Molecular Biology and Epidemiology of Dianthoviruses

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Abstract

The genus *Dianthovirus* is one of eight genera in the family *Tombusviridae*. All the genera have monopartite positive-stranded RNA genomes, except the dianthoviruses which have bipartite genomes. The dianthoviruses are distributed worldwide. Although they share common structural features with the other *Tombusviridae* viruses in their virions and the terminal structure of the genomic RNAs, the bipartite nature of the dianthovirus genome offers an ideal experimental system with which to study basic issues of virology. The two genomic RNAs seem to use distinct strategies to regulate their translation, transcription, genome replication, genome packaging, and cell-to-cell movement during infection. This review summarizes the current state of our knowledge of the dianthoviruses, with its main emphasis on the molecular biology of the virus, including the viral and host factors required for its infection of host plants. The epidemiology of the virus and the possible viral impacts on agriculture and the environment are also discussed.

1. INTRODUCTION

Since the dianthoviruses were established as a new plant virus group by the International Committee on Taxonomy of Viruses (ICTV) in 1981 (Matthews, 1982), much information has been collected not only on their basic characteristics but also on the effects of their infection of various plant species and the behavior of the viruses in different environments. While members of this group are small in number, they cause characteristic necrosis, both local and systemic, which is often associated with severe dwarfing, a frequent cause of serious reductions in crop yields. The number of *Dianthovirus* species may increase as the molecular genetic characterization of suspected viruses' advances. The viral particles are stable and abundant in reproduction, and are therefore suitable for experimental studies in many aspects.

Our knowledge of the molecular mechanisms underlying the infection processes of the dianthoviruses has increased over the past two decades, after their infectious cDNA clones became available (Ge, Hiruki, & Roy, 1993; Sit, Haikal, Callaway, & Lommel, 2001; Xiong & Lommel, 1991) since the complete nucleotide sequences of their genomes were determined (Ge, Hiruki, & Roy, 1992; Ge et al., 1993; Kendall & Lommel, 1992; Lommel, Weston–Fina, Xiong, & Lomonossoff, 1988; Ryabov, Generozov, Kendall, Lommel, & Zavriev, 1994; Xiong & Lommel, 1989).

This review summarizes the current state of our knowledge of the dianthoviruses, with its main emphasis on recent advances in our

understanding of the molecular mechanisms underlying the genome strategies used for translation, replication, and cell-to-cell movement during infection. The epidemiology and possible impacts of the dianthoviruses on agriculture and the environment are also discussed.

2. DIANTHOVIRUSES

The group consists of four viruses: the type member is *Carnation ringspot virus* (CRSV) and the other members are *Red clover necrotic mosaic virus* (RCNMV), *Sweet clover necrotic mosaic virus* (SCNMV) (Hiruki, 1987); *Furcraea necrotic streak virus* (FNSV) is a tentative member of the genus at present (Morales, Castaño, Calvert, & Arroyave, 1992).

2.1. CRSV

Symptoms such as leaf mottling and ringspotting, plant stunting, and flower distortion persist after the infection of *Dianthus caryophyllus* and *D. barbatus* by CRSV. CRSV probably occurs worldwide, wherever *Dianthus* species are grown. Virions are distributed in all parts of the infected plant, including in the cell cytoplasm and nuclei. Several species are susceptible to CRSV, showing systemic or local symptoms (Tremaine & Dodds, 1985). Its transmission by nematodes *Longidorus elongatus*, *L. macrosoma*, and *Xiphinema diversicaudatum* has been reported (Fritzsche, Kegler, Thiele, & Gruber, 1979) but is still contentious (Brunt, Crabtree, Dallwitz, Gibbs, & Watson, 1996).

2.2. RCNMV

Symptoms such as necrotic lesions accompanied with mosaic and leaf distortion persist in *Trifolium pratense*, *T. repens*, *Medicago sativa*, and *Melilotus officinalis* infected with RCNMV. The virus was first reported in the former Czechoslovakia (Musil, 1969; Musil & Matisová, 1967) but is known to occur in the United Kingdom, Australia, Canada, New Zealand, Poland, Sweden, and the United States. The virus was reported to be experimentally transmissible by the chytrid *Olpidium radicale* but is not transmitted by seed (Gerhardson & Insunza, 1979).

2.3. SCNMV

The main symptoms of SCNMV are mosaic, ringspots, and systemic veinal necrosis in the leaves of *M. officinalis* and *M. alba* (Hiruki, Rao, Chen,

Okuno, & Figueiredo, 1984). The most severe symptoms include stunting and distortion. SCNMV is widely spread in sweet clover growing in regions of dark-gray soil and black soil in Alberta, Canada (Hiruki, 1986a, 1986b, 1987). A different strain of the virus occurs in *M. sativa* (Pappu & Hiruki, 1989; Pappu, Hiruki, & Inouye, 1988). SCNMV is transmitted by contact and by drainage water but not by *Olpidium brassicae* (Hiruki, 1986b, 1994). Sixteen of 25 species in 6 dicotyledonous families were infected by inoculation with sap (Hiruki, 1986a, 1987).

2.4. FNSV

The major symptoms of FNSV are plant stunting and chlorotic streaks, which later coalesce to produce necrosis, drastically reducing the length and quality of the fiber of *Furcraea* (fique) spp., the most important fiber crops in Colombia. The virus is transmitted by mechanical inoculation and by grafting but not transmitted by *Planococcus citri*, *Saissetia coffeae*, *Xiphinema*, or *Trichodorus* spp. (Morales et al., 1992). The virus is consistently detected in the roots of infected fique plants (Morales et al., 1992).

