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Replication mechanisms of plant RNA viruses

-Current understanding and perspective-

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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, with very few exceptions for animal viral diseases. Although virus resistance genes have been used to minimize the loss of crop production in several important plant 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 caused in part by 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 to, and rearranging the 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 the *Carnation ring spot virus* (CRSV) as the type member and the *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) with 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

58 between RNA1 and RNA2 (Sit et al. 1998; Tatsuta et al. 2005). RNA2 is a
59 monocistronic RNA that encodes a movement protein (MP), which is required for viral
60 cell-to-cell movement in plants (Kaido et al. 2011; Xiong et al. 1993).
61
62 **Translation of RCNMV replicase proteins**
63
64 Many plant RNA viruses, including members of the *Tombusviridae* and the
65 *Luteoviridae* families, lack both a 5′ cap and a 3′ poly(A) tail. Instead, they have
66 cap-independent translation elements (CITEs) in the 3′ untranslated region (UTR) of
67 their genomic RNAs (Nicholson and White 2011). In some of these viruses, 3′CITEs
68 bind the eukaryotic translation initiation factor complex eIF4F or eIFiso4F (Iwakawa et
69 al. 2012; Nicholson and White 2011).
70 RCNMV RNA1 and RNA2 possess neither a cap structure at the 5′ end nor a
71 poly(A) tail at the 3′ end (Mizumoto et al. 2003). Therefore, RCNMV proteins must be
72 translated via cap-independent translation mechanisms. Two RNA elements play
73 essential roles in the cap-independent translation of the replicase proteins encoded in
74 RNA1. One element is the 3′CITE that consists of five stem-loop structures in the 3′
75 UTR of RNA1 (Mizumoto et al. 2003). Another element is an A-rich sequence
76 (ARS) located upstream of the 3′CITE (Iwakawa et al. 2012). A search for cellular

77 factors that bind the 3′ UTR of RNA1 using RNA aptamer-based one-step affinity
78 chromatography and mass spectrometry analysis led to the identification of the ARS as
79 an RNA element that binds to poly(A)-binding protein (PABP) (Iwakawa et al. 2012).
80 Mutagenesis and a tethering assay revealed that the direct interaction between PABP
81 and ARS stimulates the 3′CITE-mediated translation of RCNMV RNA1, and that the
82 PABP–ARS interaction is required for the recruitment of eukaryotic translation
83 initiation factors (eIFs), such as eIF4s or eIF(iso)4Fs, to the 3′ UTR and of the 40S
84 ribosomal subunit to the viral mRNA (Iwakawa et al. 2012). Dianthoviruses might have
85 evolved the ARS and 3′CITE as substitutes for the 3′ poly(A) tail and the 5′ cap of
86 eukaryotic mRNAs for the efficient recruitment of eIFs, PABP, and ribosomes to the
87 uncapped/nonpolyadenylated viral mRNA.

88 p88, which overlaps N-terminally with p27, is produced by programmed –1
89 ribosomal frameshifting (–1 PRF) (Kim and Lommel 1994). In addition, the production
90 of p88 requires the long-distance base pairing between a bulge sequence in the
91 stem-loop structure predicted just downstream of the –1 PRF site and the loop sequence
92 of a small stem-loop structure predicted between the ARS and the 3′CITE (Tajima et al.
93 2011). Because p88, but not p27, is required in *cis* for the replication of RNA1
94 (Okamoto et al. 2008), the existence of RNA elements responsible for −1 PRF and
95 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 the 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. 2010b) 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

One of the useful approaches 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 the 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 Arabidopsis mutant 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. 2010a). The purified fractions contained viral and host proteins and retained two types

153 of RdRP activities. One was an endogenous template-bound RdRP activity that
154 synthesizes virus-related RNAs without adding RNA templates. The other was an
155 exogenous template-dependent RdRP activity capable of *de novo* initiation of
156 complementary RNA synthesis from selected RNA templates. The RCNMV replication
157 complexes are bound tightly with membranes and their apparent molecular weight is
158 480 kDa. The complex with a template-dependent RdRP synthesizes RNA fragments by
159 specifically recognizing the 3′ terminal core promoter sequences of RCNMV RNAs.
160 Using mass spectrometry, we identified possible host proteins present in the
161 affinity-purified RCNMV RNA-dependent RNA polymerase fraction, or in the 480 kDa
162 complex fraction that was purified further via BN–PAGE. The former fraction
163 contained Hsp70, Hsp90, ubiquitin, actin, hin1-like protein, several ribosomal proteins,
164 ARF1, and other proteins in addition to viral replication proteins and CP (Mine et al.
165 2010a; A. Mine and T. Okuno, unpublished data). The latter fraction contained
166 ubiquitin, ATP synthase subunit α, hin1-like protein, and other proteins, in addition to
167 viral replicase proteins (Mine et al. 2010a). Among these candidate proteins, we
168 analyzed Hsp70, Hsp90, and ARF1 for their contribution to RCNMV RNA replication.
169 Hsp70 and Hsp90 are well-known protein chaperones and ARF1 is a highly conserved,
170 ubiquitous, small GTPase that is implicated in the formation of the COPI vesicles on
171 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-phenylethynesulfonamide (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 (A. Mine, 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

191 preferential recruitment of phosphatidylinositol-4-kinase IIIb (PI4KIIIb) to membranes,
192 yielding a phosphatidylinositol 4-phosphate (PI4P) lipid-enriched microenvironment
193 that differs from that found in uninfected cells (Hsu et al. 2010). Thus, plant and animal
194 viruses might use common host factors to create a favorable environment for viral
195 replication. Conversely, many host proteins used by viruses seem to differ among
196 viruses. TBSV is affected by a set of host factors that is vastly different from that
197 observed for BMV (Li et al. 2009). Down regulation of GAPDH, which inhibits TBSV
198 accumulation, does not affect TMV accumulation in GAPDH-silenced plants (Wang
199 and Nagy 2008).
200
201 **Perspective**
202
203 Over the past 10 years, a large amount of information has been accumulated on
204 *cis*-acting RNA elements and host proteins that play important roles in the replication of
205 positive-strand RNA viruses. The cumulative evidence supports the idea that viral
206 genomic RNAs that encode replication proteins can be conductors that orchestrate
207 multiple processes using viral and host proteins and viral RNA elements. However, our
208 current understanding of viral RNA replication and translation mechanisms is far from
209 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 of the approaches 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.

229

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