

Replication mechanisms of plant RNA viruses: current understanding and perspectives

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Introduction

Viruses cause numerous diseases in economically important plants and animals, including humans. However, there are no efficient measures to counter viral infection, except for a very few animal viral diseases. Although virus resistance genes have been used to minimize losses for several important crop species, the number of genes that have been used to confer virus resistance is very limited. The delay in the development of efficient measures for the control of viral diseases might be due in part to the lack of critical information on the mechanisms underlying viral replication, especially regarding the host proteins used by viruses.

Positive-strand RNA viruses with a genome composed of messenger-sense single-stranded RNAs, represent the largest group among the seven genetic classes of all viruses. Upon entry into host cells, the genomic RNAs of these viruses serve as mRNAs, and viral replication proteins are translated by exploiting the host translational machinery via diverse strategies, which include cap- and poly(A)-independent translation mechanisms. Subsequently, viral genomes are synthesized on intracellular membranes in infected cells by RNA replicase complexes that consist of viral RNA-dependent RNA polymerase (RdRp), viral auxiliary proteins, host-encoded proteins, and viral RNAs (Ahlquist et al. 2003; den Boon et al. 2010; Nagy and Pogany 2012). Increasing evidence has demonstrated that

viral replication proteins are multifunctional and play critical roles in recruiting the viral genomic RNAs and rearranging specific cellular membranes, which are the sites of replication of positive-strand RNA viruses. This paper presents a brief review of viral RNA translation and replication mechanisms and refers to recent data obtained in the study of the *Red clover necrotic mosaic virus* (RCNMV), which is used by our group as a model virus.

Red clover necrotic mosaic virus

RCNMV is a positive-strand RNA plant virus and a member of the genus *Dianthovirus* in the family *Tombusviridae*. This genus includes *Carnation ring spot virus* (CRSV) as the type member and *Sweet clover necrotic mosaic virus* (SCNMV) (Hiruki 1987). The genome of RCNMV consists of two RNAs (RNA1 and RNA2). The bipartite genome is unique among viruses of the family *Tombusviridae*, the genome of which is monopartite. RNA1 encodes putative RNA replicase components, an auxiliary 27-kDa protein (p27) and an 88-kDa protein (p88), which has an RdRP motif. RNA1 also encodes a 37-kDa coat protein (CP), that is expressed from a subgenomic RNA (CPsgRNA). Transcription of the CPsgRNA requires an intermolecular interaction between RNA1 and RNA2 (Sit et al. 1998; Tatsuta et al. 2005). RNA2 is a monocistronic RNA that encodes a movement protein (MP), which is required for viral cell-to-cell movement in plants (Kaido et al. 2011; Xiong et al. 1993).

Translation of RCNMV replicase proteins

Many plant RNA viruses, including members of the *Tombusviridae* and the *Luteoviridae* families, lack both a 5' cap

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and a 3' poly(A) tail. Instead, they have cap-independent translation elements (CITEs) in the 3' untranslated region (UTR) of their genomic RNAs (Nicholson and White 2011). In some of these viruses, 3'CITEs bind the eukaryotic translation initiation factor complex eIF4F or eIFiso4F (Iwakawa et al. 2012; Nicholson and White 2011).

RCNMV RNA1 and RNA2 possess neither a cap structure at the 5' end nor a poly(A) tail at the 3' end (Mizumoto et al. 2003). Therefore, RCNMV proteins must be translated via cap-independent translation mechanisms. Two RNA elements play essential roles in the cap-independent translation of the replicase proteins encoded in RNA1. One element is the 3'CITE that consists of five stem-loop structures in the 3' UTR of RNA1 (Mizumoto et al. 2003). Another element is an A-rich sequence (ARS) located upstream of the 3'CITE (Iwakawa et al. 2012). A search for cellular factors that bind the 3' UTR of RNA1 using RNA aptamer-based one-step affinity chromatography and mass spectrometry analysis led to the identification of the ARS as an RNA element that binds to poly(A)-binding protein (PABP) (Iwakawa et al. 2012). Mutagenesis and a tethering assay revealed that the direct interaction between PABP and ARS stimulates the 3'CITE-mediated translation of RCNMV RNA1 and that the PABP–ARS interaction is required for the recruitment of eukaryotic translation initiation factors (eIFs), such as eIF4s or eIF(iso)4Fs, to the 3' UTR and of the 40S ribosomal subunit to the viral mRNA (Iwakawa et al. 2012). Dianthoviruses might have evolved the ARS and 3'CITE as substitutes for the 3' poly(A) tail and the 5' cap of eukaryotic mRNAs for the efficient recruitment of eIFs, PABP, and ribosomes to the uncapped/nonpolyadenylated viral mRNA.

