimonova and colleagues (2010) were field isolates that caused visible diseases in citrus orchards. Therefore, cross protection is likely a special situation of a more common mechanism that functions to ensure closely related viral variants do not multiply in the same plants.

10.2.4 Cross Protection Is Likely Manifestation of Superinfection Resistance

In examples of cross protection, the pre-existence of the protector variants in the plants shields the plants from invasion by challenger variants. Furthermore, in a small number of cases where the challenger variant did manage to co-exist with the

protector, there is ample evidence to suggest that they are spatially separated, meaning that they do not enter the same cells/leaf areas (Hall et al. 2001; Ziebell and Carr 2009). Nevertheless, it is unclear whether this spatial separation arose from cellular level expulsion.

However, other studies published during the same period of time suggest that closely related viral isolates exclude each other at the cellular level. In one of the earlier studies, Dietrich and Maiss (2003) found that plum pox virus (PPV) variants labelled with different fluorescent protein markers (GFP and RFP), when mixed in the same inoculum before being inoculated onto plants, could co-exist in the same leaves but each colonize its own cell clusters. Furthermore, these distinctly colored cell clusters can be next to each other, yet only a one-cell wide borderline could support the expression of both GFP and RFP. It was reasoned that this one-cell wide borderline represents cells that received both viral variants at about the same time. To put it differently, given a short time lag, cells that received one of the variants first would become resistant to the other variant.

This observation was subsequently confirmed with a number of diverse viruses. For example, Miyashita and Kishino (2010) found that soil-borne wheat mosaic virus (SBWMV) variants labelled with different fluorescent markers (YFP and CFP) likewise form non-overlapping cell clusters. Similar observations have been made for apple latent spherical virus (ALSV), tobacco mosaic virus (TMV) and turnip mosaic virus (TuMV) (Takahashi et al. 2007; Julve et al. 2013; Gutierrez et al. 2015). Finally, Zhang and colleagues (2015) established recently that cells pre-infected with TCV became inaccessible to a GFP-labelled TCV variant. Together these studies strongly suggest that variants of the same virus exclude each other at the level of individual cells. While other factors cannot be completely ruled out at this point, we propose that this cellular level exclusion provides adequate explanation for cross protection.

It should be noted that this cellular level exclusion, designated superinfection exclusion or superinfection resistance, is not unique to plant viruses. Indeed, it has been observed with many human and animal pathogenic viruses, including hepatitis C virus (HCV), bovine viral diarrhea virus (BVDV), West Nile virus (WNV), and the reverse-transcribing human immunodeficiency virus (HIV) (Nethe et al. 2005; Schaller et al. 2007; Tscherne et al. 2007, 2008; Zou et al. 2009). More recent studies reported examples of superinfection resistance occurring during infections of larger, double-stranded DNA viruses like herpesviruses and poxviruses (Kobiler et al. 2010; Laliberte and Moss 2014). This level of widespread functional conservation suggests that superinfection resistance, once better understood, could be targeted to treat virus diseases.

10.2.5 Cross Protection/ Superinfection Resistance Is Likely Determined by One or a Few Virus-Encoded Proteins

The discussions in the previous section established a strong mechanistic link between cross protection and superinfection resistance. However, it remains to be

resolved what made viruses or infected cells to resist subsequent invasion of the same or closely related viruses. For starters, the plant virus-based studies cited above did not even delineate the exact step of resistance: it could function to prevent the entry of the challenger virus, or interfere with the translation of viral replicase genes, or stopping viral replicases from engaging in productive replication.

Fortunately, studies with animal virus models of superinfection resistance provided some clues in this respect. Significantly, Tscherne and colleagues (2007) was able to map HCV superinfection resistance to a post-entry step that block the multiplication of secondary HCV variant after the translation of the polyprotein from the secondary HCV genome. Significantly, this finding was independently verified by Schaller and colleagues (2007). Separately, Zou and colleagues (2009) determined that resistance to superinfection by WNV inhibited the replication of the superinfecting WNV replicon. Therefore, at least for animal viruses with single-stranded, positive sense RNA genomes, the existing evidence supports the assertion that superinfection resistance occurred after the superinfecting virus entered the cells occupied by the primary viral variant.

Several studies further mapped the superinfection resistance function to one or a few viral proteins. For example, the study by Zou and colleagues (2009) was able to isolate WNV mutants that overcame superinfection resistance through serial passages, and mapped the mutations specifically responsible for overcoming superinfection resistance to the genome region that encodes the non-structural protein NS4a and the neighboring 2K peptide. Similarly, mutations in HCV genome that led to the subversion of superinfection resistance were also mapped to just three of the HCV-encoded proteins, with the mutation within NS5A having the most pronouncing effect (Webster et al. 2013). Therefore, it is probably not surprising that the superinfection resistance determinant of CTV was found to be one single protein encoded by this virus (p33; Folimonova 2012).

To summarize, new research published during the last decade not only linked cross protection to superinfection resistance, but also mapped the step of resistance to after the entry of the superinfecting (or challenging) viruses. Furthermore, a number of studies were able to map this function to a limited number of viral proteins that likely play important roles in viral replication. These new discoveries lay the foundation for further explorations aimed at a mechanistic understanding of both cross protection and superinfection resistance. In the next section, we will discuss the potential challenges faced by researchers, and suggest a few new approaches to solve this puzzle.

10.3 Possible Mechanisms

The evidence outlined above collectively suggests that superinfection resistance is primarily a mechanism encoded in viruses themselves. This in turn raises two important questions. First, considering superinfection resistance is strongest

when both the pre-existing virus and the challenge virus are very closely related, it is puzzling as to how viruses can distinguish between primary and secondary viral genomes. Second, even if we solve the question of “how”, it remains to be resolved as to what evolutionary advantages would such a mechanism confer to viruses.

Before we set out to answer these two questions, it is imperative for us to thoroughly evaluate the potential participation of possible host cell factors. Even though previous studies appear to discount a major role of the host cells in the process of cross protection or superinfection resistance, the possibility of host involvement cannot be completely ruled out. To test this, an *in vitro* system that recapitulates major aspects of superinfection resistance would be enormously helpful. Alternatively, for viruses from which a superinfection resistance determinant has been identified, it could prove to be informative to examine whether the determinant interacts with factors of host cells.

Next, to resolve the “how” question, it is important to determine the replication kinetics of the pre-existing virus (the protector in cross protection). Is it still replicating new genomes at the time of challenger entry? Is the replication linear or exponential? Do newly synthesized genomes reiterate the replication process? These questions become important if one considers the possibility that the challenger genome might be recognized as a progeny of the pre-existing virus.

Finding answers to these questions may also offer insights as to what evolutionary advantages would a virus gain by resisting secondary invasion of the same or closely related viruses. It is important for viruses to balance replication and spread. Superinfection resistance may be a manifestation of a viral mechanism that maintains such a balance.

10.4 Concluding Remarks

Cross protection occurring in virus-infected plants has been used for decades to control viruses associated with serious diseases of crop plants, including CTV and papaya ringspot virus (Gonsalves 1998; Folimonova 2013). Basic research during the last 15 years succeeded in establishing a strong link between cross protection and the well conserved superinfection resistance that accompanies infections of diverse plant, animal, and human viruses. A better mechanistic understanding of cross protection/superinfection resistance is expected to translate into improved management of viral diseases of plants and animals.

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Chapter 11

Research Advances in Geminiviruses

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Abstract Geminiviruses are a group of single-stranded DNA viruses that infect a broad range of crops and cause extensive losses worldwide. Their small, circular DNA genomes have limited coding capacities for 5–7 proteins. Consequently, essential life cycles of geminiviruses have to be supported by geminivirus-encoded proteins together with host factors. Recent findings have contributed significantly towards our understanding of the mechanisms for how a productive infection is established. This chapter offers a brief description of the biological functions of the viral proteins. The host factors involved in reprogramming plant cellular processes are also summarized.

11.1 Introduction

Viruses of the family *Geminiviridae* have circular, single-stranded DNA (ssDNA) genomes that are encapsidated in characteristic twinned icosahedral particles. Geminiviruses are rapidly becoming the major plant pathogens in tropical and subtropical countries, they are transmitted through insect feeding in the nature to infect a wide range of both monocotyledonous and dicotyledonous plants. The

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Geminiviridae is currently divided into seven genera, *Mastrevirus*, *Begomovirus*, *Curtovirus*, *Topocuvirus*, *Becurtovirus*, *Eragrovirus* and *Turncurtovirus*, on the basis of their genome organizations, host ranges, and insect vectors (Brown et al. 2015; Varsani et al. 2014). With the exception of the genus *Begomovirus*, most of the geminiviruses have a single genome component, encoding all the necessary information for virus replication, gene expression, particle encapsidation, and virus spread (Fig. 11.1). Begomoviruses have either bipartite genome components (DNA A and DNA B) or a monopartite genome component that is similar to the DNA A of the bipartite ones. For the bipartite begomoviruses, the movement function is coordinated by the nuclear shuttle protein (NSP) and the movement protein (MP) encoded by DNA B. Despite differences in the number of virus-encoded proteins, open reading frames (ORFs) in begomoviruses, curtoviruses, topocuviruses, and turncurtoviruses are oriented bi-directionally. They extend away from an intergenic region that contains essential regulatory elements for virus replication. Genes involved in viral replication and transcription are located on the complementary strand of the genomic component and are expressed at an early stage of viral infection. Others responsible for encapsidation and movement are located on the viral strand, and are synthesized during the late stage of viral infection. For mastreviruses, eragroviruses and becurtoviruses, bi-directionally transcribed genes are separated by a large intergenic region and a small intergenic region, which comprise replication origin and transcription termination signals, respectively (Fig. 11.1).

Geminiviruses replicate in the nuclei of infected plant cells through rolling-circle or recombination-dependent replication mechanisms. Due to their limited coding capacities, geminiviruses are exclusively dependent on host cellular machineries for replication and movement. A set of complex host regulatory pathways are reprogramed by geminiviruses during plant-geminivirus coevolution. As reviewed extensively by Hanley-Bowdoin et al. (2013), geminiviruses are reported to modify host cell cycle to create conditions suitable for their proliferation. They also regulate plant signaling pathways, ubiquitination and ubiquitination-like pathways, and counteract RNA silencing-based plant defense to achieve a productive infection (Hanley-Bowdoin et al. 2013). The above described functions rely on the crosstalk between geminivirus-encoded proteins and host factors. Many geminivirus proteins are proved to be multi-functional and interact with host factors to manipulate diverse cellular processes. In this chapter, we provide a summary and update of advances in the functions of geminivirus

Fig. 11.1 (continued) largest genus, have either monopartite or bipartite genomes designated DNA A and DNA B. Many monopartite begomoviruses are associated with alphasatellite or betasatellite. ORFs encoded by geminiviruses are represented by *arrows* and are named according to the DNA component and the DNA strand on which they are encoded (viral sense, V or complementary sense, C). Abbreviations: *LIR* long intergenic region, *SIR* short intergenic region, *CR* common region, *IR* intergenic region, *A-rich* adenine-rich region, *SCR* satellite conserved region. Note that for bipartite begomoviruses, the V2 ORF is present only in Old world begomoviruses. AC5 protein is present in some of the begomoviruses?

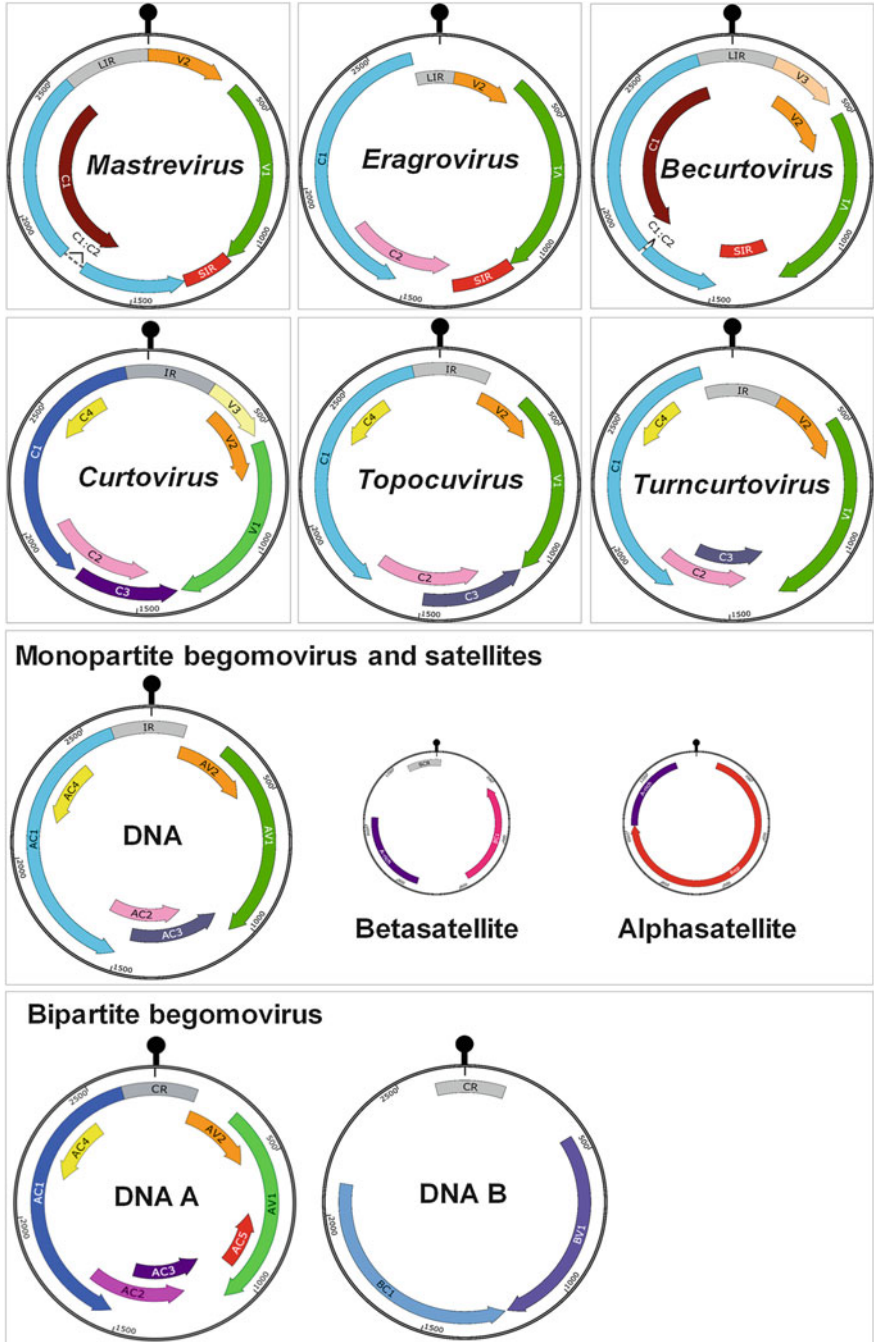


Fig. 11.1 Genome organization of geminiviruses. The family has been classified into seven genera: *Mastrevirus*, *Begomovirus*, *Curtovirus*, *Topocovirus*, *Becurtovirus*, *Eragrovirus* and *Turncurtovirus*. Except for some begomoviruses, which are composed of two genome components, all geminiviruses have a single genome component. Begomoviruses, which constitute the

proteins, especially the viral proteins encoded by members of the three well characterized genera, *Mastrevirus*, *Begomovirus* and *Curtovirus*. Increasing knowledge of the interactions between geminivirus proteins and host factors is also documented.

11.2 Functions of Virus Encoded Proteins

11.2.1 *ACI/CI*

Rep, the product of *ACI/CI* and the only viral protein essential for replication, is a multifunctional protein with site-specific nicking and ligation, DNA binding, ATPase and helicase activities that enable it to initiate, elongate and terminate rolling circle replication. A detailed geminivirus replication process has been reviewed (Hanley-Bowdoin et al. 2000; Gutierrez 1999; Jeske 2009). During this process, Rep interacts with a variety of cellular proteins, including those involved in geminivirus replication or modulation of the cellular environments.