2.5. Other suspected dianthoviruses

In 1996, Editors of *Viruses of Plant Viruses* included the following statement (Brunt et al., 1996, p. 1069) that was apparently collated by Dr. M. Hollings in 1980 under *Taxonomy and Relationships* of *Red clover necrotic mosaic dianthovirus*. Virus(es) with serologically unrelated virions: 60 strains of 45 isometric viruses. Some isolates induce different symptoms, for example, with those from England and Scotland are different. *Tetragonia expansa* is infected systemically only by RCNMV strain TpM–34.

An RNA with a gene organization very similar to that of dianthovirus RNA1 was reported in grassy stunt–diseased rice plants, although no virions were isolated nor any biological characters investigated (Miranda, Aliyar, & Shirako, 2001).

3. ECONOMIC IMPORTANCE OF DISEASES CAUSED BY DIANTHOVIRUSES

The economic importance of the diseases caused by the dianthoviruses varies according to the kind of crop affected. CRSV, the type virus of the dianthovirus group, is fairly common, infecting *Dianthus* spp. worldwide, spreading across the Central Asian region and India, the Eurasian region,

and the North American region. It has been found in Australia but with no evidence of field spread (Brunt et al., 1996).

RCNMV has been reported to occur in the former Czechoslovakia (Musil, 1969; Musil & Matisová, 1967), Sweden (Gerhardson & Lindsten, 1973), and the United Kingdom (Bowen & Plumb, 1979). It has been found in Australia, Canada, Poland, and New Zealand but with no evidence of field spread (Brunt et al., 1996).

In Alberta, Canada, the production of forage seeds, as well as hay, is very important in supporting the $3 billion cattle industry in the province, which is a major source of income in agricultural industry. Sweet clover is also important to beekeeping as a source of nectar and pollen, and beekeeping is an important enterprise in Alberta's agriculture diversification strategy. It contributes almost $15 million to the agricultural economy. SCNMV infection of sweet clover not only hampers plant growth but also significantly diminishes the production of nectar and pollen.

The genus *Furcraea* (Agavaceae) contains several species found primarily in the American tropics. In Colombia, South America, four varieties of *Furcraea* spp., collectively known as "fique," constitute the major fiber crop with over 40,000 tons of "cabuya" fiber produced annually by small farmers. Among the various biotic constraints that affect commercial *Furcraea* production, necrotic streak caused by FNSV is the most difficult to control because no reliable control methods are available (Morales et al., 1992).

4. MOLECULAR BIOLOGY OF DIANTHOVIRUS

Dianthovirus belongs to the family *Tombusviridae*. The unifying feature of this family is that each member has a highly conserved RNA-dependent RNA polymerase (RdRP) motif. The family *Tombusviridae* is classified into the supergroup that includes the families *Flaviviridae* and *Luteoviridae* and the phage lineage (Koonin, 1991; Koonin & Dolja, 1993). The molecular mechanisms underlying the infection processes of *Dianthovirus*, including its translation, RNA replication, packaging, cell-to-cell movement, and suppression of RNA silencing, have been investigated predominantly in RCNMV (Basnayake, Sit, & Lommel, 2006; Giesman-Cookmeyer & Lommel, 1993; Mine & Okuno, 2012; Okuno, 2012; Takeda et al., 2005). Recent studies have increased our understanding of how the dianthoviruses replicate in host cells during successful infection. They use several unique strategies to regulate the expression of viral genes, to form viral replication factories by

recruiting host proteins on cellular membranes, and to counteract host defense mechanisms. Here, we comprehensively review the molecular biology of *Dianthovirus*.

4.1. Genome organization

The genomes of the dianthoviruses consist of two positive-sense single-stranded RNAs, RNA1 and RNA2 (Gould, Francki, Hatta, & Hollings, 1981; Hiruki, 1987; Okuno, Hiruki, Rao, & Figueiredo, 1983). This bipartite nature of the genome is unique among the viruses of the family *Tombusviridae*, the genomes of which are usually monopartite. RNA1 (3.9 kb) encodes the RNA replicase components, an auxiliary 27-kDa protein (p27), and an N-terminally overlapping 88-kDa protein (p88) (Xiong, Kim, Kendall, & Lommel, 1993; Xiong & Lommel, 1989) that contains RdRP motif (Koonin & Dolja, 1993). RNA1 also encodes a coat protein (CP), which is translated from subgenomic RNA (sgRNA) (Sit, Vaewhongs, & Lommel, 1998; Tatsuta, Mizumoto, Kaido, Mise, & Okuno, 2005; Zavriev, Hickey, & Lommel, 1996). RNA1 can replicate in a single cell in the absence of RNA2 (Osman & Buck, 1987; Paje-Manalo & Lommel, 1989; Pappu & Hiruki, 1988). RNA2 encodes a 35-kDa movement protein (MP), which is essential for the cell–to-cell and systemic movement in plants (Osman & Buck, 1987; Xiong, Kim, Giesman-Cookmeyer, & Lommel, 1993). In addition to the genomic RNAs, a small noncoding RNA (0.4 kb) that consists of nearly the entire region of the 3′-untranslated region (UTR) of RNA1 accumulates in dianthovirus-infected plants and protoplasts (Iwakawa et al., 2008; H. Nagano, K. Omote, & T. Okuno, unpublished data). The genomic organization of RCNMV, several RNA elements of which are described in this review, is shown in Fig. 2.1.