Protein p88, which overlaps N-terminally with p27, is produced by programmed –1 ribosomal frameshifting (–1 PRF) (Kim and Lommel 1994). In addition, the production of p88 requires long-distance base pairing between a bulge sequence in the stem-loop structure predicted just downstream of the –1 PRF site and the loop sequence of a small stem-loop structure predicted between the ARS and the 3'CITE (Tajima et al. 2011). Because p88, but not p27, is required in *cis* for the replication of RNA1 (Okamoto et al. 2008), the existence of RNA elements responsible for –1 PRF and 3'CITE-mediated translation in the 3' UTR of RNA1 might be important for switching translation to replication of RNA1 and for regulating the ratio of p88 to p27.

Roles of viral replication proteins in RNA replication

Viral RdRP is a key enzyme in the synthesis of viral RNAs. In addition to the RdRP, many positive-strand RNA viruses encode an auxiliary replication protein. Accumulating

evidence indicates that the viral auxiliary replication proteins play crucial roles in recognizing viral RNA templates, targeting them to RNA replication sites, and assembling the viral replicase complexes. Thus, the auxiliary replication proteins play multiple roles in the accomplishment of viral RNA replication.

For example, the auxiliary replicase protein p33 of *Tombusvirus* binds directly to an internal replication element present in the viral replicon RNA (Pogany et al. 2005), interacts with p92 RdRP (p92^{pol}) via a protein–protein interaction, and recruits the replicon RNA and p92^{pol} to peroxisomal membranes (Panavas et al. 2005). These protein–RNA and protein–protein interactions are required for the assembly of the *Tombusvirus* replicase complex. In Brome mosaic virus (BMV), which has a tripartite genome, the 1a auxiliary replication protein recognizes RNA elements that are present at the 5' end of RNA2 and in the intergenic region of RNA3 (Chen et al. 2001; Schwartz et al. 2002). The 1a protein, 2a RdRP (2a^{pol}), and viral nucleotide sequences are required for the assembly of the functional BMV replicase complex. The 1a protein recruits replication templates and 2a^{pol} to the endoplasmic reticulum (ER) membrane, which is the site of BMV RNA replication (Liu et al. 2009; Schwartz et al. 2002).

In RCNMV, the p27 auxiliary replication protein binds specifically and directly to a Y-shaped RNA element (YRE) located in the 3' UTR of RNA2. The replicase–YRE interaction is critical for recruiting RNA2 to the membrane fraction (Hyodo et al. 2011; Iwakawa et al. 2011). The domains in p27 that are required for YRE binding were mapped to its central and C-terminal regions, and the critical amino acids of these regions were identified (Hyodo et al. 2011). The C-terminal half of p27 is also involved in the interaction with p88 (Mine et al. 2010a) and localizes at the ER membrane, together with p88 (Turner et al. 2004). The p27–p88 interaction is required for the formation of RCNMV replication complexes, as described below.

Viral RNA replication complexes and host proteins involved in RNA replication

A useful approach to identify host genes affecting virus RNA replication is a systematic genome-wide screen using yeast single-gene deletion or essential-gene libraries, when the viruses of interest can replicate in yeast (Nagy 2008). Using yeast systems, about 130 and 100 genes that affect the replication of tomato bushy stunt virus (TBSV) and BMV, respectively, have been identified (Nagy 2008). Functional analyses in several of these genes demonstrated that the encoded proteins include RNA-binding proteins, cellular chaperons, membrane-shaping proteins, proteins

associated with membrane remodeling and lipid synthesis, and others (Nagy and Pogany 2012). Screens of mutant *Arabidopsis* plants also led to the identification of several genes, such as TOM1 and TOM3, that encode membrane proteins and affect tomato mosaic virus replication (Yamanaka et al. 2000).