Retinoblastoma-related proteins (RBs), key regulators of the plant cell cycle, have been reported to interact with Reps of several geminiviruses (Ach et al. 1997; Kong et al. 2000; Xie et al. 1996). It is thought that binding of geminiviral Rep proteins to RBR interferes with the interaction between RBR and E2F, allowing E2F-dependent transcription to occur and enforcing the cell cycle to go through M phase (Hanley-Bowdoin et al. 2013). In this context, proliferating cell nuclear antigen (PCNA) is activated and has been shown to directly interact with Rep, possibly to recruit the “sliding clamp” to the viral origin and the replisome (Bagewadi et al. 2004; Castillo et al. 2003). Rep also interacts with the large subunit of the replication protein C, which forms a complex with PCNA and assists in loading of PCNA onto DNA, and the subunit of replication protein A (RPA), a ssDNA-binding protein that down- and up-regulates the nicking and ATPase activities of Rep, respectively (Luque et al. 2002; Singh et al. 2007). Moreover, Rep binds to RAD51 and RAD54, which are involved in homologous recombination and might have a role in recombination-dependent replication of geminivirus (Kaliappan et al. 2012; Suyal et al. 2013).

In addition, Rep interacts with E2-SUMO-conjugating enzyme (SCE1). The silencing of SCE1 or the disruption of Rep-SCE1 interaction reduced viral DNA accumulation, indicating that this interaction is required for viral replication (Sanchez-Duran et al. 2011; Castillo et al. 2004). Furthermore, Reps of several geminiviruses have been identified to be responsible for the repression of plant maintenance DNA methyltransferases, METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) in both locally and systemically viral infected tissues, thus displaying suppression activities of transcriptional gene silencing (TGS) (Rodriguez-Negrete et al. 2013). Other Rep-interacting host factors include mitotic kinesin, geminivirus Rep-interacting kinase 1 (GRIK1) and GRIK2,

minichromosome maintenance protein 2, NAC domain-containing protein, and histone H3 (Hanley-Bowdoin et al. 2013), but the precise roles of these host proteins during geminivirus infection remains to be elucidated.

11.2.2 AC2/C2

AC2/C2 genes of begomoviruses and curtoviruses encode a key pathogenicity factor (Bisaro 2006). They were also reported to have roles in transcriptional control, RNA silencing, regulation of basal biosynthesis, and even in viral multiplication.

Originally, the AC2/C2 product of begomoviruses was known as a transcriptional activator protein (TrAP), since it is required for transcription activation of late viral genes and regulation of host genes. Unlike the canonical transcription factors, AC2 binds ssDNA in a sequence-independent manner and weakly binds double-stranded DNA (dsDNA). Thus, it is believed that its transcription activity relies on the interaction with a plant-specific DNA binding protein, PEAPOD2 (also known as TIFY4B), which forms a complex with AC2 at the coat protein (CP) promoter sequence and activate CP gene expression (Lacatus and Sunter 2009).

AC2/C2 is also well characterized for its silencing suppression activity in both post-transcriptional gene silencing (PTGS) and TGS. The detailed mechanism is rather complicated. Failure to bind siRNAs rules out the possibility that AC2/C2 suppresses PTGS by direct binding or sequestration of siRNAs. AC2/C2 might interact with or regulate the host factors involved in RNA silencing. Transcriptional profiling of *Arabidopsis* protoplasts infected with African cassava mosaic virus (ACMV) and mungbean yellow mosaic virus (MYMV) AC2 indicates that AC2 activates expression of *Werner exonuclease-like 1* (*WEL1*), which is capable of suppressing RNA silencing in *Nicotiana benthamiana* line 16c (Trinks et al. 2005). AC2 has been shown to interact with a calmodulin-like protein (rgs-CaM), an endogenous regulator of gene silencing (Yong Chung et al. 2014). Moreover, AC2 of mungbean yellow mosaic Indian virus (MYMIV) interacts with RDR6 and AGO1 to suppress siRNA biogenesis and abrogate the RISC activity, respectively (Kumar et al. 2015). AC2/C2 employs different strategies to evade TGS. It can specifically interact with and inhibit the activity of adenosine kinase (ADK), a nucleotide kinase that plays an important role in sustaining the methyl cycle and consequently S-adenosyl-methionine (SAM)-dependent methyltransferase activity (Wang et al. 2003). Beet severe curly top virus (BSCTV) C2 interferes with the host epigenetic defense by attenuating the activity of 26S proteasome-mediated degradation of S-adenosyl-methionine decarboxylase 1 (SAMDC1) (Zhang et al. 2011). Furthermore, AC2/C2 interacts with, and inhibits the H3K9 histone methyltransferase SUVH4/KYP to attenuate TGS (Castillo-Gonzalez et al. 2015; Sun et al. 2015).

AC2/C2 proteins have been found to modulate cellular pathways to increase infectivity (Lozano-Duran et al. 2012). C2 proteins can interact and interfere with SNF1-related kinase and COP9 signalosome, leading to changes in several

signaling pathways (Caracuel et al. 2012; Soitamo et al. 2012; Lozano-Duran et al. 2011; Hao et al. 2003). In a recent study, a new function of AC2 in replication control is reported (Krenz et al. 2015).

11.2.3 AC3/C3

AC3/C3, the replication enhancer protein (REn), is a geminivirus protein conserved in all begomoviruses, curtoviruses, topocoviruses and turncurtoviruses. Although not essential for virus replication, REn greatly enhances viral DNA accumulation during infection and thus indirectly influences the extent of symptom development (Fondong 2013). This replication-enhancing activity is possibly achieved by modifying the activity of Rep and/or aiding the recruitment of host replication enzymes. Several studies showed that REn interacts with itself (oligomerization), with C1, and with host proteins, PCNA, pRBR and SINAC1 (Castillo et al. 2003; Selth et al. 2005; Settlage et al. 2001). *In vitro* experiments indicate that binding of REn to Rep increases the ATPase activity of Rep (Pasumarthy et al. 2010). Analysis of the functional regions of REn indicates that C3 oligomerization and interaction with C1 and PCNA are required for its replication-enhancing activity, but the pRBR binding is not essential for this activity (Settlage et al. 2005). The mechanism by which REn increases viral DNA accumulation involves its induction of and binding to SINAC1. Induction of SINAC1 is dependent on REn and occurs specifically in tomato leaf curl virus-infected cells. Expression of SINAC1 considerably enhanced viral ssDNA accumulation. However, it is still not known at which stage the binding of REn/SINAC1 is involved in geminivirus replication (Selth et al. 2005). Yet, exactly how do REn, Rep, PCNA, pRBR and SINAC1 concert to assure efficient geminivirus DNA replication remains to be elucidated.

11.2.4 AC4/C4

The AC4/C4 ORF is embedded entirely within the Rep coding sequence, but in a different reading frame. Despite the conservation of Rep, AC4/C4 is one of the least conserved geminivirus proteins and has enigmatic biological functions in the monopartite begomoviruses and curtoviruses as opposed to the New World bipartite begomoviruses.

Although the AC4 protein in some bipartite begomoviruses, such as tomato golden mosaic virus, has no significant effect on symptom induction, the C4 protein has been proposed as a major determinant of pathogenesis in curtoviruses and some monopartite begomoviruses, as mutagenesis and/or transgenic expression of their C4 protein produced phenotypes consistent with its role of a symptom determinant (Latham et al. 1997; Mills-Lujan et al. 2015; Piroux et al. 2007). Consequently, ectopic expression of C4 of curtoviruses beet curly top virus (BCTV) or BSCTV,

and monopartite tomato leaf curl Yunnan virus is sufficient for inducing cell division and abnormal plant development that mimics the phenotypes induced by virus infection. Consistent with these symptoms, BSCTV C4 activated the expression of a host RING finger protein (RKP), which triggered the proteosomal degradation of ICK/KRPs, thereby enhancing the activity of cell cycle-dependent kinases to accelerate G1/S cell-cycle transitions (Lai et al. 2009). In agreement with this, BSCTV infection and overexpression of C4 resulted in increased expression of cell-cycle-related genes, such as *CYC*s, *CDK*s and *PCNA*, and decreased expression of CDK inhibitor, *ICK1* and *RBRI* (Park et al. 2010). Moreover, C4 interacted with *Arabidopsis* shaggy-like protein kinase (AtSKs), and interfered with AtSK-directed phosphorylation and inactivation of BES1/BZR1, a family of transcription factors involved in the BR-regulated gene expression. It is possible that C4 induces hyperplasia in a manner recapitulated by bikini, an ATP competitor that specifically inhibits the kinase activity of the seven C4-interacting AtSKs (Deom and Mills-Lujan 2015).

C4 of monopartite begomoviruses or curtoviruses was reported to be involved in virus movement. It can bind ss/dsDNA in a non-specific manner and localizes to the nuclei and cytosol in both *Arabidopsis* protoplasts and *N. benthamiana* leaves. BSCTV mutants containing a disrupted C4 ORF retained the ability to replicate in *Arabidopsis* protoplasts, but did not accumulate viral DNA in the newly emerged leaves of infected *N. benthamiana* and *Arabidopsis*. Expression of BSCTV C4 can complement the movement function of C4-deficient BSCTV mutants in plants, suggesting that C4 may facilitate virus movement (Rojas et al. 2001; Saeed et al. 2007; Teng et al. 2010).

The functionally divergent AC4 and C4 of bipartite and monopartite geminiviruses converge in acting as a silencing suppressor (Sunitha et al. 2013; Vanitharani et al. 2004; Xie et al. 2013). Suppression of PTGS by ACMV AC4 allows enhanced disease phenotypes and promotes viral invasion, resulting in a synergistic phenomenon, when mixed-infection of ACMV with East African cassava mosaic virus (EACMV) was applied (Vanitharani et al. 2004). ACMV AC4 was also shown to specifically bind to single-stranded forms of miRNAs and siRNAs, and presumably inactivated mature miRNAs, thus blocking the normal miRNA-mediated cleavage of target mRNA. The resultant upregulation of the target mRNAs caused developmental defects in *Arabidopsis* (Chellappan et al. 2005).

11.2.5 AC5/C5

AC5/C5, the fifth ORF (AC5/C5) on the complementary sense of viral DNA, has been increasingly described and annotated in about 350 isolates belonging to at least 28 distinct begomovirus species. It locates downstream of AC3/C3, and overlaps a portion of the CP ORF. Mutational analyses of MYMIV AC5 showed that AC5 played a critical role in MYMIV DNA replication both in yeast and in

plants (Li et al. 2015; Raghavan et al. 2004). Overexpression of AC5 from a PVX vector produced severe mosaic symptoms followed by a hypersensitive-like response in *N. benthamiana*. MYMIV AC5 was also demonstrated to effectively suppress single-stranded RNA (ssRNA)-induced PTGS and to reverse TGS of a GFP transgene, probably by inhibiting expression of a CHH cytosine methyltransferase in *N. benthamiana* (Li et al. 2015). Nevertheless, the function of AC5/C5 may vary in different viruses and even among different isolates within the same viral species. The AC5 proteins of two bipartite begomoviruses, tomato chlorotic mottle virus and watermelon chlorotic stunt virus, and C5 of two isolates of tomato leaf deformation virus, appeared to be not essential for infection, whereas a C5-null mutant of the PA10-3 isolate induced less severe symptoms in plants (Fontenelle et al. 2007; Kheyr-Pour et al. 2000; Melgarejo et al. 2013). Hence, roles of AC5/C5 in viral infection between different begomoviruses or even among different isolates within the same viral species are worthy for further study.

11.2.6 AV1/V1

The AV1/V1 ORF of geminiviruses encodes the CP protein that forms the characteristic geminate particles of geminiviruses. It is also involved in a number of processes during geminivirus life cycle.

CP is the only viral protein involved in insect transmission. In begomovirus-transmitting *Bemisia tabaci*, CP of tomato leaf curl virus (TYLCV) or cotton leaf curl virus interacts with a chaperonin, GroEL, homologue of the insect's endosymbiont, preventing virions from rapid proteolysis and ensuring a safe circulative transmission of the virus (Morin et al. 1999, 2000; Rana et al. 2012). TYLCV CP also specifically interacts with the heat shock protein 70 (HSP70) of *B. tabaci*, which was shown to be induced upon TYLCV infection. Feeding whiteflies with anti-HSP70 antibodies enhanced TYLCV transmission rate, suggesting that HSP70 restricts virus transmission, thereby protecting the insect from deleterious effects of the virus (Gotz et al. 2012). Interestingly, HSP70 from tomato plants is involved in TYLCV CP intracellular movement (Gorovits et al. 2013).

To encapsidate viral DNA, CP must enter the nucleus. CP of TYLCV and other monopartite begomoviruses has been detected to localize to the nucleus and be associated with the nucleolus (Rojas et al. 2001; Kunik et al. 1998). It binds ss- and dsDNA and was assumed to be a functional homologue of the NSP of bipartite begomoviruses that shuttle viral DNA between nucleus and cytoplasm (Kunik et al. 1998; Liu et al. 1997). This was supported by the fact that CP of squash leaf curl virus can functionally replace NSP. Experimental observations showed that TYLCV and MYMV CPs interacted with importin alpha, a crucial component of the nuclear pore-targeting complex, and transportation of CP into the nucleus occurs via an importin alpha-dependent pathway (Guerra-Peraza et al. 2005; Kunik et al. 1999). In a recent study, CP was reported to interact with HSP70 from tomato plants. HSP70 formed aggregates during the development of TYLCV infection of

tomato plants, and CP was shown to sequentially co-localize with the cytosolic aggregates, and then the nuclear aggregates. HSP70 inactivation decreased nuclear CP aggregates and re-localized CP from the nucleus to the cytoplasm, as well as showing a decrease in TYLCV DNA levels, indicating that recruitment of the host HSP70 by CP is required for TYLCV infection (Gorovits et al. 2013).

In addition to nuclear shuttling, CPs of monopartite geminiviruses are absolutely essential for virus movement and long-distance transportation of viral DNA (Bridson et al. 1989; Padidam et al. 1996). These functions are presumably mediated by interaction with the movement protein in mastreviruses or the V2 and C4 proteins in monopartite begomoviruses (Rojas et al. 2001; Poornima Priyadarshini et al. 2011). By contrast, the CP of bipartite geminiviruses is dispensable for systemic spread but does attenuate symptoms and decrease viral DNA accumulation, indicating their possible role in enhancing cell-to-cell movement or systemic spread of the bipartite begomoviruses (Fondong 2013).

11.2.7 AV2/V2

The AV2/V2 ORF, located in front of CP, is found in members of different geminivirus genera, but not in the New World bipartite begomoviruses. As reviewed previously, V2 ORF codes for the movement protein of mastrevirus, and appears to be a symptom determinant for curtovirus BCTV (Fondong 2013). For the V2/AV2 ORFs of begomoviruses, there is no consistent description for their function, implying that their function may vary among different viruses.

V2 has been reported to have a role in escaping RNA silencing-based plant defense. For example, the V2 protein of TYLCV was reported to act as a suppressor that counteracts PTGS (Zrachya et al. 2007). This activity is accomplished by a direct interaction with SGS3, a protein that is required to convert ssRNA to double-stranded RNA (dsRNA) in the RNA silencing pathway to produce siRNAs (Glick et al. 2008). Like SGS3, TYLCV V2 is a dsRNA-binding protein that selectively favors 5'-overhang-containing substrate dsRNA. Mutant with C84S/C86S amino acid substitutions not only lost the ability to interact with SGS3, but also failed to overcome SGS3 binding and to suppress PTGS, demonstrating that V2 functions as a PTGS suppressor by preventing SGS3 from accessing substrate RNAs (Fukunaga and Doudna 2009). TYLCV V2 is also functional in TGS suppression (Wang et al. 2014). Expression of V2 from a PVX-based vector restored established TGS of a transcriptionally silenced GFP transgene in *N. benthamiana* line 16-TGS, leading to a substantial reduction of DNA methylation in its 35S promoter sequences. Transgenic expression of V2 in *Arabidopsis* displayed late flowering phenotypes and interfered with the DNA methylation levels of the endogenous loci. Interestingly, the C84S/C86S amino acid substitutions, which were shown to be essential for PTGS suppression and competition of siRNA binding with SGS3, did not affect V2-mediated TGS suppression activity. Therefore, the mechanism by which V2 suppresses TGS remains to be further elucidated.