4.2. Cap-independent translation

The genomic RNAs of RCNMV lack both a 5′-cap structure (Mizumoto, Tatsuta, Kaido, Mise, & Okuno, 2003) and a 3′ poly(A) tail (Lommel et al., 1988; Mizumoto, Hikichi, & Okuno, 2002; Mizumoto et al., 2003; Xiong & Lommel, 1989). Mutagenesis studies using a reporter mRNA that included the firefly luciferase (*Luc*) gene revealed that RNA1 contains an RNA element essential for the cap-independent translation in the 3′-UTR (Mizumoto et al., 2003). The element designated "3′TE-DR1" is predicted to have five stem-loop (SL) structures (Mizumoto et al., 2003;
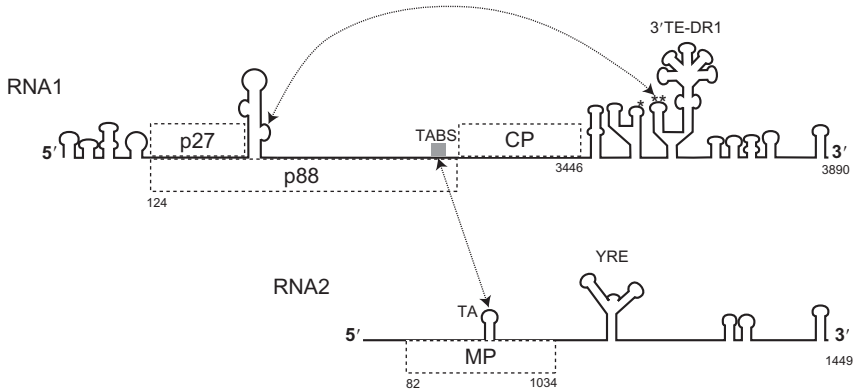
Figure 2.1 Schematic representation of the genome structure of RCNMV, with the predicted RNA structures. TABS, *trans*-activator-binding site; 3′TE-DR1, cap-independent translational enhancer; TA, *trans*-activator; YRE, Y-shaped RNA element. *A-rich sequence (ARS): the PABP-binding site. **Loop sequences required for the long-distance RNA–RNA interaction that facilitates −1PFS. Nucleotide numbers from the 5′ end are shown below the lines.

Sarawaneeyaruk et al., 2009), and this overall structure has been confirmed by structure probing (Wang, Kraft, Hui, & Miller, 2010; Y. Tajima & T. Okuno, unpublished data). Of these SLs, the 5′-proximal SL and two 3′-proximal SLs are required for its efficient translation (Mizumoto et al., 2003; Sarawaneeyaruk et al., 2009).

Cap–independent translation enhancers like 3′TE-DR1 have been identified in the 3′-UTRs of many viral RNA genomes (Kneller, Rakotondrafara, & Miller, 2006; Nicholson & White, 2011). These RNA elements replace the cap–structure and poly(A) tail in facilitating the translation of viral proteins and are termed as 3′ cap–independent translation elements (3′CITEs). The 3′CITEs of plant RNA viruses are classified into several structural classes (Miller, Wang, & Treder, 2007; Nicholson & White, 2011). The 5′-proximal SL of 3′TE-DR1 that is conserved in dianthoviruses, including SCNMV and CRSV, is very similar to an essential SL structure in the 3′CITE of *Barley yellow dwarf virus* (BYDV) (Guo, Allen, & Miller, 2000; Mizumoto et al., 2003; Shen & Miller, 2004).

3′CITEs interact with the eukaryotic initiation factors eIF4F and eIFiso4F (Gazo, Murphy, Gatchel, & Browning, 2004; Nicholson, Wu, Chevtchenko, & White, 2010; Treder et al., 2008; Wang, Treder, & Miller, 2009) or the 60S ribosomal subunits (Stupina et al., 2008). The 3′-UTR of RCNMV RNA1 interacted with the eIF4F/iso4F components in an RNA-aptamer (Strepto-Tag) affinity assay in a cell-free extract of

evacuolated BY-2 protoplasts (BYL) (Iwakawa et al., 2012). BYL (Komoda, Naito, & Ishikawa, 2004) efficiently supports the 3′TE-DR1-mediated cap-independent translation and negative-strand RNA synthesis of RCNMV (Iwakawa, Kaido, Mise, & Okuno, 2007). Mutations in the 5′-proximal SL of 3′TE-DR1 abolish the association of these translation initiation factors with the 3′-UTR.

Poly(A)-binding protein (PABP) also coprecipitates with the Strepto-Tag-fused 3′-UTR (Iwakawa et al., 2012). The binding site of PABP resides in an adenine-rich sequence (ARS) (3518-AAACAGUAAAAUUGC AAAAAA-3538) located 60 nucleotides upstream from 3′TE-DR1 in the 3′-UTR. Interestingly, the mutations in ARS that compromise PABP binding also compromise the binding of the eIF4F/iso4F components and abolish 3′TE-DR1-mediated cap-independent translation, whereas the mutations in 3′TE-DR1 that abolish translation have no effect on PABP binding. Therefore, PABP binding seems to be required for the recruitment of the eIF4F/iso4F components to 3′TE-DR1. The binding of both the eIF4F/iso4F components and PABP to the 3′ RNA elements is required for the efficient recruitment of the 48S and 80S ribosome complexes to the viral RNA (Iwakawa et al., 2012).

Some form of communication must occur between the 5′-UTR and the 3′CITE of the viral RNAs to deliver translation factors for recruiting ribosomes to the 5′ translation initiation sites. Such communications could be mediated by long-distance RNA–RNA base pairing, as reported for BYDV (Guo, Allen, & Miller, 2001), *Black currant reversion nepovirus* (Karetnikov & Lehto, 2008), *Carnation Italian ringspot tombusvirus* (Nicholson & White, 2008), *Cucumber leaf spot aureusvirus* (Xu & White, 2009), *Saguaro cactus virus* (Chattopadhyay, Shi, Yuan, & Simon, 2011), and *Tomato bushy stunt virus* (TBSV) (Fabian & White, 2004, 2006). Alternatively and additionally, mechanisms other than RNA–RNA base pairing could mediate the 5′–3′ interaction of viral RNAs. This is the case for RCNMV RNA1.