Host proteins required for plant RNA virus replication have also been identified by purifying membrane-associated viral RNA replication complexes using biochemical and immunological methods, followed by mass spectrometry and other methods. This approach led to the identification of several proteins, including a yeast homologue of mammalian glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for TBSV, the eukaryotic translation elongation factor 1A for TBSV and *Turnip mosaic virus*, heat shock protein 70 (Hsp70) for TBSV (Nagy and Pogany 2012), and Hsp70, Hsp90, and ADP-ribosylation factor 1 (ARF1) for RCNMV, as described below.

RCNMV RNA replication complexes were isolated from virus-infected plant tissues using immunoprecipitation and the membrane-associated replication complexes were analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE) (Mine et al. 2010b). The purified fractions contained viral and host proteins and retained two types of RdRP activities. One was an endogenous template-bound RdRP activity that synthesizes virus-related RNAs without adding RNA templates. The other was an exogenous template-dependent RdRP activity capable of de novo initiation of complementary RNA synthesis from selected RNA templates. The RCNMV replication complexes are bound tightly with membranes, and their apparent molecular weight is 480 kDa. The complex with a template-dependent RdRP synthesizes RNA fragments by specifically recognizing the 3' terminal core promoter sequences of RCNMV RNAs.

Using mass spectrometry, we identified possible host proteins present in the affinity-purified RCNMV RNA-dependent RNA polymerase fraction and in the 480-kDa complex fraction that was purified further via BN-PAGE. The former fraction contained Hsp70, Hsp90, ubiquitin, actin, hin1-like protein, several ribosomal proteins, ARF1, and other proteins in addition to viral replication proteins and CP (Mine et al. 2010b; A. Mine and T. Okuno, unpublished data). The latter fraction contained ubiquitin, ATP synthase subunit α , hin1-like protein, and other proteins, in addition to viral replicase proteins (Mine et al. 2010b). Among these candidate proteins, we analyzed Hsp70, Hsp90, and ARF1 for their contribution to RCNMV RNA replication. Hsp70 and Hsp90 are well-known protein chaperones, and ARF1 is a highly conserved, ubiquitous, small GTPase that is implicated in the formation of the COPI vesicles on Golgi membranes. Our recent experimental data suggest that these three proteins interact

directly and colocalize with p27 within the virus-induced large punctate structures of ER membranes. Downregulation of these proteins by virus-induced gene silencing decreased RCNMV RNA accumulation in plants. Furthermore, specific inhibitors of Hsp70, Hsp90, and ARF1, such as 2-phenylethanesulfonamide (PES), geldanamycin, and brefeldin A, respectively, inhibited the formation of the 480-kDa replication complex, and the synthesis of RCNMV RNA in an in vitro cell-free viral translation/replication system and in protoplast experiments (Mine et al. 2012; K. Hyodo and T. Okuno, unpublished data). These data suggest that Hsp70, Hsp90, and ARF1 play important roles in the formation of replication complexes and viral RNA synthesis. Confocal microscopy using GFP-fused Arf1 suggested that p27 induces the redistribution of Arf1 to large aggregate structures from small punctate structures that could be the Golgi apparatus (K. Hyodo and T. Okuno, unpublished data). Interestingly, inhibition of the nucleotide exchange activity of ARF1 by brefeldin A disrupts the ER-localization of p27 in RCNMV-infected cells. Although the precise role of Arf1 in the replication of RCNMV RNA is unclear at present, it is tempting to speculate that p27 functions as the recruiter of Arf1 to the replication site and acts in viral RNA replication via membrane modification and the formation of RNA replication complexes at the membrane. It is noteworthy that enteroviral 3A proteins bind and modulate Arf1 and its guanine nucleotide exchange factor GBF1 to enhance the preferential recruitment of phosphatidylinositol-4-kinase IIIb (PI4KIIIb) to membranes, yielding a phosphatidylinositol 4-phosphate (PI4P) lipid-enriched microenvironment that differs from that found in uninfected cells (Hsu et al. 2010). Thus, plant and animal viruses might use common host factors to create a favorable environment for viral replication. Conversely, many host proteins used by viruses seem to differ among viruses. TBSV is affected by a set of host factors that is vastly different from that observed for BMV (Li et al. 2009). Downregulation of GAPDH, which inhibits TBSV accumulation, does not affect TMV accumulation in GAPDH-silenced plants (Wang and Nagy 2008).