Like many RNA silencing suppressors, some begomovirus V2 ORFs are regarded as an essential part of virulence determination. Transient expression of tomato leaf curl Java virus V2 in *N. benthamiana* elicited hypersensitive response (HR)-like lesions at the site of inoculation (Sharma and Ikegami 2010). V2 of several begomoviruses induced necrosis, chlorotic spots or systemic HR in *N. benthamiana* leaves when expressed from a PVX vector (Chowda-Reddy et al. 2008; Zhang et al. 2012a; Mubin et al. 2010). V2 was also reported to interact with CYP1, a tomato papain-like cysteine protease that is involved in plant immunity against pathogens via HR (Bar-Ziv et al. 2012). The V2-CYP1 interaction did not interfere with the post-translational maturation of CYP1, but inhibited the proteolytic activity of this protein (Bar-Ziv et al. 2015).

Discrepancies have been discussed regarding the role of begomoviruses V2/AV2 in cell-to-cell movement. Previously, disturbance of V2 in TYLCV or related viruses induced symptomless infection with attenuated viral DNA levels in plants, while accumulated viral DNA at levels close to that of the wild-type virus in protoplasts, suggesting the requirement of V2 for virus movement (Padidam et al. 1996; Rigden et al. 1993). In agreement with this, two TYLCV mutants, one impaired in the V2 silencing-suppression activity, and another carrying a non-translatable V2, led to decreased viral DNA accumulation (Hak et al. 2015). However, both mutants spread to newly emerged leaves at the same rate as the wild-type virus, indicating that the movement of TYLCV *in planta* does not require a functional V2 protein, and the setback in virus proliferation is probably due to the lack of silencing suppression activity (Hak et al. 2015).

11.2.8 BVI

The product of *BVI* from bipartite geminiviruses is an NSP, which binds and shuttles viral genome DNA between the nucleus and the cytoplasm. NSP binds ssDNA and dsDNA on the basis of size and form, without sequence-specificity, and this provides specificity for viral DNA forms as well as strict maintenance of geminiviral genome size (Gilbertson et al. 2003; Hehnle et al. 2004; Rojas et al. 1998). Several studies have provided strong evidence supporting the model that NSP binds to viral DNA and moves it across the nuclear envelope, where MP traffics them across plasmodesmata (PD) and mediates cell-to-cell transportation as well as long-distance transfer via the phloem (Jeske 2009; Rojas et al. 2005).

To date, several NSP-interacting host partners have been identified. Cabbage leaf curl virus (CaLCuV) NSP interacts with the *Arabidopsis* nuclear acetyltransferase, AtNSI, and this interaction is necessary for infection and pathogenicity, indicating that post-translational modification on NSP might affect viral movement or infection (Carvalho and Lazarowitz 2004). CaLCuV NSP could also interact with a cellular NSP-interacting GTPase (NIG), a cytosolic GTP-binding protein that accumulates around the nuclear envelope and possesses intrinsic

GTPase activity. The interaction between NSP and NIG might facilitate NSP transit into the cytosol through the nuclear pore and redirect the viral protein from the nucleus to the cytoplasm (Carvalho et al. 2008). Bean dwarf mosaic virus (BDMV) NSP could interact and co-localize with a host nucleoprotein, histone H3, in the nucleus and nucleolus (Zhou et al. 2011). In addition, NSP could bind and interact with NIK, a member of the leucine-rich repeat receptor-like kinase subfamily that transduces plant defense signaling (Santos et al. 2010). Although NIK functions in defense against geminivirus infections, its kinase activity can be suppressed by NSP (Florentino et al. 2006; Fontes et al. 2004; Santos et al. 2009).

11.2.9 *BC1*

The product of *BC1* from bipartite geminiviruses is an MP, which coordinates with NSP to mediate viral DNA movement into and out of the nucleus and between cells. Except for its involvement in viral cell-to-cell transport, MP facilitates long-distance transport within plants and influences viral pathogenicity (Rojas et al. 2005).

In a currently proposed model for cell-to-cell transport of bipartite begomoviruses, the NSP binds to and mediates the export of nascent viral DNA from the nucleus. At the nuclear periphery, the microsomal vesicles, or the plasma membranes, MP binds the NSP/DNA complex and mediates its transfer into the adjacent cell either along the endoplasmic reticulum or the plasma membrane that traffics through PD (Rojas et al. 2005). However, how do NSP and MP coordinate this process was not understood. Histone H3, a nucleoprotein, has been shown to interact with both the NSP and MP of BDMV, and co-localize with NSP in the nucleus and nucleolus, with MP in the cell periphery and PD. Interestingly, histone H3, NSP, MP and viral DNA formed a complex during the BDMV infection, suggesting that histone H3 may play a role in geminivirus cell-to-cell movement through the formation of a movement-competent complex (Zhou et al. 2011). An *Arabidopsis* synaptotagmin protein (SYTA) which regulates endocytosis could interact with the CaLCuV MP. Knockdown of SYTA delayed CaLCuV infection and inhibits cell-to-cell spread of CaLCuV MP, suggesting that the viral MP transports its cargos to PD for cell-to-cell spread via an endocytic recycling pathway (Lewis and Lazarowitz 2010). The abutilon mosaic virus MP could interact with the nuclear-encoded and plastid-targeted heat shock cognate 70 kDa protein (cpHSC70-1). Silencing of this gene exhibited a spatial restriction to small areas adjacent to veins (Krenz et al. 2010). Therefore, along with the identification of many host factors, it becomes possible to unveil the mechanism of geminivirus movement in the near future.

11.3 Function of Betasatellite

Betasatellites are circular ssDNA molecules that have been identified frequently in association with monopartite begomoviruses and require their helper viruses for replication, movement and encapsidation. Betasatellites have been proved to perform a variety of functions in diverse cellular processes. A remarkable feature of betasatellites is that most of them are required for their helper viruses to induce typical disease symptoms and can enhance viral DNA accumulation in infected host plants (Saunders et al. 2000; Briddon et al. 2001; Cui et al. 2004). Analysis of betasatellite mutants containing a disrupted β C1 ORF and expression of β C1 from a transgene or using a PVX vector indicate that the unique β C1 protein is a pathogenicity determinant (Cui et al. 2004; Qazi et al. 2007; Saeed et al. 2005; Saunders et al. 2004; Sharma et al. 2010). Expression of β C1 in transgenic plants or using a PVX vector reduced the expression of developmental miRNAs, such as miR165/166, and elevated the expression of the targets of development-related miRNAs, which also explained why these plants often exhibited developmental abnormalities (Yang et al. 2008; Amin et al. 2011b). The β C1 protein of tomato yellow leaf curl China betasatellite (TYLCCNB) has been shown to mimic *ASYMMETRIC LEAVES 2* (AS2) and compete with AS2 to form a complex with *ASYMMETRIC LEAVES 1* (AS1) in *Arabidopsis* (Yang et al. 2008). AS1 is a conserved regulator for polar development of leaves and plant immune response, interaction between TYLCCNB β C1 and AS1 caused alternations in leaf morphology and repressed expression of some of the jasmonic acid (JA)-responsive genes (Yang et al. 2008). In addition, the β C1 protein of cotton leaf curl Multan betasatellite (CLCuMB) interacts with a tomato ubiquitin-conjugating enzyme 3 (SIUBC3), leading to a global reduction of polyubiquitylated proteins and a manifestation of strong symptoms. Accordingly, mutation of a specific Gly103 residue within the predicted myristoylation-like motif (103GMDVNE108) of CLCuMB β C1, which is required for interaction with SIUBC3, failed to induce CLCuMB-specific symptoms in host plants (Eini et al. 2009).

Besides its obvious feature in symptom induction, β C1 encoded by betasatellite is capable for the suppression of PTGS and TGS (Zhou 2013). The β C1 protein of several betasatellites functions as a suppressor of PTGS. TYLCCNB β C1 binds ssDNA and dsDNA in a sequence non-specific manner and localizes primarily to the nucleus, and its suppression activity requires the nuclear localization signal (Cui et al. 2005). TYLCCNB β C1 upregulates the transcription level of *rgs-CaM*, a host calmodulin-like protein and endogenous suppressor of RNA silencing, to suppress PTGS and to repress RDR6-mediated anti-geminivirus silencing defense (Li et al. 2014a). At the transcriptional level, TYLCCNB β C1 has been demonstrated to reverse TGS through the interaction with S-adenosyl homocysteine hydrolase (SAHH), a methyl cycle enzyme that is also required for TGS. Physical interactions between β C1 and SAHH resulted in stoichiometric and substantial reductions in SAHH activities, which indirectly blocked the methyl cycle, and

thereby interfered with the epigenetic modification of the viral genome (Yang et al. 2011).

Invasive whiteflies have developed indirect mutualism with TYLCCNV and tobacco curly shoot virus (TbCSV) via host plants (Jiu et al. 2007). This vector-virus mutualism was later demonstrated to be achieved through repression of JA defenses by betasatellite in plants (Zhang et al. 2012b). Meanwhile, betasatellite is reported to suppress terpenoid-mediated plant defense against whiteflies (Luan et al. 2013). TYLCCNB β C1 interacts with the MYC2 transcription factor, a key component in the JA pathway, to subvert plant terpene synthesis and accelerate whitefly performance. Interaction of β C1 with MYC2 interferes with MYC2 dimerization, compromising the activation of MYC2-regulated terpene synthase genes, thereby reducing whitefly resistance (Li et al. 2014b).

11.4 Function of Alphasatellite

Alphasatellites are another kind of circular ssDNAs that are approximately 1,400 nt in size and require the helper begomovirus for spread in plants and insect transmission. They are frequently found to accompany with begomovirus/betasatellite complexes originating from the 'Old World' (Xie et al. 2010; Briddon et al. 2004). Initial efforts to study the aetiology of ageratum yellow vein disease showed that ageratum yellow vein alphasatellite had little effect on the accumulation of ageratum yellow vein virus in *N. benthamiana* plants (Saunders and Stanley 1999). Whereas the alphasatellites associated with TbCSV and okra leaf curl disease attenuated disease symptoms induced by begomovirus/betasatellite complex and reduced the accumulation of betasatellite (Kon et al. 2009; Wu and Zhou 2005). Different from betasatellite, alphasatellite encodes a single rolling-circle replication initiation protein (alpha-Rep) in the virion-sense strand that enables it to replicate autonomously in plant cells (Kon et al. 2009). Recently, two alphasatellites, cotton leaf curl Multan alphasatellite (CLCuMA) and guar leaf curl alphasatellite (GLCuA), have been found to be associated with wheat dwarf India virus (WDIV) in wheat (Kumar et al. 2014). CLCuMA and GLCuA enhanced viral symptoms and WDIV DNA accumulation in wheat (Kumar et al. 2014). This might be achieved through the overcome of RNA silencing-mediated host defense, as the presence of alphasatellite reduced the production of WDIV-derived small interfering RNAs in infected wheat plants, and alpha-Rep proteins encoded by GDarSLA and GMuSLA can suppress PTGS (Nawaz-UI-Rehman et al. 2010). However, no suppressor activity has been identified for CLCuMA (Amin et al. 2011a). It is possible that the role of alphasatellite in attenuating viral symptoms or overcoming host defense is species- or isolate-specific.

11.5 Conclusions and Perspectives

Geminivirus-encoded proteins have evolved to be multifunctional in order to compensate for their tiny genomes. All the functions so far described are accomplished mainly by self-interactions of viral proteins, interactions with other viral proteins, and/or host factors, thus participating in redirecting and reprogramming a variety of plant cellular processes. The conflicts and complexity of the plant-geminivirus interactions resemble warfare. As in war, plants employ various layers of defense against geminivirus infection. Accordingly, geminiviruses battle to evolve diverse countermeasures. Although a considerable progress has been made in understanding the plant-geminivirus interaction, the sophisticated network of interaction has made the task of developing geminivirus-resistant crops much more difficult. A future breakthrough in identification of the yet unknown cellular regulators during geminivirus infection will aid us in understanding the ongoing arms race between plants and geminiviruses, which may ultimately provide clues for the development of strategies against geminivirus infection.

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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 antisense 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 antisense

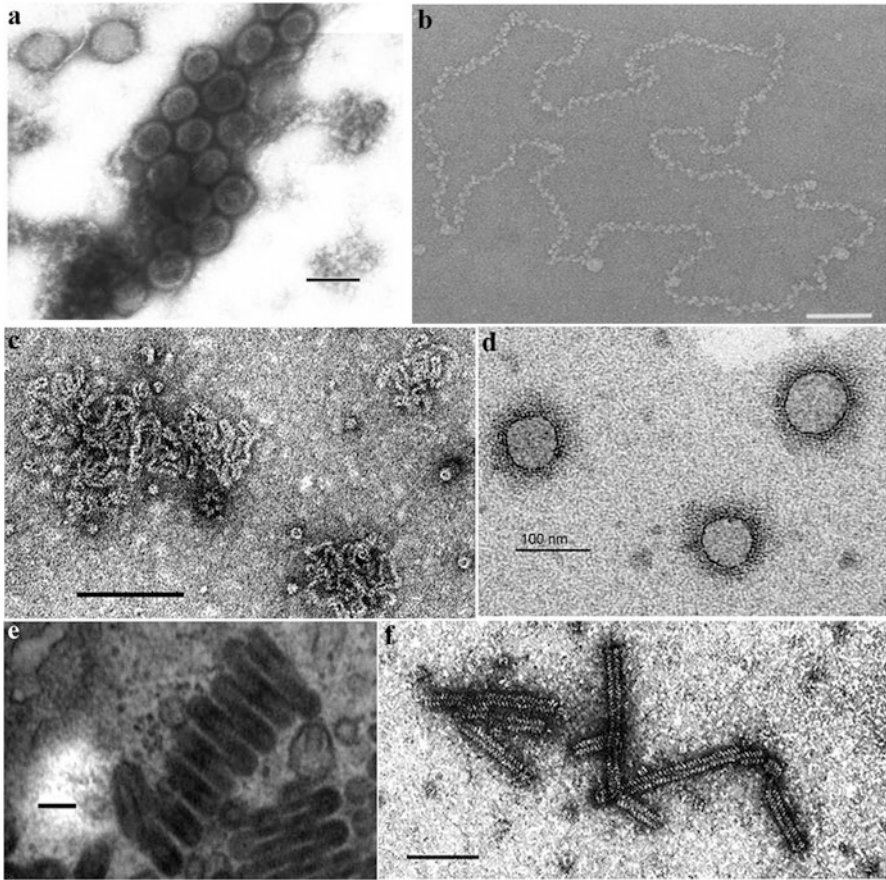


Fig. 12.1 Electron micrographs for particles of TSWV [a; provided by Prof. Zhongkai Zhang], RSV [b; cited from (Ishikawa et al. 1989)], MiLBVV [c; cited from (Vaira et al. 2012)], EMARV [d; cited from (Mühlbach and Mielke-Ehret 2012)]; SYNIV [e; provided by Prof Zhenghe Li]; LBVaV [f; cited from (Walsh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

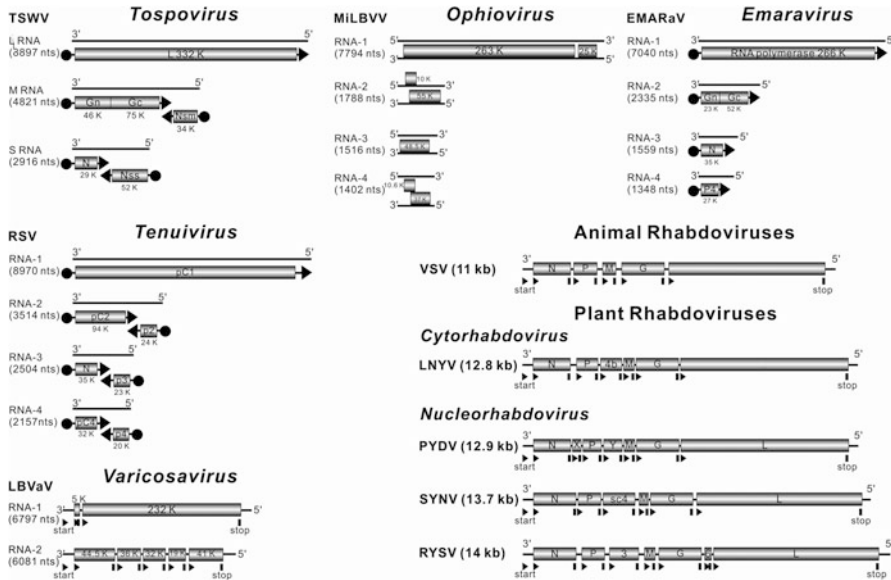


Fig. 12.2 Genome structure of TSWV in *Tospovirus*, RSV in *Tenuivirus*, MiLBVV in *Ophiiovirus*, EMARaV in *Emaravirus*, VSV in animal *Rhabdovirus*, LNYV in plant *Cytorhabdovirus*, PYDV, SYN and RYSV in plant *Nucleorhabdovirus* and LBVaV in *Varicosavirus*

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).