The requirement for the RNA1 5′-UTR in 3′TE-DR1-mediated cap-independent translation differs greatly between plant species (Sarawaneeyaruk et al., 2009). The deletion of any one of the four SLs predicted in the 5′-UTR of RNA1 inhibited the 3′TE-DR1-mediated cap-independent activity of reporter mRNAs in BY-2 tobacco protoplasts, whereas their deletion had no effect in cowpea protoplasts. The RNA1 5′-UTR contributes to RNA stability in BY-2 protoplasts. A compensatory mutagenesis analysis used to identify possible interactions between the 5′- and 3′-UTRs of RNA1 in BY-2 protoplasts suggested that no long-distance RNA–RNA interaction is essential for 3′–5′ communication during

3′TE-DR1-mediated cap-independent translation (Sarawaneeyaruk et al., 2009; S. Sarawaneeyaruk & T. Okuno, unpublished data). Similarly, little or no involvement of RNA–RNA interactions between the 5′- and 3′-UTRs has been reported in the 3′CITE-mediated cap-independent translation of *Pea enation mosaic virus* RNA 2 (Wang et al., 2009), satellite *Tobacco necrosis virus* (Gazo et al., 2004), or *Turnip crinkle virus* (TCV) (Qu & Morris, 2000; Stupina et al., 2008). The mechanisms for delivering the translation initiation factors and translation initiation ribosome complexes to the 5′-UTR of the viral RNAs in these viruses, including RCNMV, remain to be resolved.

CP is translated from CPsgRNA, which is transcribed from RNA1 and coterminates with RNA1. This implies that CP is translated in a 3′TE-DR1-mediated cap-independent manner. Indeed, uncapped chimeric CPsgRNA, in which the CP open reading frame (ORF) is substituted with the Luc ORF, is translated as efficiently as RNA1 (Sarawaneeyaruk et al., 2009).

In contrast to RNA1 and CPsgRNA, RNA2 lacks RNA elements, such as ARS and 3′TE-DR1, that function efficiently in a reporter mRNA. Reporter Luc mRNAs with the 5′- and 3′-UTRs of RNA2 (R2–UTR–Luc) or with the Luc ORF inserted between the 5′-UTR and the MP ORF (RNA2–Luc) in RNA2 are not translated efficiently in protoplasts in the absence of the 5′-cap structure (Mizumoto, Iwakawa, Kaido, Mise, & Okuno, 2006). The cap-independent translational activities of R2–UTR–Luc and RNA2–Luc are less than 1% of 3′TE-DR1-mediated cap-independent translation. Instead, RNA2–Luc, which can replicate in the presence of RCNMV replicase proteins, functions as an efficient mRNA when replicated (Mizumoto et al., 2006). This suggests a strong link between the cap-independent translation of RNA2 and RNA replication and a difference in the translational mechanisms of RNA1 and RNA2. Host factors associated with the viral RNA replication process might facilitate the translation of RNA2. Alternatively, RNA2 may have a silencer element(s) that suppresses the translation of RNA2. The low translational activity of RNA2 may be comprehensible, if MP is only required in the late stage of replication to move the virus to neighboring cells. The difference in the translation strategy of RNA1 and RNA2 may be important for the temporal, spatial, and quantitative regulation of RCNMV gene expression during infection.

4.3. Translation via −1 programmed ribosome frameshifting

−1 Programmed ribosome frameshifting is one of the translation strategies used by many RNA viruses to regulate viral gene expression (Brierley, 1995). p88 is translated by −1PRF from RCNMV RNA1 (Xiong, Kim, Kendall, et al., 1993). The *cis*-acting RNA elements required for −1PRF

have been mapped to a shifty heptanucleotide sequence GGAUUUU at the slippage site (Kim & Lommel, 1994) and a highly-structured bulged SL structure predicted just downstream from the slippage site in RNA1 (Kim & Lommel, 1998). In addition to these elements, a third *cis*-acting RNA element that facilitates −1PRF was identified using an *in vitro* translation/replication system (Tajima, Iwakawa, Kaido, Mise, & Okuno, 2011). This third RNA element is a small stable SL structure predicted between ARS and 3′TE-DR1 in the 3′-UTR of RNA1. The loop sequence of this element can base pair with the bulge of the SL adjacent to the shifty site. Such long-distance base pairing is possible in all dianthoviruses. Disruption and restoration mutagenesis analyses have demonstrated the importance of long-distance base pairing for efficient −1PRF in RCNMV RNA1 (Tajima et al., 2011). A similar requirement for long-distant RNA–RNA communication has been reported for the −1PRF of BYDV (Barry & Miller, 2002).