Perspectives

Over the past 10 years, a large amount of information has accumulated on *cis*-acting RNA elements and host proteins that play important roles in the replication of positive-strand RNA viruses. The cumulative evidence supports the idea that viral genomic RNAs that encode replication proteins can be conductors that orchestrate multiple processes using viral and host proteins and viral RNA elements. However, our current understanding of viral RNA replication and translation mechanisms is far from

complete. Viral RNAs appear to change their structure regarding either local or global structures in a spatially and temporally regulated manner, probably via the concerted action of viral and host proteins.

Screenings of host proteins that bind viral RNAs may represent a way to study the regulatory mechanisms of viral gene expression. One approach is the use of RNA aptamer-based one-step affinity chromatography and mass spectrometry analysis to identify host proteins that bind specifically to viral RNAs. Several host proteins have been identified for RCNMV (Iwakawa et al. 2012). Another approach is a high-throughput method using yeast protein microarrays. This method has led to the identification of as many as 50 viral RNA-binding host proteins in the studies of BMV and TBSV (Li et al. 2009; Zhu et al. 2007). Further studies using proteomics and genome wide-screens of co-opted host factors will also expand this field of research. Functional analyses of the candidate host proteins using biochemical methods and single-molecule techniques in cell-free systems in combination with analyses in living cells will be needed to test the roles of candidate proteins in virus replication processes. Confocal and electron microscopy experiments will also be useful to study the manner via which the candidate proteins function spatially and temporally in virus replication processes. Our understanding of the roles of viral and host proteins and viral RNA elements in viral replication may lead to the development of novel strategies aimed at controlling viral diseases.

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References

- Ahlquist P, Noueiry AO, Lee WM, Kushner DB, Dye BT (2003) Host factors in positive-strand RNA virus genome replication. *J Virol* 77:8181–8186
- Chen J, Noueiry A, Ahlquist P (2001) *Brome mosaic virus* protein 1a recruits viral RNA2 to RNA replication through a 5' proximal RNA2 signal. *J Virol* 75:3207–3219
- den Boon JA, Diaz A, Ahlquist P (2010) Cytoplasmic viral replication complexes. *Cell Host Microbe* 8:77–85
- Hiruki C (1987) The dianthoviruses: a distinct group of isometric plant viruses with bipartite genome. *Adv Virus Res* 33:257–300
- Hsu NY, Ilnytska O, Belov G, Santiana M, Chen YH, Takvorian PM, Pau C, van der Schaar H, Kaushik-Basu N, Balla T, Cameron CE, Ehrenfeld E, van Kuppeveld FJM, Altan-Bonnet N (2010) Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell* 141:799–811
- Hyodo K, Mine A, Iwakawa H-O, Kaido M, Mise K, Okuno T (2011) Identification of amino acids in auxiliary replicase protein p27 critical for its RNA-binding activity and the assembly of the replicase complex in *Red clover necrotic mosaic virus*. *Virology* 413:300–309
- Iwakawa H-O, Mine A, Hyodo K, An M, Kaido M, Mise K, Okuno T (2011) Template recognition mechanisms by replicase proteins differ between bipartite positive-strand genomic RNAs of a plant virus. *J Virol* 85:497–509
- Iwakawa H-O, Tajima Y, Taniguchi T, Kaido M, Mise K, Taniguchi H, Okuno T (2012) Poly(A)-binding protein facilitates translation of an uncapped/nonpolyadenylated viral RNA by binding to the 3' untranslated region. *J Virol* 86:7836–7849
- Kaido M, Funatsu N, Tsuno Y, Mise K, Okuno T (2011) Viral cell-to-cell movement requires formation of cortical punctate structures containing Red clover necrotic mosaic virus movement protein. *Virology* 413:205–215
- Kim KH, Lommel SA (1994) Identification and analysis of the site of –1 ribosomal frameshifting in red clover necrotic mosaic virus. *Virology* 200:574–582
- Li Z, Pogany J, Panavas T, Xu K, Esposito AM, Kinzy TG, Nagy PD (2009) Translation elongation factor 1A is a component of the tombusvirus replicase complex and affects the stability of the p33 replication co-factor. *Virology* 385:245–260
- Liu L, Westler WM, den Boon JA, Wang X, Diaz A, Steinberg HA, Ahlquist P (2009) An amphipathic α -helix controls multiple roles of Brome mosaic virus protein 1a in RNA replication complex assembly and function. *PLoS Pathog* 5(3):e1000351
- Mine A, Hyodo K, Takeda A, Kaido M, Mise K, Okuno T (2010a) Interactions between p27 and p88 replicase proteins of *Red clover necrotic mosaic virus* play an essential role in viral RNA replication and suppression of RNA silencing via the 480-kDa viral replicase complex assembly. *Virology* 407:213–224
- Mine A, Takeda A, Taniguchi T, Taniguchi H, Kaido M, Mise K, Okuno T (2010b) Identification and characterization of the 480-kilodalton template-specific RNA-dependent RNA polymerase complex of *Red clover necrotic mosaic virus*. *J Virol* 84:6070–6081
- Mine A, Hyodo K, Tajima Y, Kusumanegara K, Taniguchi T, Kaido M, Mise K, Taniguchi H, Okuno T (2012) Differential roles of Hsp70 and Hsp90 in the assembly of the replicase complex of a positive-strand RNA plant virus. *J Virol* (in press)
- Mizumoto H, Tatsuta M, Kaido M, Mise K, Okuno T (2003) Cap-independent translational enhancement by the 3' untranslated region of *Red clover necrotic mosaic virus* RNA1. *J Virol* 77:12113–12121
- Nagy PD (2008) Yeast as a model host to explore plant virus–host interactions. *Ann Rev Phytopathol* 46:217–242
- Nagy PD, Pogany J (2012) The dependence of viral RNA replication on co-opted host factors. *Nat Rev Microbiol* 10:137–149
- Nicholson BL, White KA (2011) 3'Cap-independent translation enhancers of positive-strand RNA plant viruses. *Curr Opin Virol* 1:373–380
- Okamoto K, Nagano H, Iwakawa H, Mizumoto H, Takeda A, Kaido M, Mise K, Okuno T (2008) *cis*-Preferential requirement of a-1 frameshift product p88 for the replication of *Red clover necrotic mosaic virus* RNA1. *Virology* 375:205–212
- Panavas T, Hawkins CM, Panaviene Z, Nagy PD (2005) The role of the p33:p33/p92 interaction domain in RNA replication and intracellular localization of p33 and p92 proteins of *Cucumber necrosis* tombusvirus. *Virology* 338:81–95
- Pogany J, White KA, Nagy PD (2005) Specific binding of tombusvirus replication protein p33 to an internal replication element in the viral RNA is essential for replication. *J Virol* 79:4859–4869
- Schwartz M, Chen J, Janda M, Sullivan M, den Boon J, Ahlquist P (2002) A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol Cell* 9:505–514