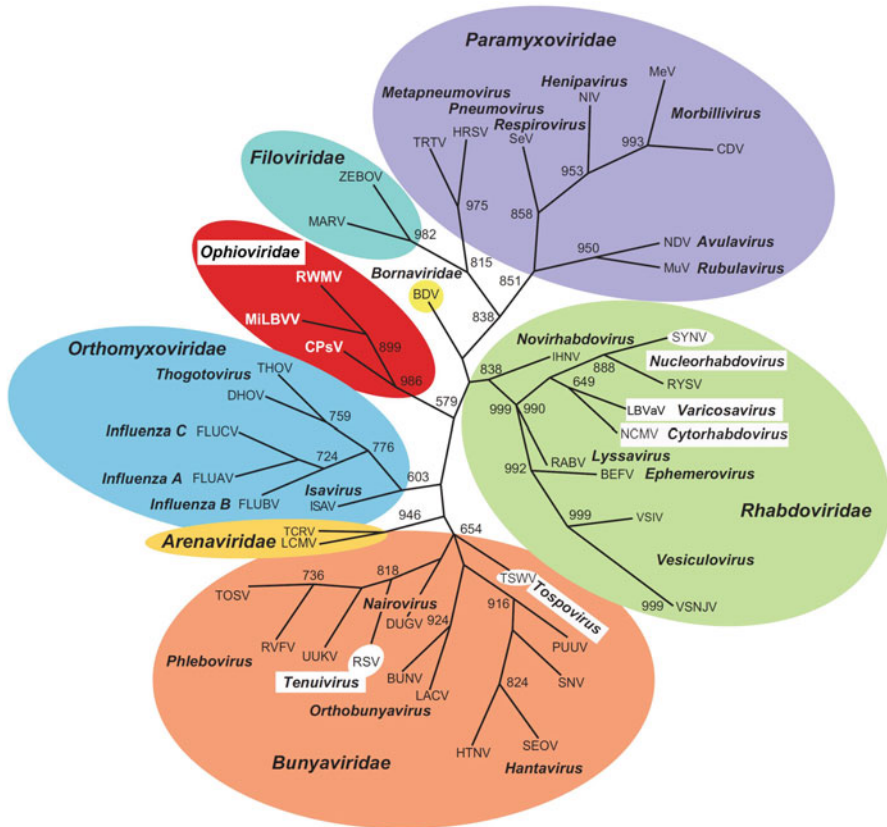


Fig. 12.3 Unrooted phylogenetic tree of the negative stranded RNA viruses based on their conserved RdRp modules. The figure was modified from Ophiovirus genus description in Virus Taxonomy. Oxford: Elsevier, 2011, pp. 743–748. The family and genera described in this article are highlighted. Viruses included 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], SYN [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 *Laodelphax striatellus* were identified by deep sequencing (Xu et al. 2012). The number and size distributions of vsRNAs 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, *STVII*, 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 nucleocapsid 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. Zaneck 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 (Zaneck 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 rhabdovirus 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 nucleocapsid 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 SYN. 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 SYN 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 SYN 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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Chapter 13

Viroids: Small Noncoding Infectious RNAs with the Remarkable Ability of Autonomous Replication

José-Antonio Daròs

Abstract Viroids are infectious agents of plants, constituted exclusively by a non-coding small (246–401 nucleotides) circular RNA molecule. When this RNA manages to enter a cell of an appropriate host plant, it moves to the subcellular replication site and replicates through an RNA-to-RNA rolling circle mechanism. Viroid progeny is then able to move cell-to-cell through plasmodesmata and long distances through the phloem to invade distal parts of host plants. Two types of viroids exist, classified into the families *Pospiviroidae* and *Avsunviroidae*. They replicate in the nucleus (*Pospiviroidae*) and chloroplast (*Avsunviroidae*), hijacking host enzymes. Members of the family *Pospiviroidae* recruit host DNA-dependent RNA polymerase II, RNase III and DNA ligase 1, while members of the *Avsunviroidae* (which contain embedded hammerhead ribozymes for self-cleavage) use host nuclear-encoded chloroplastic RNA polymerase and the chloroplastic isoform of tRNA ligase. Viroids are mainly transmitted mechanically from plant to plant, and frequently exert a pathogenic effect on infected plants. Some symptoms in viroid infections are induced by the viroid-derived small RNAs produced by the host defensive RNA silencing machinery. Interestingly, viroids are targets of the host Dicer-like and RNA-dependent RNA polymerase enzymes, but are particularly resistant to the action of the RNA-induced silencing complex.

13.1 Introduction

Viroids are a particular type of pathogens that affect plants, since they exclusively consist of a small circular single-stranded RNA molecule. In the species known to date, this molecule ranges from 246 to 401 nucleotides (nt); indeed very few

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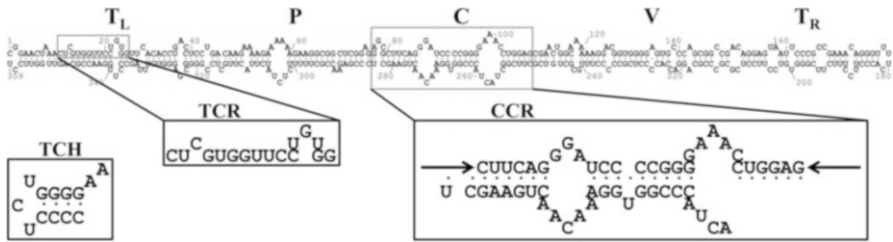


Fig. 13.1 Sequence of PSTVd (sequence variant U23058) and secondary structure as obtained by the Mfold algorithm. The division in domains T_L, P, C, V and T_R domains is indicated. Conserved motifs (CCR and TCR) are boxed. TCH motif, not present in PSTVd but conserved in viroids belonging to the genera *Hostuviroid* and *Cocadviroid* within the family *Avsunviroidae*, is also indicated

sequence variants are longer in length, but contain block-duplications of parts of their genomes. Viroid RNAs are extensively base-paired and adopt compact secondary structures of minimum free energy (Fig. 13.1). Most viroid molecules can adopt rod-like or quasi-rod-like conformations (Giguère et al. 2014b), as confirmed in viroid preparations under electron microscopy (Sogo et al. 1973). However, very few viroids adopt branched conformations of minimum free energy, which also include tertiary structure elements (Giguère et al. 2014a). Remarkably, there is no evidence that viroid RNAs code for proteins, which means that with only a small RNA molecule, viroids are able to replicate when they enter the appropriate host cell, move cell-to-cell, move long distances through the plant, and somehow, circumvent the host defensive response. How they manage to do this is still a mystery that scientists have been trying to decipher since their discovery in the 1960/1970s.

During this time, many articles and some books have exhaustively reviewed different aspects of viroid biology. The following are some recent examples Diener 2003; Tabler and Tsagris 2004; Ding et al. 2005; Flores et al. 2005; Daròs et al. 2006; Ding and Itaya 2007; Tsagris et al. 2008; Ding 2009; Sano et al. 2010; Navarro et al. 2012b; Palukaitis 2014; Flores et al. 2015. This chapter is a personal overview of the field and attempts to highlight the classic findings that have shaped current knowledge about viroids and recent tendencies in research into these fascinating pathogens.

13.2 Viroid Discovery

While working at the U.S. Department of Agriculture in Beltsville, Maryland (USA), Theodor O. Diener found that the properties of the causal agent of potato spindle tuber disease, at that time believed to be a virus, were not conventional at all. In late 1960s early 1970s, he published a series of thorough articles that pinpointed the atypical properties of this pathogen (see the following as examples Diener and Raymer 1967; Diener 1972). The causal agent of potato spindle tuber

disease behaved as a single naked RNA molecule, which was far too small to contain sufficient genetic information for an autonomously replicating virus. The infectivity of the agent was not affected by DNase, protease or phenol treatment, but was inactivated by RNase treatment under low ionic strength conditions. In 1971 he proposed the term “viroid” to designate the new class of pathogens (Diener 1971a). Soon the viroid concept was reinforced by the characterization of the causal agent of exocortis in citrus (Semancik and Weathers 1972). A few years later, Heinz L. Sänger (Justus Liebig-Universität, Giessen, Germany) and collaborators first discovered that viroids were covalently closed RNA molecules, which existed as highly base-paired rod-like structures (Sänger et al. 1976). They were also able to elucidate the full sequence and secondary structure of *Potato spindle tuber viroid* (PSTVd) (Gross et al. 1978), the first pathogen of a eukaryotic organism from which the complete molecular structure was established. An analysis of the PSTVd sequence reinforced previous results, which suggested that viroids might not encode proteins (Davies et al. 1974), and this notion has not been refuted to date. In 1981 at the University of Adelaide (Australia), Robert H. Symons determined the sequence of *Avocado sunblotch viroid* (ASBVd) and observed its very low homology with the other viroid sequences known at the time (Symons 1981) (Fig. 13.2). A few years later, he and his collaborators showed that ASBVd strands of both polarities contained ribozymes, which were able to self-cleave dimeric transcripts of this viroid (Hutchins et al. 1986) (Fig. 13.2). Interestingly, these small noncoding

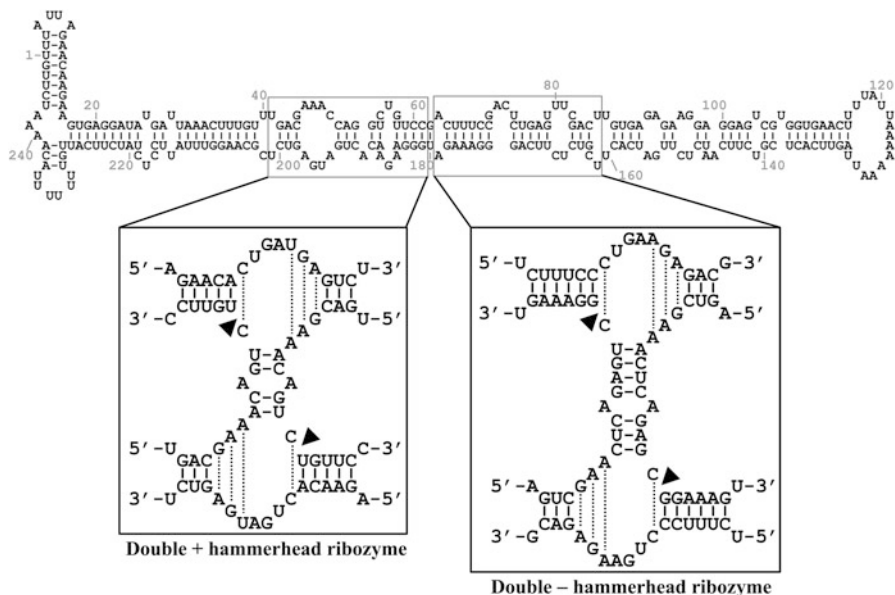


Fig. 13.2 Sequence of ASBVd (sequence variant X52041) and secondary structure as obtained by the Mfold algorithm. The domains forming the double hammerhead ribozyme structures in oligomeric strands of + and - polarities are boxed. In the ribozyme schemes, *arrowheads* indicate the self-cleavage sites and *dotted lines* non-canonical interactions

RNAs, able to replicate and move autonomously in plants, remained as an oddity in biology until recent years, when a myriad of small and long noncoding RNAs have been discovered, which play crucial roles in the regulation of almost all biological processes (Morris and Mattick 2014).

13.3 Viroid Species

The 9th report the International Committee on Taxonomy of Viruses (ICTV) currently recognizes 32 different viroid species (Owens et al. 2012a) (Table 13.1), and many sequence variants have been described to belong to most of these species. Viroid sequence variants have been collected together in the Subviral RNA Database (<http://subviral.med.uottawa.ca>) (Rocheleau and Pelchat 2006). Initially, a criterion of less than 90% sequence identity along the whole molecule was arbitrarily established to demarcate species in viroid taxonomy. However, to better adapt viroid taxonomy to currently accepted criteria in virus taxonomy, different and non-overlapping biological properties are also required to establish new species. This change led to two previously established species, *Tomato chlorotic dwarf viroid* (TCDVd) and *Mexican papita viroid* (MPVd) (Table 13.1), to be currently under consideration (Di Serio et al. 2014).

An early analysis of viroid phylogeny showed two clearly different viroid lineages (Elena et al. 1991). These two lineages are currently recognized as two different viroid families, *Pospiviroidae* and *Avsunviroidae* (Table 13.1), named after the type species PSTVd (Fig. 13.1) and ASBVd (Fig. 13.2), respectively. Most viroids known to date belong to the family *Pospiviroidae*. A common property shared by these viroids is the presence of a conserved region, approximately at the center of their structures of minimum free energy, known as the central conserved region (CCR) (Fig. 13.1). This is a quasi-double-stranded structure with an upper and a lower strand flanked by two imperfect inverted repeats that allow the formation of a cruciform structure, an alternative to that of minimum free energy. Specific sequences in the CCR, and the presence or absence of two other conserved elements in the molecule, the terminal conserved region (TCR) and the terminal conserved hairpin (TCH) (Fig. 13.1), are used to allocate different species to five genera: *Pospiviroid* (type member PSTVd), *Hostuviroid* (type member *Hop stunt viroid*, HSVd), *Cocadviroid* (type member *Coconut cadang-cadang viroid*, CCCVd), *Apscaviroid* (type member *Apple scar skin viroid*, ASSVd) and *Coleviroid* (type member *Coleus blumei viroid 1*, CbVd-1) (Table 13.1). Keese and Symons (1985) proposed a model for the presence of five structural domains in viroids. This model has survived to date for family *Pospiviroidae* members: terminal-left (T_L), pathogenic (P), central (C), variable (V) and terminal-right (T_R) (Fig. 13.1). The CCR is in the C domain and the alternative TCR or TCH are in the T_L domain.