4.4. *cis*-Preferential requirement of replicase protein

An RNA1 mutant expressing p88 could be replicated efficiently in protoplasts when p27 was supplied in *trans*, but an RNA1 mutant expressing p27 alone could not be replicated when p88 was supplied in *trans* (Okamoto et al., 2008). Thus, only RCNMV RNA1, from which p88 is translated, is an effective template, on which viral RNA replicase can initiate RNA synthesis in the presence of p27. The *cis*-Preferential function of viral-encoded replication proteins or the coupling of translation and replication has been reported for several viruses, including *Alfalfa mosaic virus* (Neeleman & Bol, 1999; van Rossum, Garcia, & Bol, 1996), *Brome mosaic virus* (Yi & Kao, 2008), *Clover yellow mosaic virus* (White, Bancroft, & Mackie, 1992), Coronavirus (Chang, Hofmann, Sethna, & Brian, 1994), *Cowpea mosaic virus* (Van Bokhoven et al., 1993), Poliovirus (Hagino-Yamagishi & Nomoto, 1989; Johnson & Sarnow, 1991; Novak & Kirkegaard, 1994), TCV (White, Skuzeski, Li, Wei, & Morris, 1995), *Tobacco etch virus* (Mahajan, Dolja, & Carrington, 1996; Schaad, Haldeman-Cahill, Cronin, & Carrington, 1996), *Tobacco mosaic virus* (TMV) (Lewandowski & Dawson, 2000), TBSV (Oster, Wu, & White, 1998), *Turnip yellow mosaic virus* (Weiland & Dreher, 1993), and *Rubella virus* (Liang & Gillam, 2001).

4.5. RNA elements required for genome replication

The *cis*-acting RNA elements required for viral RNA replication have been identified for many viruses with mutagenesis studies, when the RNA can be

replicated by a viral replicase supplied in *trans*. However, it is difficult to apply this method to viral RNAs that encode replication proteins that are required in *cis* for RNA replication. This is the case for RCNMV RNA1.

The RNA elements required for the negative-strand synthesis of RNA1 have been determined in its 3′-UTR using capped viral RNA transcripts in BYL and BY-2 protoplasts (Iwakawa et al., 2007). The use of capped viral RNA transcripts in BYL allowed the effects of introduced mutations on cap-independent translation and the negative-strand RNA synthesis of RNA1 to be distinguished. Two SL structures at the 3′ end of RNA1 and the intervening sequence between the two SLs are essential for negative-strand synthesis (Iwakawa et al., 2007) (see Fig. 2.1). These RNA elements are conserved between RNA1 and RNA2 and between SCNMV and CRSV. Interestingly, the core RNA element of 3′TE-DR1, which is essential for cap-independent translation of RNA1, is not essential for negative-strand RNA synthesis (Iwakawa et al., 2007). Thus, the RNA elements in the 3′-UTR of RCNMV RNA1 that are required for negative-strand RNA synthesis are separated from those required for cap-independent translation.

RCNMV-Australian (Aus) and RCNMV-Canadian (Can) strains have different temperature sensitivities during infection (Mizumoto et al., 2002). RCNMV-Can does not replicate in protoplasts at temperatures higher than 22 °C, indicating that replication processes are involved in the temperature-sensitive phenotype. Mutagenesis studies have shown that the temperature sensitivity of RCNMV is attributable to at least the 3′ terminal SL of RNA1, because a single-nucleotide substitution from U to C, which changes a U–G wobble pair to a stable C–G pair in the stem of the 3′ terminal SL in RNA1-Can allows the RNA to replicate and to support RNA2 accumulation at nonpermissive temperatures (Mizumoto et al., 2002). The lack of temperature sensitivity in RNA synthesis activity in the crude membrane-bound cellular fraction prepared from RCNMV–Can–infected plants or protoplasts suggests that the promoter elements are distorted in the initiation of minus-strand synthesis at the nonpermissive temperatures (K. Shimada & T. Okuno, unpublished data).

In contrast to RNA1, RNA2 is replicated efficiently by the RCNMV replicase proteins supplied in *trans*. The core promoter of negative-strand RNA synthesis is located in the 3′ proximal region of RNA2, which is homologous to that of RNA1, except for the presence of 66 extra nucleotides upstream from the 3′ terminal SL, which are not essential for replication (Iwakawa et al., 2007; Turner & Buck, 1999). Three discontinuous

nucleotides in the loop of the 3′ terminal SL are thought to be involved in the interaction with the RCNMV RNA replicase (Weng & Xiong, 2009).

In addition to the core promoter, RNA2 has other unique replication elements. These elements include a *trans*-activator (TA) in the MP ORF (Tatsuta et al., 2005) and a Y-shaped RNA element (YRE) in the 5′ proximal region of the 3′-UTR (An et al., 2010; Iwakawa et al., 2011; Fig. 2.1). Interestingly, TA is the RNA element that interacts with RNA1 and enhances the RNA-mediated transcription of CPsgRNA (Sit et al., 1998) (see Section 4.10). Both TA and YRE play crucial roles in the negative-strand RNA synthesis of RNA2 (An et al., 2010).

YRE consists of two small SLs with a short intervening region between them on the basal stem structure (An et al., 2010). This structure is conserved among the dianthoviruses. The entire Y-shaped structure (84 nucleotides), including the two small SLs, is important for the negative-strand RNA synthesis of RNA2 (An et al., 2010). YRE is the only RNA element of RCNMV that interacted with p27 supplied in *trans*, when assessed with the RNA-aptamer (Strepto-Tag) affinity and immunoprecipitation assay in BYL (Iwakawa et al., 2011). YRE also interacts with the 480-kDa replicase complex via p27 (Iwakawa et al., 2011). The 480-kDa replicase complex contains p88 and host proteins, and is thought to be a key player in RCNMV RNA replication (Mine, Takeda, et al., 2010) (see Sections 4.7 and 4.8). The interaction between YRE and p27 is required for the recruitment of RNA2 to the endoplasmic reticulum (ER) membrane (Hyodo et al., 2011), which is the site of RCNMV RNA replication (Hyodo et al., 2013; Turner, Sit, Callaway, Allen, & Lommel, 2004).