- Sit TL, Vaewhongs AA, Lommel SA (1998) RNA-mediated trans-activation of transcription from a viral RNA. *Science* 281:829–832
- Tajima Y, Iwakawa H, Kaido M, Mise K, Okuno T (2011) A long-distance RNA–RNA interaction plays an important role in programmed –1 ribosomal frameshifting in the translation of p88 replicase protein of *Red clover necrotic mosaic virus*. *Virology* 417:169–178
- Tatsuta M, Mizumoto H, Kaido M, Mise K, Okuno T (2005) The *Red clover necrotic mosaic virus* RNA2 trans-activator is also a cis-acting RNA2 replication element. *J Virol* 79:978–986
- Turner KA, Sit TL, Callaway AS, Allen NS, Lommel SA (2004) Red clover necrotic mosaic virus replication proteins accumulate at the endoplasmic reticulum. *Virology* 320:276–290
- Wang RY-L, Nagy PD (2008) *Tomato bushy stunt virus* co-opts the RNA-binding function of a host metabolic enzyme for viral genomic RNA synthesis. *Cell Host Microbes* 3:178–187
- Xiong Z, Kim KH, Giesman-Cookmeyer D, Lommel SA (1993) The roles of the red clover necrotic mosaic virus capsid and cell-to-cell movement proteins in systemic infection. *Virology* 192:27–32
- Yamanaka T, Ohta T, Takahashi M, Meshi T, Schmidt R, Dean C, Naito S, Ishikawa M (2000) *TOM1*, an *Arabidopsis* gene required for efficient multiplication of a tobamovirus, encodes a putative transmembrane protein. *Proc Natl Acad Sci USA* 97:10107–10112
- Zhu J, Gopinath K, Murali A, Yi G, Hayward SD, Zhu H, Kao C (2007) RNA-binding proteins that inhibit RNA virus infection. *Proc Natl Acad Sci USA* 104:3129–3134