Unlike many species in the family *Pospiviroidae*, only four species are presently recognized in the family *Avsunviroidae* (Flores et al. 2000; Fadda et al. 2003). The

Table 13.1 Viroid species recognized by ICTV in its 9th report

Family	Genus	Species	Acronym
<i>Pospiviroidae</i>	<i>Pospiviroid</i>	<i>Chrysanthemum stunt viroid</i>	CSVd
		<i>Citrus exocortis viroid</i>	CEVd
		<i>Columnnea latent viroid</i>	CLVd
		<i>Iresine viroid 1</i>	IrVd-1
		<i>Mexican papita viroid</i>	MPVd
		<i>Pepper chat fruit viroid</i>	PCFVd
		<i>Potato spindle tuber viroid</i>	PSTVd
		<i>Tomato apical stunt viroid</i>	TASVd
		<i>Tomato chlorotic dwarf viroid</i>	TCDVd
		<i>Tomato planta macho viroid</i>	TPMVd
	<i>Hostuviroid</i>	<i>Hop stunt viroid</i>	HpSVd ^a
		<i>Dahlia latent viroid</i> ^b	DLVd
	<i>Cocadviroid</i>	<i>Citrus bark cracking viroid</i>	CBCVd
		<i>Coconut cadang-cadang viroid</i>	CCCVd
		<i>Coconut tinangaja viroid</i>	CTiVd
		<i>Hop latent viroid</i>	HpLVd
	<i>Apscaviroid</i>	<i>Apple dimple fruit viroid</i>	ADFVd
		<i>Apple scar skin viroid</i>	ASSVd
		<i>Australian grapevine viroid</i>	AGVd
		<i>Citrus bent leaf viroid</i>	CBLVd
		<i>Citrus dwarfing viroid</i>	CDVd
		<i>Citrus viroid V</i>	CVd V
		<i>Citrus viroid VI</i>	CVd VI
		<i>Grapevine yellow speckle viroid 1</i>	GYSVd-1
		<i>Grapevine yellow speckle viroid 2</i>	GYSVd-2
		<i>Pear blister canker viroid</i>	PBCVd
		<i>Apple fruit crinckle viroid</i> ^b	AFCVd
		<i>Grapevine yellow speckle viroid 3</i> ^b	GYSVd-3
	<i>Persimmon latent viroid</i> ^b	PLVd	
	<i>Coleviroid</i>	<i>Coleus blumei viroid 1</i>	CbVd-1
		<i>Coleus blumei viroid 2</i>	CbVd-2
		<i>Coleus blumei viroid 3</i>	CbVd-3
		<i>Coleus blumei viroid 4</i> ^b	CbVd-4
<i>Coleus blumei viroid 5</i> ^b		CbVd-5	
<i>Coleus blumei viroid 6</i> ^b		CbVd-6	
<i>Avsunviroidae</i>	<i>Avocado sunblotch viroid</i>	ASBVd	
	<i>Pelamoviroid</i>	<i>Chrysanthemum chlorotic mottle viroid</i>	CChMVd
		<i>Peach latent mosaic viroid</i>	PLMVd
	<i>Elaviroid</i>	<i>Eggplant latent viroid</i>	ELVd

Type species of the different genera are highlighted on *gray background*

^aAlthough official acronym is HpSVd, this species is commonly abbreviated HSVd

^bSpecies not yet approved by ICTV

molecules of these viroids do not contain a CCR, rather embedded hammerhead ribozyme structures in their strands of both polarities (Fig. 13.2), which are able to self-cleave the viroid oligomeric RNAs. The four viroids in the family *Avsunviroidae* are allocated to three genera, *Avsunviroid* (type species ASBVd), *Pelamoviroid* (type species *Peach latent mosaic viroid*, PLMVd) and *Elaviroid* (type species *Eggplant latent viroid*, ELVd), based on specific sequences in hammerhead domains, guanosine plus cytosine (G + C) content, and solubility in 2 M LiCl, which most probably reflects how compact tertiary structure is.

New viroid species are continuously being discovered. Some recent examples include: *Citrus viroid V* (CVd-V), found in the citrus relative *Atalantia citroides* (Serra et al. 2008); two viroids that infect Japanese and American persimmon (Nakaune and Nakano 2008; Ito et al. 2013), *Pepper chat fruit viroid* (PCFVd), *Dahlia latent viroid* (DLVd), and a symptomless viroid related to *Iresine viroid 1* (IrVd-1) that has been isolated from portulaca (Verhoeven et al. 2009, 2013, 2015); a new grapevine viroid isolated from China (Jiang et al. 2009); two tentative new species of coleoviroids (CbVd-5 and CbVd-6) found in *Coleus blumei* (Hou et al. 2009a, b). Deep sequencing approaches are greatly influencing the identification of new viroid and viroid-like RNAs. The full genomic sequence of a viroid that resembles *Apple dimple fruit viroid* (ADFVd) has been assembled from a library of small RNAs obtained from fig (Chiumenti et al. 2014). Hence algorithms (PFOR, and its improved version PFOR2) have been recently developed to assemble the circular genomes of viroid and viroid-like RNAs from deep sequencing data. These algorithms are homology-independent and can reveal viroid and viroid-like genomes that do not necessarily resemble any currently known species, like *Grapevine latent viroid* (GLVd) and two viroid-like RNAs with hammerhead ribozymes from grapevine and apple (Wu et al. 2012; Zhang et al. 2014).

13.4 Viroid Relatives

Viroid properties are quite unique. In fact some such properties suggest that they might be survivors from the RNA world (Diener 1989; Flores et al. 2014). However, some other RNAs share properties with viroids. The main one is human hepatitis delta virus (HDV), a satellite RNA virus. HDV consists of a single-stranded circular RNA with ribozymes (not of the hammerhead-type) in the strands of both polarities, and very much resemble viroids (Flores et al. 2012; Taylor 2014). The main differences are that HDV depends on a helper virus, hepatitis B virus (HBV), in whose virions it is encapsidated, and it encodes a protein in the antigenomic strand, the delta antigen. Some satellite RNAs of plant viruses probably relate more to viroids, and are also noncoding RNAs; interestingly however, the coding properties of the satellite RNA of *Rice yellow mottle virus* have been recently reported (AbouHaidar et al. 2014). Some of these satellite RNAs are circular, or undergo a circular phase during replication, and they contain ribozymes (hammerhead-type, but not only hammerhead-type) (Hu et al. 2009; Rao and

Kalantidis 2015). Circular satellites are known as virusoids, although the ICTV no longer supports this category.

Another RNA that is related to viroids, with a somewhat puzzling biological nature, is cherry small circular RNA (cscRNA). This is a viroid-like RNA with hammerhead ribozymes in the strands of both polarities (Di Serio et al. 1997). Recent research has suggested that it is a satellite RNA of the mycoviruses that induce the leaf scorch disease of cherries (Minoia et al. 2014b). What is even more puzzling is the biological nature of a retroviroid-like element found in carnations. This circular viroid-like RNA, with hammerhead ribozymes in the strands of both polarities, cannot be transmitted from plant to plant, but a DNA counterpart has been found to be directly fused to DNA sequences of *Carnation etched ring virus*, a pararetrovirus, most likely in the form of an extrachromosomal element that is transmitted to descendants (Daròs and Flores 1995).

13.5 Viroid Replication

Viroids replicate through an RNA-to-RNA rolling circle mechanism. Since viroids do not code for proteins, the polarity of viroid RNAs is arbitrarily assigned. In most species, the viroid molecule with the highest accumulation in infected tissues is circular. This form is considered the viroid genome and is attributed positive, or plus (+), polarity. Consequently, complementary forms are considered to take negative, or minus (–), polarity. Viroid RNAs of + and – polarities accumulate asymmetrically in infected tissues. Strands of + polarity accumulate in larger amounts than those of – polarity. Differential accumulation depends on viroid species and is generally greater in the members of *Pospiviroidae* than in *Avsunviroidae*. However, two species of *Avsunviroidae*, PLMVd and, particularly *Chrysanthemum chlorotic mottle viroid* (CChMVd; both of which belong to the genus *Pelamoviroid*), are peculiar because monomeric linear forms are predominant, probably due to very active hammerhead ribozymes.

13.5.1 Replication Mechanism

One major finding to help decipher viroid replication was the detection of *Citrus exocortis viroid* (CEVd) RNAs of – polarity in *Gynura aurantiaca* infected tissues (Grill and Semancik 1978) and lack of evidence of viroid DNA intermediates. Oligomeric RNAs of + polarity were also detected in both PSTVd-infected potato cells (Spiesmacher et al. 1983) and tissues infected by ASBVd, CEVd and CCCVd (Hutchins et al. 1985). Another main observation was the unambiguous detection of monomeric circular viroid RNAs of – polarity in avocado tissues infected by ASBVd, unlike what occurred in tissues infected by PSTVd and other members of its family (Hutchins et al. 1985; Daròs et al. 1994). In order to understand the

mechanism of viroid replication, presence of hammerhead ribozymes in the RNAs of both polarities in all the viroids of family *Avsunviroidae* is highly relevant (Hutchins et al. 1986; Flores et al. 2001). These ribozymes are also considered to likely act *in vivo* during replication because linear RNAs opened at the ribozyme self-cleavage site have been identified in ASBVd-infected avocado tissue (Daròs et al. 1994). For all these reasons, two different versions of a rolling-circle mechanism have been proposed to explain viroid replication in the members of *Pospiviroidae* and *Avsunviroidae* (Branch and Robertson 1984; Branch et al. 1988).

Members of the family *Pospiviroidae* are considered to replicate via an asymmetric rolling circle mechanism (Fig. 13.3). In this mechanism, viroid circular RNA of + polarity acts as a template for the synthesis of oligomeric RNAs of – polarity by reiterative transcription. Oligomeric – RNAs act as templates to produce complementary oligomeric RNAs of + polarity. According to the asymmetric model, only + oligomeric RNAs are cleaved to monomers which, in the last instance, are ligated to render viroid + circular progeny (Fig. 13.3). The symmetric model explains the replication of the viroids belonging to the family *Avsunviroidae*. In this model (Fig. 13.3), viroid oligomeric – RNAs are also produced from a circular template of + polarity. However, these oligomeric RNAs undergo self-cleavage through hammerhead ribozymes and the resulting monomeric RNAs are circularized to produce monomeric circular viroid RNAs of – polarity. This species is the template to produce oligomeric + RNAs in a second rolling circle, which is symmetrical to the first (Fig. 13.3). Oligomeric + RNAs, which also contain hammerhead ribozymes, self-cleave to produce monomers that are finally circularized.

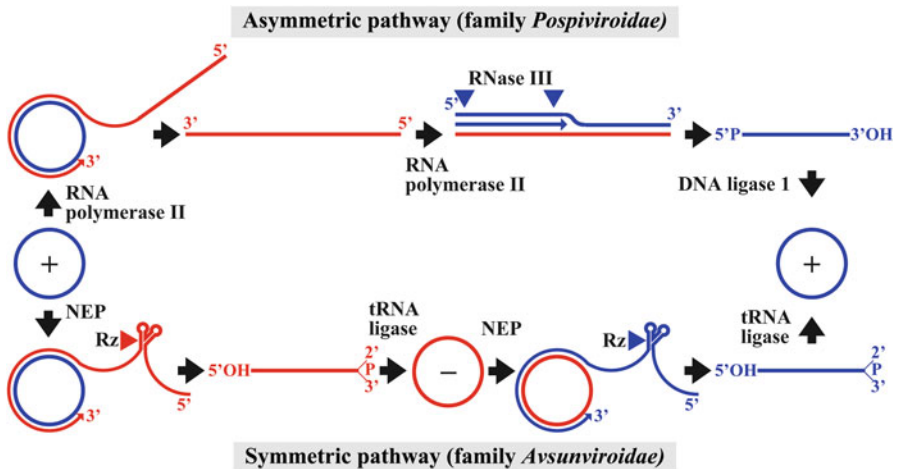


Fig. 13.3 Schematic representation of the viroid RNA-to-RNA rolling circle replication mechanism. Asymmetric and symmetric pathways are followed by the members of the family *Pospiviroidae* in the nucleus and by the members of the *Avsunviroidae* in chloroplasts, respectively. Viroid RNAs of + and – polarities are represented with blue and red lines, respectively. Arrowheads represent cleavage sites. P and OH indicate phosphoester and hydroxyl terminal groups

13.5.2 *Replication Site*

One important question is where exactly all these replication steps take place. Early works localized PSTVd and its replication intermediates in the nuclei of infected cells (Diener 1971b; Spiesmacher et al. 1983), and all evidence obtained to date indicates that the replication and accumulation of the members of *Pospiviroidae* occur in this subcellular location. More specifically, an *in situ* hybridization analysis of *Nicotiana benthamiana* tissues infected with PSTVd has revealed that viroid – strands localize in the nucleoplasm, while + strands localize in both the nucleoplasm and nucleolus (Qi and Ding 2003). In contrast, an *in situ* hybridization analysis with electron microscopy has indicated that ASBVd localizes in the chloroplasts of infected cells, mostly on thylakoid membranes (Bonfiglioli et al. 1994; Lima et al. 1994). The same occurred with PLMVd in infected peach leaves (Bussi ere et al. 1999). Multistranded ASBVd complexes, considered viroid replication intermediates, have also been localized in chloroplasts (Navarro et al. 1999). Thus, chloroplasts are accepted as the replication and accumulation site of *Avsunviroidae*. However, a recent intriguing research conducted with ELVd has shown that this viroid RNA is able to traffic from the cytoplasm to the nucleus, and from the nucleus to the chloroplast (G omez and Pall as 2012), so reality could be more intricate.

13.5.3 *RNA Transcription*

Another important question is what enzymes are involved in all these replication steps. This is a particularly interesting question, because viroids, unlike viruses, do not encode any replication protein. Pioneering viroid transcription analyses, done with replication complexes that were partially purified from infected tissues, have demonstrated that the synthesis of HSVd and CEVd strands is not affected by DNase or actinomycin D, but is sensitive to fungal toxin α -amanitin at the low concentrations that typically inhibit eukaryotic DNA-dependent RNA polymerase II (M uhlbach and S anger 1979; Flores and Semancik 1982). This conclusion is quite outstanding because RNA polymerase II typically uses a DNA template in host cells. However, viroids somehow manage to subvert its activity to recognize an RNA template. Involvement of DNA-dependent RNA polymerase II in the transcription of the members of the family *Pospiviroidae* has been further supported through an immunoprecipitation analysis done with an antibody against the carboxy-terminal domain of this enzyme (Warrilow and Symons 1999), and by showing direct binding between the enzyme and PSTVd in a tomato nuclear extract (Bojic et al. 2012). By also analyzing the effect of a fungal toxin, this time tagetitoxin, upon transcription using partially purified replication complexes from avocado chloroplasts infected by ASBVd, it has been concluded that the polymerase which mediates the synthesis of these viroid RNAs is chloroplastic nuclear-

encoded polymerase (NEP) (Navarro et al. 2000). This is a single-subunit enzyme that resembles bacteriophage RNA polymerases. However, *in vitro* analyses, which used PLMVd RNAs and purified *Escherichia coli* RNA polymerase, have suggested that bacterial-like RNA polymerase from peach chloroplasts may catalyze PLMVd replication (Pelchat et al. 2001). This is a plastid-encoded polymerase (PEP) that consists in several subunits and resembles prokaryotic RNA polymerases. In any case, both host enzymes are DNA-dependent RNA polymerases, which means that the members of the *Pospiviroidae* and *Avsunviroidae* are capable of changing the substrate specificity of some of their replication enzymes.

Another intriguing question in viroid replication is whether viroid transcription starts at random in the circular template or if, on the contrary, transcription promoters exist in viroid molecules. A pioneering research work by Navarro and collaborators, which labeled the 5'-triphosphate groups of linear ASBVd RNAs of both polarities isolated from infected avocado tissues, has demonstrated that this viroid transcripts start with a UAAAA sequence, which maps to similar A + U-rich terminal loops in their predicted quasi-rod-like secondary structures. Moreover, the sequences around initiation sites have been highlighted as being similar to the promoters used by chloroplastic NEP (Navarro and Flores 2000), which further supports the involvement of this polymerase in ASBVd replication. Other studies done into PLMVd have also suggested that the transcription of + and – strands starts at definite positions in the corresponding templates. More specifically, they map at similar double-stranded motifs, which contain the conserved GUC triplet that precede the self-cleavage site in both polarity strands (Delgado et al. 2005; Motard et al. 2008). For the members of the family *Pospiviroidae*, an analysis that employed PSTVd molecules and a potato nuclear extract, and which allowed the *de novo* synthesis of viroid transcripts, has revealed that – strands also start at a single site located in the hairpin loop of the viroid left terminal region (Kolonko et al. 2006). Finally, although viroid RNA turnover has not received much attention to date, a recent research work, which used PSTVd-infected eggplant tissues, has provided a mechanistic insight into how viroid decay may proceed *in vivo* during replication (Minoia et al. 2015).