4.6. Template recognition mechanisms of replicase proteins

RNA1 lacks the RNA elements that interact with p27 and p88 supplied in *trans*, as determined in the Strepto-Tag affinity and immunoprecipitation assay in BYL (Iwakawa et al., 2011). Interestingly, however, a protein-mediated coimmunoprecipitation analysis showed that both p27 and p88 can interact with their translating template RNA1, which are associated with ribosomes. The interaction between p27 or p88 and its translation templates is abolished or compromised, respectively, by puromycin treatment, which induces the dissociation of polyribosomes from mRNA (Blobel & Sabatini, 1971; Lehninger, Nelson, & Cox, 1993). These results imply that p27 binds to the template RNA1 via a polyribosome-dependent and puromycin-sensitive mechanism, whereas p88 mainly binds to the template RNA in

a translation-coupled, polyribosome-independent, and puromycin-tolerant manner. The interaction mechanism unique to p88 is maintained after the dissociation of the polyribosomes. p88 seems to bind specifically to the 3'-UTR of RNA1 in this translation-coupled mechanism, because SR1f, a degradation product of the 3'-UTR of RNA1 (see Section 4.11), is coimmunoprecipitated with p88 but not with p27. The translation-coupled binding of p88 may partly explain the strong *cis*-preferential requirement for p88 in the replication of RNA1 (Okamoto et al., 2008).

4.7. Composition of RNA replicase complexes

A template-bound solubilized RNA polymerase has been isolated from RCNMV-infected *Nicotiana clevelandii* plants (Bates, Farjah, Osman, & Buck, 1995). The polymerase becomes template dependent after removing endogenous RNA templates with micrococcal nuclease. The RdRP contains p27 and p88 with several unknown host proteins and produces double-stranded RNA in a template-specific manner (Bates et al., 1995).

The *Agrobacterium*-mediated expression of RCNMV replicase proteins and RNAs in *N. benthamiana* leaves allowed large detergent-solubilized membrane-associated protein complexes to be isolated, with an apparent molecular mass of 480-kDa on blue-native polyacrylamide gel electrophoresis (BN–PAGE) (Mine, Takeda, et al., 2010). The 480-kDa integral membrane complex contains both p27 and p88, and is associated with possible host proteins. In sucrose gradient sedimentation, the 480-kDa complex cofractionates with both endogenous template-bound and exogenous template-dependent RdRP activities. The 480-kDa complex corresponds predominantly to the exogenous template-dependent RdRP activity, and specifically recognizes the 3' core promoter sequences of the RCNMV genomic RNAs to produce viral RNA fragments. In contrast, the endogenous template-bound RdRP produces genome-sized RNAs without the addition of RNA templates (Mine, Takeda, et al., 2010).

Analysis of the affinity-purified solubilized membrane-bound RdRP complexes using two-dimensional BN/SDS–PAGE and mass spectrometry showed that the RdRP complexes contain many host proteins, including Hsp70, Hsp90, glyceraldehyde 3-phosphate dehydrogenase, ADP-ribosylation factor 1 (Arf1), histone deacetylase 1, ubiquitin, and several ribosomal proteins, in addition to viral replicase proteins (Hyodo et al., 2013; Mine, Takeda, et al., 2010).

In addition to the 480-kDa complex, a 380-kDa complex is formed in BYL and RCNMV-infected plant tissues expressing p27 alone or both p27 and p88

(Mine, Hyodo, et al., 2010; Mine, Takeda, et al., 2010). The 380-kDa complex could be a p27 oligomer with some host proteins.

4.8. Assembly of the replicase complex

The assembly of the viral replicase complexes of eukaryotic positive-strand RNA viruses is a regulated process: multiple viral and host proteins and template RNAs must be assembled on intracellular membranes and organized into quaternary complexes capable of synthesizing viral RNAs (Mine & Okuno, 2012; Nagy & Pogany, 2012).

p27 interacts with both p27 and p88 through direct protein–protein contacts. The C-terminal half of p27 is responsible for these interactions, whereas the nonoverlapping region unique to p88 is responsible for the p27–p88 interaction (Mine, Hyodo, et al., 2010). Both the p27–p27 and p27–p88 interactions are required for the formation of the 480-kDa complex *in vitro* and *in planta* (Mine, Hyodo, et al., 2010). A mutant p27 incapable of interacting with p27, but capable of interacting with p88, fails to form the 480-kDa complex. Another mutant p27 capable of interacting with p27, but incapable of interacting with p88, formed the 380-kDa complex but failed to form the 480-kDa complex. Thus, p27 oligomerization is a critical step in the formation of the 480-kDa complex. It appears that the formation of the 480-kDa complex is directed by the p27-oligomer (the 380-kDa complex), which interacts with the p88 protein(s).

The formation of the 480-kDa replicase complex is enhanced by the presence of the RCNMV genomic RNAs (Mine, Takeda, et al., 2010). The RNA-binding domain of p27 was identified using an RNA-aptamer (Strepto-Tag)-fused YRE that binds p27 in an affinity and immunoprecipitation assay in BYL (Hyodo et al., 2011). Deletion and alanine-scanning mutation analyses indicated that the main functional domains required for RNA binding differ from those required for protein binding, although they partially overlap.

There is a robust correlation between the RNA-binding activity of p27 and its RNA–recruiting activity to the ER membrane (Hyodo et al., 2011). Interestingly, several p27 mutants that retain the ability to bind to RNA2, to recruit RNA2 to the membrane, and to interact with both p27 and p88, fail to form the 480-kDa complex or to support RNA replication (Hyodo et al., 2011). These p27 mutants might lack the ability to interact, directly or indirectly, with the host proteins that are required for the proper assembly of the 480-kDa replicase complex.