13.5.4 Viroid RNA Cleavage

The oligomeric transcripts of both polarities in the viroids of the family *Avsunviroidae* self-cleave through the hammerhead ribozymes embedded in these molecules (Hutchins et al. 1986; Prody et al. 1986; Flores et al. 2001). Self-cleavage occurs in the absence of proteins. However, host RNA chaperons, like proteins PARBP33 and PARBP35 from avocado chloroplasts, may facilitate self-cleavage *in vivo* (Daròs and Flores 2002). Interestingly, tertiary interactions between peripheral regions in hammerhead structures have proven crucial for activity at the low magnesium concentrations which exist *in vivo* (De la Peña et al. 2003; Khvorova et al. 2003). During replication, viroid oligomeric transcripts

self-cleave very efficiently. In fact, a mutational analysis that used ELVd + hammerhead ribozyme has suggested that natural viroid ribozymes have been evolutionary selected to cleave RNAs co-transcriptionally (Carbonell et al. 2006). Then, after cleavage and circularization, viroids must have some kind of regulatory mechanism to avoid the cleavage of circular viroid progeny. An early work conducted after the discovery of the hammerhead ribozyme has demonstrated that ASBVd achieves this regulation thanks to thermodynamically unstable hammerhead ribozymes that contain short helices III capped with short loops. The cleavage of ASBVd hammerhead ribozymes is efficient by the formation of double hammerhead structures only in oligomeric transcripts, while single hammerhead ribozymes are poorly active (Forster et al. 1988; Davies et al. 1991). PLMVd and CChMVd may regulate the activity of their hammerhead ribozymes in circular progeny by engaging their corresponding sequences in thermodynamically very stable quasi-double-stranded arms. Nonetheless, these two viroids, particularly CChMVd, have the lower ratio of circular to linear forms in infected tissues. Finally, ELVd hammerhead ribozyme of + polarity shows efficient co-transcriptional cleavage, as mentioned above, and a poor self-cleavage rate constant after transcription (Carbonell et al. 2006).

Despite a thorough search along this lines (Tsagris et al. 1987), there is no evidence to prove that the oligomeric RNAs of the members of the family *Pospiviroidae* undergo autocatalytic cleavage. This means that a specific host enzyme must recognize the replication intermediate of + polarity and cleaves precisely to produce the monomeric linear RNAs. A pioneering work that used CEVd has identified the upper CCR strand as the processing site and advanced hairpin I, or an alternative double-stranded palindrome structure formed by two contiguous hairpins I, as the RNA motifs that direct cleavage (Visvader et al. 1985). It was not long before a viroid processing model, which involved this thermodynamically double-stranded structure that can be adopted by the oligomers of all the members of the *Pospiviroidae*, was soon proposed (Diener 1986). A research work, done with PSTVd RNAs and nuclear extracts from potato cell suspensions, has mapped an equivalent cleavage site in the upper strand of the CCR, but also proposed a multi-branched structure that would undergo conformational transition to mediate viroid RNA cleavage and ligation (Baumstark et al. 1997). It is noteworthy that the sequence motifs which support this last model do not exist beyond the genus *Pospiviroid*. Gas and collaborators used an *Arabidopsis thaliana* experimental system in which viroid replication intermediates were expressed (Daròs and Flores 2004), to map the processing site of three viroids (CEVd, ASSVd and HSVd) that belong to three different genera in the family *Pospiviroidae*. They found equivalent positions in loop capping hairpin I, more specifically between the third and fourth nucleotides of this tetraloop (Gas et al. 2007). From the effect on the cleavage and ligation of a series of mutations around the CEVd processing site, it has been concluded that the substrate for cleavage is the double-stranded structure adopted by the hybridization of two hairpin I domains, which belong to two contiguous units in viroid oligomeric intermediate, whereas ligation is determined by loop E and the flanking nucleotides of the two CCR strands (Gas et al. 2007).

Another analysis of monomeric linear CEVd RNAs isolated from *A. thaliana* plants expressing oligomeric +RNAs has identified 5'-phosphomonoester and 3'-hydroxyl terminal groups in this replication intermediate. The nature of these termini and the double-stranded structure, previously proposed to be the substrate for cleavage *in vivo*, suggests that a type III RNase catalyzes cleavage, and an RNA ligase that recognizes these termini, promotes circularization (Gas et al. 2008). All these results led to a model to explain RNA processing during the replication of the members of the *Pospiviroidae* (Fig. 13.4). In this model the four nucleotides in the loops of two contiguous hairpins I in the oligomeric replication intermediate –the sequence of this tetraloop in all known members of *Pospiviroidae* is palindromic–interact to trigger a conformational transition that forms the double-stranded intermediate. This intermediate that contains the two cleavage sites in opposite strands, separated by two nucleotides in a 3' protruding manner, is the substrate for host RNase III that produces the monomers with 5'-phosphomonoester and 3'-hydroxyl terminal groups. Finally, these monomers undergo conformational transition to form a ligation intermediate that is sealed by an RNA ligase activity (DNA ligase 1, see below) recognizing these 5'-phosphomonoester and 3'-hydroxyl terminal groups (Fig. 13.4).

13.5.5 Viroid RNA Circularization

With the genuine monomeric linear replication intermediate at hand (which in the case of PSTVd is opened at position G95-G96 and contains 5'-phosphomonoester and 3'-hydroxyl terminal groups), Nohales and collaborators purified a tomato protein fraction capable of efficient circularizing this RNA (Nohales et al. 2012a). A mass spectrometry analysis of this fraction highlighted the presence of tomato DNA ligase 1. A recombinant version of this protein produced in *E. coli* has demonstrated the efficient circularization of representative viroids in the family *Pospiviroidae*, opened at their physiological processing sites. Finally a virus-induced gene silencing (VIGS) approach has demonstrated the involvement of this host enzyme in viroid circularization in PSTVd-infected *N. benthamiana* plants (Nohales et al. 2012a). This remarkable finding indicates that, similarly to what occurs in transcription, viroids (*Pospiviroidae*) also subvert a DNA enzyme to mediate an RNA reaction in the last replication step (Fig. 13.3).

Unlike the monomeric linear replication intermediates of the members of the *Pospiviroidae*, those from *Avsunviroidae* are produced by the activity of hammerhead ribozymes, and contain 5'-hydroxyl and 2',3'-cyclic phosphodiester termini. These are the typical terminal groups recognized by tRNA ligase, an enzyme conserved in all the eukaryotes involved in tRNA maturation (Abelson et al. 1998). After considering a work showing that this enzyme in plants, in addition to the nucleus, also localizes in the cytoplasm and chloroplast (Englert et al. 2007), Nohales and collaborators cloned the cDNA corresponding to this enzyme from eggplant and produced a recombinant version of the protein in *E. coli*. This recombinant protein

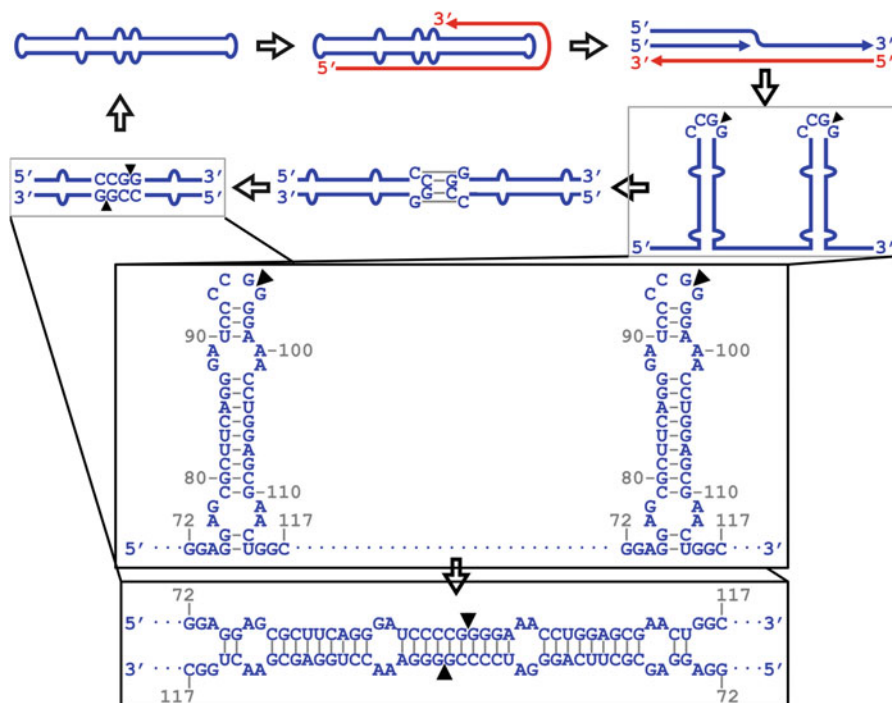


Fig. 13.4 Model to explain RNA processing during the replication of the viroids belonging to the family *Pospiviroidae*. The nucleotides in the loop of two contiguous hairpins I in the oligomeric replication intermediate interact to trigger formation of a palindromic quasi-double-stranded structure, which is the substrate for a host RNase III. After cleavage, linear monomers refold to a conformation recognized by DNA ligase I. Blue and red lines and sequences correspond to + and - polarities, respectively. Gray lines indicate the kissing loop interaction and arrowheads indicate the processing sites. Sequences and numbering correspond to PSTVd sequence variant U23058

efficiently circularizes all the monomeric linear forms of both polarities of the four species in the family *Avsunviroidae*. A VIGS assay has been done to silence *N. benthamiana* endogenous tRNA ligase, and it also supports the involvement of this enzyme in ELVd circularization in plants (Nohales et al. 2012b).

13.6 Viroid Traffic

When viroid molecules manage to enter the host cell, they must move towards the appropriate subcellular location for replication which entails, according to current knowledge, the nucleus for the members of the family *Pospiviroidae* and chloroplasts for the members of the *Avsunviroidae*. Then the viroid progeny, like that of plant viruses, should move to neighboring cells to continue replication, and then to distal plant parts once they reach vascular tissue (Ding et al. 2005). There is a remarkable difference between viroids and viruses in terms of the systemic invasion

of host plants. Viroid infections can be considered slow (several weeks) when compared to the pace of most plant viruses, which can reach distal plant parts in just a few days. The molecular mechanisms that underlie this difference are currently unknown.

Analyzing viroid movement is extremely difficult when compared to plant viruses. There are no viroid-encoded proteins to which reporter moieties, like green fluorescence protein (GFP), can be fused for tracking purposes. What makes things worse is that viroid genomes are highly compact and do not admit the insertion of reporter genes while maintaining viability. Nonetheless, an ingenious experimental approach, which used *Potato virus X* (PVX) as a vector to launch PSTVd fragments embedded in an intron-containing GFP mRNA has demonstrated that hairpin I, the palindromic structure present in the upper strand of the C domain in all the species of the family *Pospiviroidae*, suffices to mediate entry of RNA into the nucleus of *N. benthamiana* cells (Zhao et al. 2001; Abraitienė et al. 2008). This import is independent of the cytoskeleton, uncoupled to the Ran GTPase cycle and facilitated by a receptor (Woo et al. 1999). A bromodomain-containing protein, which also interacts with PSTVd, has recently proposed to mediate nuclear importation of satellite RNA of *Cucumber mosaic virus* (CMV) (Chaturvedi et al. 2014). As mentioned above, satellite RNAs differ from viroids in which the former need a helper virus to assist some steps in the infection process. On the other side, fusion of the ELVd sequences in the 5' untranslated region (5'UTR) of a GFP reporter construct expressed in *N. benthamiana* tissues using *Agrobacterium tumefaciens* leads to GFP translation and accumulation in chloroplasts, thus evidencing the remarkable capability of ELVd RNA to translocate into chloroplasts (Gómez and Pallás 2010). Further research with ELVd, which incorporated the intron-containing PVX expression tool this time, has indicated that the scenario of *Avsunviroidae* intracellular movement might be more complex than initially expected because upon entry into the cell, this viroid may move first from the cytoplasm to the nucleus, and then from the nucleus to the chloroplast for replication, to finally move back to the nucleus and the cytoplasm to continue its systemic spread through the plant (Gómez and Pallás 2012).

Viroids are considered to move cell-to-cell through plasmodesmata. When they reach vascular tissue, they translocate into the phloem to move long distance through the plant. Thanks to a genome-wide mutational analysis, Zhong and collaborators have revealed a series of motifs along the PSTVd molecule involved in systemic movement (Zhong et al. 2008). With more details, a bipartite motif that included U201 and U309, together with U47/A313 of the PSTVd molecule, has been found to be involved in viroid movement from bundle sheath to mesophyll cells (Qi et al. 2004). Bulge 7 was shown to be involved in the translocation from bundle sheath cells into the phloem (Zhong et al. 2007), and bulge 6 in the movement from palisade to spongy mesophyll cells (Takeda et al. 2011). Once inside the vascular tissue, phloem protein 2 has been proposed to be the host factor to mediate viroid movement, as this protein has been reported to interact with HSVd RNA *in vitro* and *in vivo* (Gómez and Pallás 2001; Owens et al. 2001; Gómez and Pallás 2004). Graft experiments with citrus viroids have also highlighted the presence of a translocatable factor from Etrog citron that is capable of mediating

viroid invasion of nonvascular tissues (Bani-Hashemian et al. 2015). *N. tabacum* protein Nt-4/1 has also been suggested to be involved in PSTVd mobility (Solovvey et al. 2013).

13.7 Viroid Pathogenesis

Viroids infect angiosperm plants, monocotyledonous and dicotyledonous. To date no viroid that infects gymnosperms, pteridophytes, bryophytes or algae has been found. Among angiosperms, all kinds of plants are infected, including herbaceous and ligneous. Most of the viroids known to date are associated with cultivated plants, although this may reflect only the effect of agricultural practices on amplifying and spreading viroids worldwide. Viroids are particularly prevalent in plants cultivated in tropical and subtropical regions. This can once again be an effect of agricultural practices, or could indicate an advantage of warmer climates in viroid replication and spread. It is worth mentioning that citrus plants have the infamous honor of being hosts of many viroid species (Murcia et al. 2009), and that despite efforts made to set up such an experimental system, no complete viroid infection has been described in plant model *A. thaliana* (Daròs and Flores 2004). The effect of viroid diseases range from devastating, like cadang-cadang which killed millions of coconut palms in South East Asia (Randles et al. 1988) to asymptomatic. Some examples of the so called latent viroids exist, like ELVd, that have no apparent effect on host plants (Fadda et al. 2003). Yet in many instances, symptoms depend on the host –PSTVd sequence variants, which are strongly symptomatic in tomato, are almost symptomless in *N. benthamiana*– or interestingly on viroid sequence variants, and some examples of well characterized pathogenicity determinants exist (De la Peña and Flores 2002; Malfitano et al. 2003; Murcia et al. 2011; Wu et al. 2013).

Typical symptoms in viroid diseases are leaf chlorosis, internode shortening, bark cracking, flower discoloration, fruit skin deformation or tuber malformation. Plant stunting and leaf epinasty (downward bending from growth at the top) are common expressions of many viroid diseases. The molecular mechanisms that underlie viroid symptoms have been a mystery for a long time, and most symptoms in viroid diseases are possibly the result of a complex combination of molecular effects. However, a recent research by Navarro and collaborators has demonstrated that two viroid-derived small sRNAs (vd-sRNAs, see below), which arise from the – strand of a PLMVd variant that induces intense albinism (peach calico), target the mRNA coding for chloroplast heat-shock protein 90 (cHSP90). This protein is a molecular chaperone involved in chloroplast development and its down-regulation may abort chloroplast maturation to produce the albino phenotype (Navarro et al. 2012a). It is also interesting to note that PLMVd has been shown to accumulate to higher titers in albino sections of infected peach leaves. Consequently, targeting host cHSP90 with vd-sRNA through an RNA silencing down-regulating mechanism might be a viroid strategy to increase its progeny. One good example of

how symptoms in infected plants can arise from RNA silencing mechanisms also stems from the satellite RNA of CMV. Infection with the yellow satellite RNA (Y-sat) of CMV induces a small interfering RNA (siRNA) that down-regulates a chlorophyll biosynthetic gene (CHLI), which, in turn, promotes leaf yellowing (Shimura et al. 2011). Interestingly, the hypothesis that vd-sRNAs trigger symptoms in viroid infections was anticipated years ahead of these discoveries (Wang et al. 2004) and led to heated debate as to whether viroids, whose genomes are at least 10 times shorter than those of plant viruses, may target host genes (Navarro et al. 2012b). Another observation to support this hypothesis came from the expression of PSTVd sequences as artificial microRNAs (amiRNAs) in *N. tabacum* and *N. benthamiana*. One amiRNA, which corresponds to the virulence modulating region of this viroid and targets host soluble inorganic pyrophosphatase, induces abnormal phenotypes that closely resemble PSTVd symptoms in these plants (Eamens et al. 2014).

Despite all these examples, most symptoms in viroid diseases may still be the result of a complex combination of molecular effects. Viroids hijack host proteins to mediate replication and movement, and some symptoms may result from detracting these proteins from their physiological roles in host plants. HSVd infection has been shown to cause a significant imbalance in the expression of phenylpropanoid metabolite-affecting genes via a complex mechanism (Füssy et al. 2013). HSVd infection has also been shown to induce changes in the dynamic DNA methylation of ribosomal RNA (rRNA) genes. In infected plants, some rRNA genes are demethylated and transcriptionally reactivated (Martinez et al. 2014). Moreover, viroid infections are known to induce a strongly altered gene expression in the host plants (Itaya et al. 2002; Owens et al. 2012b; Rizza et al. 2012; Lisón et al. 2013).

13.8 Viroids and RNA Silencing

Plants use RNA silencing pathways to defend themselves from invading viruses, but viruses display RNA silencing suppressors to counteract this defensive mechanism (Ding 2010). The relation between viroids and RNA silencing has been, ever since this mechanism was discovered, intriguing for several reasons. First, viroid molecules strongly resemble the structured RNAs that are substrates of Dicer-like (DCL) enzymes and trigger RNA silencing. Viroid replication also produces, at least transiently, double-stranded RNAs. Second, as viroids are noncoding RNAs, they cannot display the repertoire of proteins with RNA silencing suppressor activity found in plant viruses (Li and Ding 2006). Third, and in quite the opposite direction, highly structured viroid molecules may be particularly resistant to the down-regulating activity of the RNA-induced silencing complex (RISC). Fourth and finally, the subcellular localization of viroid molecules (nucleus and chloroplast) very much dissembles that of RNA viruses, and could also be important to interpret how viroids circumvent RNA silencing.

Pioneering analyses have shown that plant tissues infected with viroids, regardless of them belonging to the families *Pospiviroidae* or *Avsunviroidae*, accumulate vd-sRNAs, similarly to what occurs in virus infections (Itaya et al. 2001; Papaefthimiou et al. 2001; Martínez de Alba et al. 2002). Moreover, these vd-sRNAs have been seen to be phosphorylated and methylated at the 5' and 3' ends, respectively, like genuine virus-derived small RNAs, which supports an origin from RNA silencing pathways (Martín et al. 2007). More recently, these vd-sRNAs have also been shown to be loaded by Argonaute (AGO) proteins (Minoia et al. 2014a). Furthermore, vd-sRNAs have also been reported to be functional *in vivo* down-regulating reporter genes fused to viroid sequences (Vogt et al. 2004; Gómez and Pallás 2007). However, mature viroid molecules have been reported to exhibit a remarkable resistance (Gómez and Pallás 2007; Itaya et al. 2007), but not complete immunity (Carbonell et al. 2008; Kasai et al. 2013), to RNA silencing. All these observations suggest that viroids may be able to maintain a delicate equilibrium between triggering and being targets of the plant antiviral RNA silencing pathways.

Analyses performed by deep sequencing have demonstrated that vd-sRNAs are homogeneously distributed along both strands of viroid RNAs, which suggests the involvement of RNA-dependent RNA polymerases (RDR) in the production of secondary vd-sRNAs that amplify the silencing signal (Di Serio et al. 2009; Navarro et al. 2009; Bolduc et al. 2010; Diermann et al. 2010; Martínez et al. 2010). RDR are cytoplasmic enzymes, which indicates that plants may take advantage of viroid traffic through the cytoplasm to trigger RNA silencing. In this context, RDR6 has been shown to preclude meristem invasion by PSTVd in *N. benthamiana* (Di Serio et al. 2010). One interesting and important aspect of RNA silencing, RNA-directed DNA methylation, was first discovered in viroid-infected plants (Wassenegger et al. 1994; Dalakouras et al. 2013). However, a recent work has revealed that despite PSTVd replication induces RNA-directed DNA methylation, it fails to trigger posttranscriptional gene silencing in the nucleus, the organelle where this viroid replicates (Dalakouras et al. 2015).

13.9 Viroid Transmission

The main form of viroid plant-to-plant transmission seems to be mechanical inoculation, which may be accidentally caused in some agricultural practices, such as grafting or pruning, or may be naturally occurring through injuries that result from physical contact between plants. It is worth noting that root grafting may occur naturally in high-density plantations. Seed and pollen transmission of viroids is not common, although substantial rates have been reported for some viroids like ASBVd or ELVd (Flores et al. 2000; Fadda et al. 2003). Bearing this in mind, it is clear that managing free-of-viroid germplasms is crucial to avoid spreading viroid disease, and that the exchange of contaminated material has probably been the main cause of the worldwide spread of some viroid diseases. Tool disinfection in

agricultural practices to prevent mechanical transmission is therefore important (Li et al. 2015). Attention should also be paid to plants in which viroids can occur latently, as shown in some ornamental plants (Verhoeven et al. 2008; Singh et al. 2009). All these recommendations stress the importance of viroid diagnoses in managing viroid diseases.

Fortunately, viroid transmission by vectors (beyond human beings) is not considered significant, although some cases of insect transmission have been reported for *Tomato apical stunt viroid* (TASVd) (Antignus et al. 2007) and TCDVd (Matsuura et al. 2010), and a recent worrying work has localized two viroids, PSTVd and TASVd, in stylets and stomachs of aphids feeding on infected plants (Van Bogaert et al. 2015).

13.10 Viroid Diagnosis

Reliably diagnosing viroid diseases is crucial for managing free-of-viroid vegetative material in the plant industry, and to eradicate infected plants and trees in orchards and plantations to avoid transmission. Viroid detection in plant tissues entails another difficulty when compared to viruses because viroid-encoded proteins are lacking, which precludes the use of immunological techniques like the enzyme-linked immunosorbent assay (ELISA), otherwise widely used in plant health programs.

It was only possible to accomplish early efforts made in viroid diagnoses by means of biological assays with indicator hosts, which showed characteristic infection symptoms. Biological assays are slow and costly, but very sensitive and informative, and are still used nowadays in some instances, particularly in research (Murcia et al. 2011). The analysis of RNA preparations by polyacrylamide gel electrophoresis (PAGE) has revolutionized viroid diagnoses when combining separation under two different conditions, first native and then denaturing (Schumacher et al. 1983). This technique takes advantage of the fact that viroids are circular molecules, which migrate likewise to their linear host counterparts of a similar molecular weight under native conditions, and quickly slow down migration under denaturing conditions. In this way, they are easily separated and detected in the second denaturing gel. Double or sequential PAGE, with some modifications made to the original design, is still greatly appreciated in research, particularly for the identification of new viroids, because its results are sequence-independent (Verhoeven et al. 2013). Molecular hybridization techniques have been widely used for viroid diagnoses in both dot-blot and northern versions. Using polyprobes allows the simultaneous detection of several viroids (Lin et al. 2011; Torchetti et al. 2012). Recent developments in molecular hybridization techniques have resorted to microarray chips as they are able to simultaneously detect hundreds of species of viruses, satellite RNAs and viroids (Nam et al. 2014; Adams et al. 2015). Finally, a wide variety of very sensitive reverse transcription-polymerase chain reaction (RT-PCR) techniques, including one-step, multiplex and quantitative

approaches, as well as RT loop-mediated isothermal amplification (RT-LAMP), has been also proposed for viroid diagnoses (Hajizadeh et al. 2012; Botermans et al. 2013; Thanarajoo et al. 2014; Malandraki et al. 2015).

13.11 Biotechnological Applications of Viroids

A classic biotechnological application of viroids has been cross-protection. Even before this phenomenon even began to be understood (Ziebell and Carr 2010), researchers realized that asymptomatic or mild strains of some viroids, like viruses, were able to protect plants from more severe symptoms caused by aggressive strains (Niblett et al. 1978; Khoury et al. 1988). Viroids have also been used to induce desirable agronomic traits in plants, particularly dwarfing. Citrus trees infected by certain viroid strains show some dwarfing properties that facilitate cultivation, and do not apparently affect fruit production and quality (Tessitori et al. 2013). An interesting example is an elite Brazilian cultivar of Tahiti acid lime, whose properties, which include not only tree size, but also fruit quality, are thought to be induced by a particular combination of viroids (Eiras et al. 2010). Viroids are certainly considered very interesting tools for basic research. Their unique properties have made them ideal experimental systems in many research works into the structure–function relationship, RNA replication and processing, RNA movement through the plant, evolution of RNA pathogens and, of course, RNA silencing. However, some viroid properties, mainly a very compact molecule packed with functions, have so far precluded their use as biotechnological tools. Our recent research, however, shows how the combined expression of ELVd (used as a molecular scaffold) and eggplant tRNA ligase allows production of large amounts of recombinant RNA in *E. coli* cultures (Daròs et al. 2014).

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Chapter 14

Diagnosis of Plant Viruses Using Next-Generation Sequencing and Metagenomic Analysis

Ian Adams and Adrian Fox

Abstract Next generation sequencing (NGS) is revolutionising the diagnosis of plant viral disease. This chapter describes the sequencing platforms (Illumina, 454, Pacific Biosciences, IonTorrent, Nanopore) and techniques used to produce and analyse NGS virus data. At present NGS has been used for plant viral disease diagnosis in a number of exemplar cases but, as yet, it has still to be routinely adopted for frontline diagnostic applications. The barriers to this uptake including access, cost, analysis, validation and interpretation are discussed.

14.1 Introduction

Next generation sequencing (NGS) describes a series of technologies which have revolutionised molecular biology. They allow millions or billions of DNA molecules to be sequenced simultaneously. In 2004, after a 20 year project costing three billion dollars, the sequence of the human genome was announced. Just a year later the first NGS platform was released allowing a human genome to be sequenced in a few months and for less than 50,000 dollars. This technology has continued to improve and in 2015 the latest NGS platform allows a lab to sequence 18,000 human genomes in 1 year at the cost of 1000 dollars per genome. The technology that has enabled these advances in genomics have rapidly been adopted across many biological fields including medical microbiology, plant breeding, population genetics and in the diagnosis of human, animal and plant diseases. The introduction of NGS to plant virus diagnostics has led to a large increase in the number and frequency of novel viruses being discovered. This technology is rapidly developing into a routine diagnostic tool.

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14.2 NGS Platforms

The first commercially available NGS platform was the Roche 454 released in 2005 (Margulies et al. 2005). This platform carried out pyrosequencing on beads arrayed in a multi-million well “picotitre” plate. In its most recent format, the 454 FLX+, it is capable of producing one million reads up to 750 bp long. Due to the high cost of 454 sequencing in terms of consumables and staff time and the relatively low throughput most 454 machines have now been decommissioned and technical support for this platform will be withdrawn in 2016. The next platform to be released was the Illumina Genome Analyser which utilised reversible florescent dideoxy terminators to sequence DNA clusters amplified on the surface of flow cells. This technology has continued to be developed and Illumina currently have four main sequencing platforms. The HiSeq X ten capable of sequencing 18,000 human genomes in a year. The HiSeq range capable of producing 150 bp from each end ($2 \times 150\text{bp}$) of approximately two billion DNA clusters. The NextSeq which produces approximately 200 million $2 \times 150\text{bp}$ reads and the MiSeq (Fig. 14.1a) which produces approximately 30 million $2 \times 300\text{bp}$ reads. The Illumina platforms are currently used to perform a majority of the DNA sequencing worldwide.

Life Technologies initially introduced a sequencing by ligation based platform the SoLiD but this platform has since be superseded by their Ion torrent platform which utilizes an electronic microchip to detect the addition of bases to a DNA template. The latest Ion Torrent device can produce 5 million 400 bp reads. This platform gained some popularity due to the cheap cost of devices and reagents but many core facilities with access to multiple platforms no longer use the Ion Torrent platform favouring the Illumina platforms due to higher throughput and improved data quality.

Pacific Biosciences produce a platform which utilises single molecule real time sequencing (SMRT). This involves incorporating fluorescently labelled nucleotides into the DNA molecule in small wells and measuring the fluorescence incorporated. The Pacific Biosciences platform is capable of producing up to 50,000 reads averaging 5000 bp with some reads up to 20,000 bp. The platform has a high

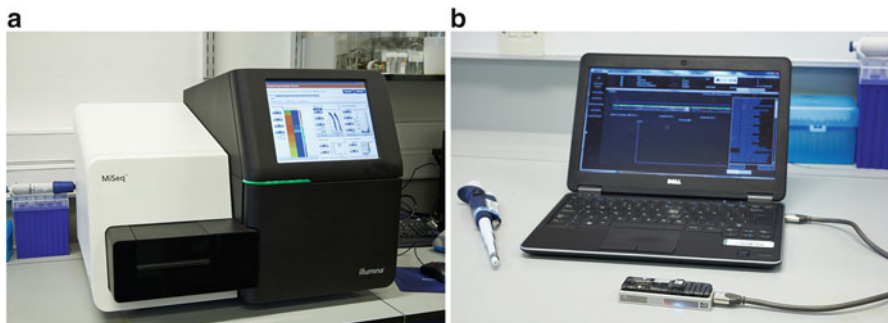


Fig. 14.1 (a) Illumina MiSeq and (b): Oxford Nanopore MinION NGS sequencers

error rate of 13 % but recently this has been compensated for by circularising the libraries allowing the same DNA to be sequenced multiple times thus giving a more accurate “consensus” sequence. This platform is often utilised to provide scaffolds to combine with other higher throughput technologies (Illumina) allowing the closing of bacterial genomes or the sequencing of complete viral genomes.

The final popular NGS platform is the Oxford Nanopore MinION (Fig. 14.1b). This is a device smaller than a smart phone which can be plugged into a laptop. The device contains 512 nanopores through which electronic current is passed. As a DNA molecule passes through one of these nanopores it changes the current through the pore and as this change in current varies with different nucleotides the DNA can be sequenced. This platform is still not commercially available but is capable of producing thousands of reads averaging over 5000 bp with some up to 150,000 bp (Laver et al. 2015). It current has a single read error rate of about 15 %. It has recently been successfully used to sequence the flu virus (Wang et al. 2015) and a number of plant viruses (unpublished).

14.3 Plant Viral Diagnostics

Plant viral diagnostics uses two broad groups of methods. The first group are the specific or targeted methods. These are serological or molecular tests capable of accurately determining the presence of a single target (Boonham et al. 2014). Such assays test for the presence/absence of a single target pathogen or group of closely related pathogens. Antibody based methods such as ELISA or Lateral Flow Devices (Boonham et al. 2014; Clark and Adams 1977) using antibodies specific to the target virus are among the most commonly used methods in routine virus diagnostics. Around the turn of the century and in the early 2000s specific molecular detection assays were rapidly developed. These assays can take the form of real time or conventional PCR (Mumford et al. 2000) or loop mediated amplification (LAMP) (Tomlinson et al. 2013) relying on amplification of targets starting from viral specific oligonucleotides. Whilst each individual assay has a specific range of detection these assays can be multiplexed and run in parallel allowing multiple viruses to be tested for simultaneously.

Microarrays which rely on hybridisation of target nucleic acid to specific probes either in solution (van Brunschot et al. 2014) or on a solid support (Boonham et al. 2007) allow the detection of hundreds or even thousands of viruses in a single assay are still fundamentally a whole series of specific single virus assays carried out in parallel. What these assays have in common is the requirement for knowledge of the virus being tested, allowing the synthesis of specific oligonucleotides or antibodies. This in itself has an inherent disadvantage in that if assays are very specific they may miss strains of viruses which have small differences in genome or coat protein.

The second group of methods consist of more general non-specific methods. This group contains methods such as inspection of visual symptoms either in the

original host or in ranges of indicator test plants as well as methods such as electron microscopy. Whilst these methods require no prior knowledge they tend not to be able to identify viral infections down to species level. They also have specific drawbacks, for instance identification of viruses on a range of indicator hosts relies on the ability to transmit the virus, and in some cases this may only be possible by specific transmission methods (e.g. polioviruses are not transmissible through mechanical inoculation).

14.4 Plant Viral NGS

NGS has opened up plant diagnostics producing a method which requires no prior knowledge of the virus being tested whilst delivering a species or even strain specific result. NGS was first carried on plant viruses in 2009 with three research groups utilising different NGS methods to produce plant virus sequence. Adams et al. (2009) used a Roche 454 FLX to produce novel viral sequence from total RNA enriched for virus sequences using subtractive hybridisation. Kreuze et al. (2009) sequenced siRNA from a sweet-potato (*Ipomoea batatas*) plant using an Illumina GAII to identify novel viruses and Rwhannih et al. (2009) sequenced double stranded RNA from grapevines using a Roche 454 FLX. Since then a large number of plant viruses have been sequenced using NGS. Barba et al. (2014) and Roossinck et al. (2015) both detail most of the plant viruses sequenced using NGS.