The roles in viral RNA replication of Hsp70 and Hsp90 detected in the affinity-purified solubilized membrane-bound RdRP complexes of RCNMV (Mine, Hyodo, et al., 2010) were investigated (Mine et al., 2012). A bimolecular fluorescence complementation (BiFC) assay using confocal microscopy showed that p27, but not p88, interacts directly with both Hsp70 and Hsp90 within the p27-induced large aggregated structures on the ER in the perinuclear region. Gene silencing and the pharmacological inhibition of Hsp70 and Hsp90 compromised RCNMV RNA replication in plant cells. The inhibition of p27–Hsp70 interaction by 2-phenylethynesulfonamide (a specific inhibitor of Hsp70) inhibited the formation of the 480-kDa complex, but instead induced the formation of nonfunctional large complexes (∼1024 kDa) in BYL. Hsp70 appears to control the proper assembly of the viral replicase complexes by preventing the aggregation of p27. Alternatively, the large complexes of p27 could be intermediates in the assembly of the RCNMV replicase complex, and Hsp70 might assist the assembly of these complexes into functional replicase complexes. In contrast, the inhibition of p27–Hsp90 interaction by geldanamycin (a specific inhibitor of Hsp90) inhibited the formation of the 480-kDa complex without inducing large complexes, and rendered p27 incapable of binding to a specific viral RNA element (YRE), which is a critical step in the assembly of the 480-kDa replicase complex. These findings suggest that Hsp70 and Hsp90 play essential roles in the assembly of the 480-kDa replicase complex mainly by regulating protein–protein interactions and protein–RNA interactions, respectively. Thus, Hsp70 and Hsp90 regulate different steps in the assembly of the RCNMV replicase complex.

4.9. Viral RNA replication factory

The replication of eukaryotic positive-strand RNA viruses occurs on membranes of selected subcellular organelles, such as the ER, chloroplasts, mitochondria, peroxisomes, or tonoplasts in infected cells (den Boon & Ahlquist, 2010; Mine & Okuno, 2012; Nagy & Pogany, 2012; Salonen, Ahola, & Kaariainen, 2005). Viral proteins play essential roles in targeting the viral replication complexes to these membranes and induce morphological changes in these membranes (Belov & van Kuppeveld, 2012; den Boon & Ahlquist, 2010; Miller & Krijnse-Locker, 2008).

Confocal microscopy using green fluorescent protein (GFP)-fused p27 and p88 showed that both proteins colocalize to the cortical and cytoplasmic

ER, and that p27 causes the restructuring and proliferation of the ER membrane (Kusumanegara et al., 2012; Mine et al., 2012; Turner et al., 2004). The domains and critical amino acids in p27 required for its association with and targeting of ER membranes were determined using a C-terminally GFP-fused p27 (p27–GFP), which supported viral RNA replication in the presence of p88. Membrane-flotation assays combined with microscopic observation revealed that the membrane association of p27 is mediated by a stretch of 20 amino acids located in its N-terminal region (amino acids 31–50) (Kusumanegara et al., 2012). These 20 amino acids are predicted to form an amphipathic α-helix, with one side having a cluster of hydrophilic, polar residues and the other side hydrophobic, nonpolar residues, and were sufficient to target the nonviral GFP to ER membranes. Mutations that impede the membrane association of p27 compromise the formation of the RCNMV RNA replication complexes and negative-strand RNA synthesis (Kusumanegara et al., 2012).

The role in viral RNA replication of the Arf1 identified in the affinity-purified RCNMV RdRP fraction was investigated (Hyodo et al., 2013). Arf1 is a ubiquitous, highly conserved, small GTPase that is implicated in the formation of the coat protein complex I (COPI) vesicles on the Golgi membranes and in the membrane transport from the Golgi to the ER (D'Souza-Schorey & Chavrier, 2006). GTP-bound Arf1 facilitates the formation of several types of vesicle coat complexes and activates lipid-modifying enzymes on the Golgi membranes (Donaldson & Jackson, 2011; Memon, 2004).

The pharmacological inhibition of the nucleotide-exchange activity of Arf1 using the inhibitor brefeldin A (BFA) and the expression of dominant-negative Arf1 mutants compromised RCNMV RNA replication in tobacco BY-2 protoplasts. *In vitro* pull-down and BiFC analyses showed that p27 interacts with Arf1 within the virus-induced large punctate structures of the ER membrane. Inhibition of Arf1 activity by BFA disrupted p27-mediated ER remodeling and the assembly of the 480-kDa viral replication complex. Thus, p27 interacts with Arf1, and recruits and relocalizes this protein to the aggregate structures on the ER membranes, where they colocalize (Hyodo et al., 2013).

BFA treatment or the expression of dominant-negative mutants of Arf1 in plant cells not only inhibited the COPI pathway but also compromised COPII vesicle trafficking (Stefano et al., 2006). Interestingly, the expression of a dominant-negative mutant of Sar1, a key regulator of the biogenesis of COPII vesicles at ER exit sites, also compromises RCNMV RNA

replication (Hyodo et al., 2013). These results suggest that the replication of RCNMV depends on the host membrane trafficking machinery or that RCNMV rewires the cellular trafficking pathways to build a viral replication factory. A model for the formation of the viral replication factory in RCNMV-infected cells is presented (Fig. 2.2).

4.10. Subgenomic RNA

RCNMV uses a unique strategy to produce sgRNA. In addition to the core promoter that is predicted to exist within a stable SL (Zavriev et al., 1996), the transcription of CPsgRNA from RNA1 requires RNA2 *in trans* and is regulated by intermolecular interactions via base pairing between the eight-nucleotide sequence in the loop of TA in RNA2 and the complementary eight-nucleotide sequence in the TA-binding site (TABS) immediately upstream from the CPsgRNA transcription start site in RNA1 (Sit et al., 1998) (refer to Fig. 2.1). Biophysical analyses, using short synthetic oligonucleotides that mimic the TA of RNA2 and the TABS of RNA1, suggested the formation of a weak but stable bimolecular complex between these two mimics (Guenther et al., 2004).