Unlike other molecular diagnostics the sensitivity and cost of performing NGS is directly related to the sensitivity determined by how much sequencing is performed. With sequencer runs costing thousands of pounds it is usual to multiplex multiple samples in the same run to reduce costs thus sacrificing sensitivity. The degree of multiplexing determines the amount of sequence produced from single samples and thus the sensitivity obtained. In order to minimise costs and increase sensitivity, methods have been developed to maximise the sequencing of plant viruses and minimise the wasteful sequencing of plants and other organisms present in the infected plant. A number of different approaches have been evaluated to solve this problem.

- **Total nucleic acid:** This approach has the advantage of not biasing the results in favour or against any particular virus and allows for simple sample processing which reduces costs. DNA sequencing identifies DNA viruses and RNA sequencing identifies RNA viruses and frequently identifies DNA viruses which replicate through an RNA intermediate. Since the first plant virus was sequenced in 2008 the sequencing per base costs have dropped by over 50 fold (Wetterstrand 2015). It is now the case that a significant proportion of the cost of sequencing any sample is the pre-sequencing processing costs, so the simple workflows of this method are an advantage. This method was first used by Rwhannih et al. (2009) and Adams et al. (2009) and was recently used to identify

a range of viruses in carrots (Adams et al. 2014b). The disadvantage of this method is that large amounts of un-necessary sequencing is carried out which needs to be paid for and processed.

- **Ribosome depletion:** A modification of the total RNA method is to use one of the ribosome subtraction technologies (RiboZero, Illumina, USA; RiboMinus, Life Technologies, USA) to remove the plant ribosomal RNA from the total RNA. As this is subtracting plant RNA it shouldn't be biased against viral RNA and can be integrated into a library prep protocol but does increase the cost and time required to do the sample prep. The use of the RiboZero plant leaf kit led to a ten-fold enrichment in virus sequence recovered in a MiSeq run (unpublished data).
- **Double Stranded (ds)RNA:** Most RNA viruses have a dsRNA stage during replication, either encapsulated in a virus particle or as an intermediate of replication. This phenomenon has long been exploited and it is possible to purify dsRNA using cellulose (Dodds et al. 1984) or using lithium chloride (Akin et al. 1998). Using this method Rwhnih et al. (2009) were able to sequence grapevine viruses. It has since been used successfully many times (Roossinck et al. 2015; Barba et al. 2014). The disadvantage of this procedure is that it is time consuming, complicated to carry out, selective in favour of certain viruses and doesn't work particularly well on negative sense single stranded viruses or at all on DNA viruses (Roossinck et al. 2015)
- **Small RNA:** One of the ways plants defend themselves against viruses is through the RNA interference pathway (Hamilton and Baulcombe 1999). The plant detects viral dsRNA and uses the enzyme dicer to cleave the dsRNA into small 21–24 nucleotide fragments. These small RNA molecules are called small interfering RNAs. This viral defence mechanism was first utilised by Kreuze et al. (2009) who identified a series of plant viruses by purifying and sequencing plant siRNA. The method has since been used successfully to sequence a whole range of viruses (Barba et al. 2014; Roossinck et al. 2015) and is described in detail by Kutnjak et al. (2015). The disadvantage of this method is that the extraction and processing can be difficult and time consuming, it favours certain viruses and sequence assembly from 21 to 24 nucleotide fragments is complex and not always possible.
- **Subtractive hybridisation:** If viral infected and healthy plant material is available it is possible to enrich for viral RNA by subtractive hybridization. This was successfully carried out by Adams et al. (2009)). The disadvantage of this method is that it requires a healthy plant for the subtraction and requires multiple processing steps making it contamination prone.
- **Viral associated nucleic acid (VANA):** The final method commonly used to enrich for viral nucleic acid is the sequencing of VANA (Roossinck et al. 2015). In this method viral particles are purified from plant material using centrifugation, precipitation or filtration, DNase and RNase treated to remove contaminating plant nucleic acid before the nucleic acid protected by the viral protein coat is extracted and sequenced. This method was successfully used in a study of viruses in Prairie grass (Thapa et al. 2012) as well in the genomic sequencing of

a number of viruses (Richards et al. 2014). The disadvantage of this method is that it requires complex sample processing and that the purification may favour certain viruses.

14.5 Sample Processing

All current sequencing platforms require some form of library preparation prior to sequencing. For an RNA sample they all include cDNA synthesis and ligation of adapters and may include an amplification step. The time required to carry out library prep varies considerably from hours to a couple of days and along with the cost of reagents is now a significant part of the overall cost of viral NGS. As an illustration, the following details the steps required to produce a library for an Illumina sequencer (MiSeq/NextSeq/HiSeq) using the ScriptSeq library prep system from Illumina.

1. **Fragmentation:** Chemical fragmentation of RNA to produce sequencer compatible fragments
2. **cDNA synthesis:** Anchored random primed first strand cDNA synthesis (leaves defined nucleotide sequence tag at 5' end of cDNA).
3. **3' Tagging:** Terminal tagging of cDNA using random terminal tagging oligonucleotide (leaves defined nucleotide sequence tag at 3' end of cDNA).
4. **cDNA purification:** Clean up of cDNA using Solid Phase Reversible Immobilization (SPRI) beads (AMPure XP, Agencourt, USA).
5. **Amplification and indexing:** Amplification of cDNA using primers to 5' and 3' tags. Sample specific index nucleotide sequences can also be added at this stage allowing multiplexing of samples.
6. **Library purification:** Clean up of cDNA library using SPRI beads.
7. **Quality control:** Library quality control usually consisting of Bioanalyser (Agilent, USA) size measurement and fluorimetry (Qubit, LifeTech) or real time PCR (KapaBioScience, USA) quantification.

14.6 Bioinformatic Analysis

Although some of this analysis can be carried out on a desktop computer, due to the growing size of the datasets it is most efficient to carry out NGS analysis on faster multicore servers with large amounts of RAM usually running a Linux operating system. Such servers can frequently be found in academic IT departments but similar computing power can be accessed reasonably cheaply by the hour from cloud providers such as Amazon web services (AWS 2015).

Before carrying out detailed analysis of DNA sequence it is important to first confirm the quality of the sequence and remove any which is of low quality. Sequenced DNA is usually stored in the fastq format. For every position in the

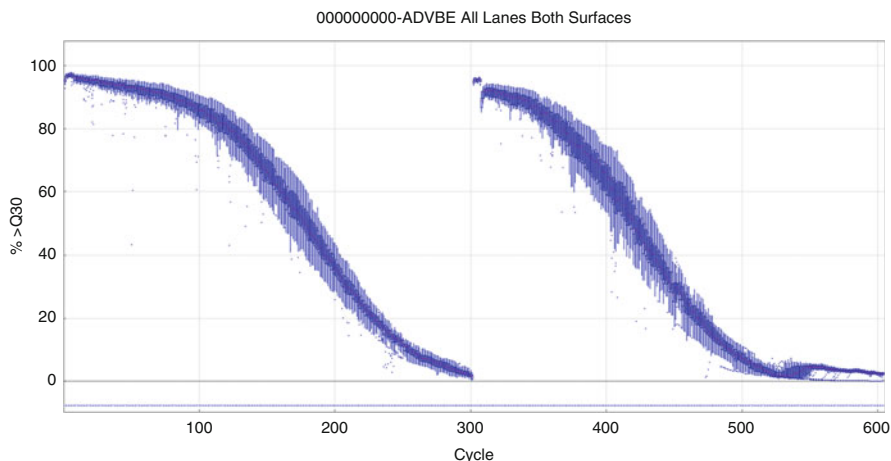


Fig. 14.2 Plot of percent bases above quality 30 against cycle for a typical MiSeq run of two 300 bp paired reads

sequence there are two pieces of data, the nucleotide base call (assignment of a nucleotide base to a particular position in a sequence) and a quality score based on the calculated accuracy of the base call. The quality score has its origins in the PHRED scores developed for automation of the human genome project (Ewing and Green 1998). It is a number usually between 1 and 40. A score of 10 equates to a 1 in 10 probability of an error in the base calling, 20 to 1 in 100, 30 to 1 in 1000 and 40 to 1 in 10,000. Thus the higher the number, the greater the quality and the higher the confidence in the DNA sequence. Low quality sequence tends to occur at the 3' end of sequences so trimming back from the 3' end until a threshold quality is reached is the accepted procedure. A quality score of 30 or 20 is usually set as the threshold. Figure 14.2 shows a typical quality profile for a MiSeq run showing a low quality tail at the 3' end of the sequences. Various software packages can be used to do this quality trimming such as Sickle (Najoshi 2011), SolexaQA (Cox et al. 2010) or FLASH (Magoc and Salzberg 2011). After trimming the next step is usually sequence assembly, finding overlaps between sequences and then combining those with repeat sequences from the same region to produce longer consensus sequences or contigs. This reduces the number of sequences that need to be taxonomically assigned and finally improves taxonomic assignment by potentially grouping together a series of motifs which together strengthen an assignment. For plant viral sequencing a range of different assemblers have been used (Wu et al. 2015) to which list Trinity (Grabherr et al. 2011) should be added (Adams et al. 2014b).

The final stage is then to identify sequences of viral origin. If the host plant has a sequenced genome this can be used to subtract any host sequences reducing the number of sequences which need to be screened. The remaining sequences then need to be identified and two fundamental approaches have been used. The first approach is to look for homology between existing viruses and sequences being

screened. This uses either Basic local Alignment Search Tool (BLAST) (Camacho et al. 2009) or one of the more recent alternatives such as LMAT (Ames et al. 2013) or Kraken (Wood and Salzberg 2014). BLAST looks for similarities between the query sequence and a sequence database. BLASTn compares nucleotide sequences and can be used to compare the query sequence with the whole or parts of the NCBI GenBank databases (Benson et al. 2010). This allows the detection of known viruses, their variants and viruses closely related to known viruses. BLASTx translates the query nucleotide sequence into the six possible amino acid sequences which could be produced from the 3 forward and 3 reverse frames and then compares these amino acid sequences to a protein database such as the NCBI nr database. This frequently allows for the detection of novel viruses which can share little or no nucleotide homology with a known virus but share conserved protein domains at the amino acid level. For instance, for *Arachacha virus B*, a Cheravirus (Adams et al. 2013a) the highest BLASTn score of RNA 2 is to 80 bp (2% of the full sequence) of *Strawberry latent virus RNA 2* and this level of homology would be unlikely to be considered significant. BLASTx of the same sequence shows that it has greater than 20% identity over greater than 70% of its length to a range of related Cheraviruses positively identifying it as the RNA 2 molecule of a Cheravirus. The major disadvantage of BLAST is the time searches take. This is particularly compounded for BLASTx searches which carry out 6 searches for each query sequence. One way to speed up BLAST analysis is to reduce the database searched to only virus sequences but this tends to lead to spurious low homology findings of virus which, when the sequences are compared to the complete database, turn out to originate as other organisms such as plants. Other approaches to improve analysis speed is to use alternative algorithms. These include LMAT (Ames et al. 2013) or Kraken (Wood and Salzberg 2014) which although faster tend to be too specific and miss variants or close relatives of existing viruses and novel viruses (Soueidan et al. 2014).

Taxonomic assignments can be visualized using the tool MEGAN (Huson et al. 2011) which uses a lowest common ancestor algorithm to assign query sequences to related organisms at the appropriate taxonomic level. Figure 14.3 shows a typical MEGAN plot of a plant infected with a number of novel Nepoviruses. The software also displays the alignments used to place a query sequence and also allow the sequences assigned to specific groups to be downloaded.



Fig. 14.3 MEGAN plot showing Nepovirus infection in a plant

The major problem with homology based sequence identification is that it relies on viruses being related to existing viruses. Anything different is unlikely to be identified. To address this a second approach is being developed to identify features or signatures of viral sequences which identify them as being viral in origin. The existing methods (Wu et al. 2015; Soueidan et al. 2014) are still in the early stages of development. This development may be hampered by the fact that viral genomes evolve within the plant cell sharing replication and transcription apparatus. Fungal and bacterial genomes on the other hand which can be separated from plant sequences evolve in their own cells with their own replication and transcription apparatus.

14.7 Plant Viral Diagnostic Use of NGS

Since its introduction in 2009 viral NGS has been used for plant disease diagnostics. Al Rwhnih et al. (2015) compared NGS and biological indexing for the detection of grapevine viruses and found NGS to be superior both in terms of comprehensiveness and speed. NGS has also been used in a number of important plant disease diagnostic problems. In 2012 there were increasing reports of total losses of maize crops in Kenya. Existing methods used both locally and in international labs were unable to diagnose the problem and it was only with the application of NGS that two known viruses (*Sugarcane mosaic virus* and *Maize chlorotic mottle virus*) were detected and shown to be divergent from the standard strains, thus explaining the failure of the existing methods (Adams et al. 2013b). As almost complete genomic sequences had been obtained for both viruses it was then possible to develop specific real time PCR assays for these divergent strains and these assays are still in use for screening for these viruses. NGS has continued to be used in tracking the spread of this disease across East Africa allowing the genotyping of fresh isolates and informing appropriate control measures (Mahuku et al. 2015; Adams et al. 2014a).

The routine adoption of NGS for plant viral diagnostics has been considered but not yet been implemented. A number of factors have slowed this implementation.

- **Platforms:** The early platforms such as the 454 and Illumina GAII while being ground breaking were complex and time consuming to use. Because of this they tended to only be found in large core facilities. The advent of benchtop sequencer (Illumina MiSeq, LifeTech Ion Torrent, Roche 454 junior) purpose built for ease of use in smaller labs has reduced this barrier to the use of NGS diagnostics.
- **Cost:** When viral NGS was first developed, due to the high cost of equipment and the large amount of staff time required to process samples, it cost thousands of dollars get a plant virus sequenced. With the simplification of sequencing equipment and workflows this price has now come down to hundreds of dollars and is now comparable to the cost of doing a suite of PCR or ELISA assays.
- **Analysis:** Initially it required a highly skilled bioinformatician with coding skills and a high powered server to analyse NGS data. With the advent of simplified pipelines and cloud computing this is no longer the case, although there is still a

requirement for bioinformatics skills in initially setting up the pipelines and cloud instances.

- **Validation:** Before any diagnostic test can be used it requires validation. In the European Union plant virus molecular diagnostic tests are validated based on guidance from the European Plant Protection Organisation (EPPO) (EPPO 2014). This guidance designed for specific molecular tests will need adapting. It calls for validation based on specificity, sensitivity, reproducibility and repeatability. The sample processing, sequencing and even the analysis of an NGS based viral diagnostic test will need to be assessed against all these criteria. Initial work is under way and Fox et al. (2015) reported on comparative sensitivity of NGS and real-time PCR when screening seeds for virus and viroid infection.
- **Incidental findings:** As noted in Hall et al. (2015) one of the concerns of diagnostics via NGS is what they describe as incidental findings. This is the discovery of viruses other than those being screened for. In the European plant health context a screen could be against the EPPO quarantine list of viruses and incidental findings would be viruses already present in the EU are more problematically novel viruses which may or may not cause symptoms. It is important before analysing a sample to agree with the customer how you will deal with such incidental viruses. Not reporting the presence of viruses is problematic and how to deal with novel viruses is difficult as they may or may not be damaging to the plant and therefore of concern. One approach (Stobbe et al. 2013) is to only search against a predefined database of target viruses.
- **Asymptomatic viruses:** The advent of NGS has led to the discovery of novel potential viruses based solely on sequence homology often with no supporting biological data. Many of these viruses many never be properly characterised and what to do with these viruses and their sequence data is under debate. The ICTV is currently discussing how to deal with this sequence only viruses with the potential for a naming convention which recognises viruses with only sequence data.

Recent technological and procedural developments mean that next generation sequencing is now ready to mature as a diagnostic technique for the detection of plant viruses and over the next couple of years is likely to become a common tool in many diagnostic labs.

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