Several lines of evidence have suggested that CPsgRNA is generated by a premature termination mechanism (Guenther et al., 2004; Sit et al., 1998; Tatsuta et al., 2005). The premature termination model is supported by the accumulation of negative-strand CPsgRNA in protoplasts (Iwakawa et al., 2008) because the production of negative-sense sgRNA templates is a fundamental step in premature termination (Eckerle, Albarino, & Ball, 2003; Lin & White, 2004; White, 2002). CPsgRNA is also unlikely to be an efficient RNA replicon because its 3′-UTR is identical to that of RNA1 whose replication depends predominantly on replication proteins translated from its own RNA in *cis* (Iwakawa et al., 2011; Okamoto et al., 2008).

4.11. A viral noncoding RNA

A small noncoding RNA (0.4 kb), designated "SR1f," which consists of nearly the entire 3′-UTR of RNA1, accumulates in RCNMV-infected plants and protoplasts, and is packaged into virions (Iwakawa et al., 2008). SR1f is neither a sgRNA nor a defective RNA replicon, but a stable degradation product generated by *cis*-RNA element-mediated protection against 5′ → 3′ decay. A 58 nucleotide sequence in the 5′ proximal region of the 3′-UTR of RNA1 is necessary and sufficient to protect against
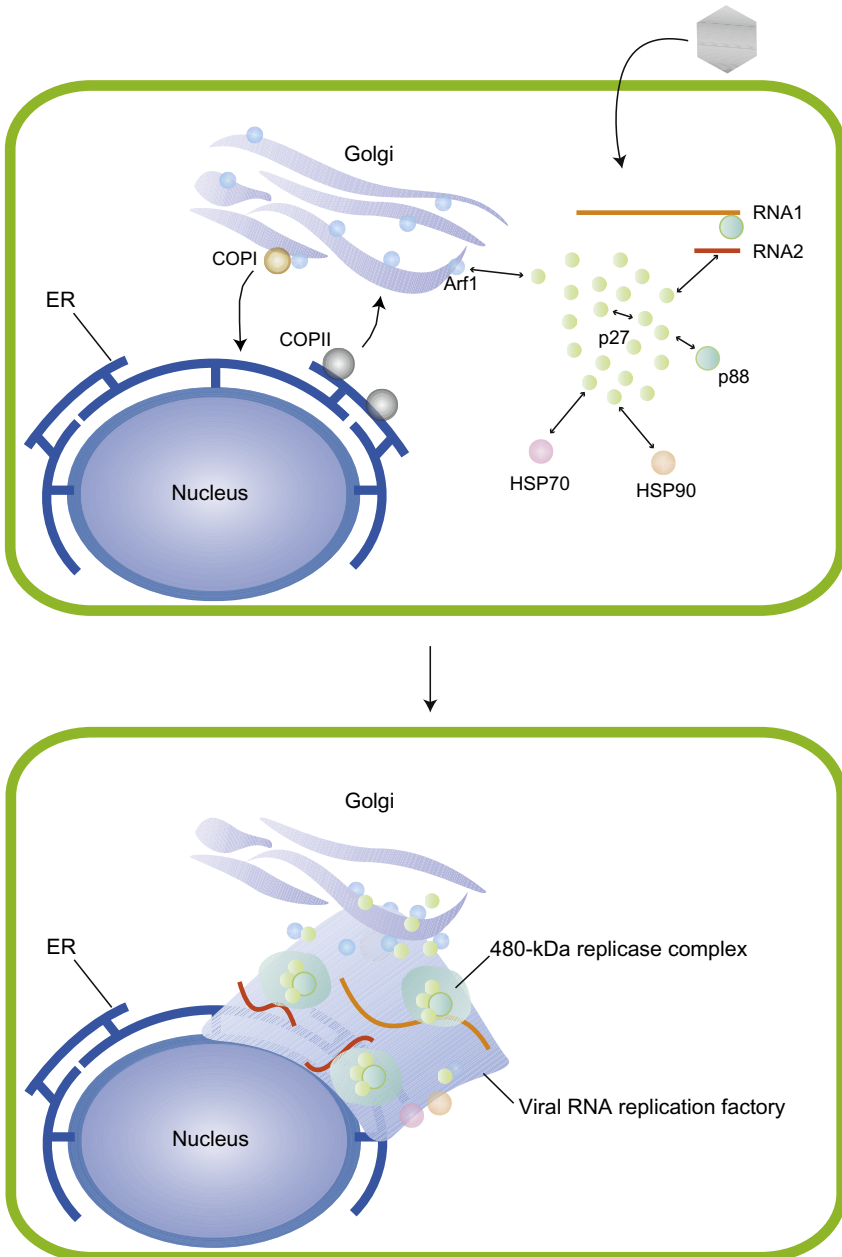
Figure 2.2 Schematic representation of the formation of the viral replication factory in RCNMV-infected cells. The abundant auxiliary replicase protein p27 interacts with p27 itself, p88, and viral RNAs, and also interacts with host proteins, such as Hsp70, Hsp90, and Arf1. These interactions are essential for the recruitment of viral genomic RNAs, viral proteins, and host factors to the ER membranes and for the reorganization of the membranes to form the viral RNA replication factory.

$5' \rightarrow 3'$ decay (Iwakawa et al., 2008). SR1f efficiently suppresses both cap–independent and cap–dependent translation, both *in vitro* and *in vivo*. SR1f *trans*-inhibits the negative-strand RNA synthesis of RCNMV genomic RNAs *via* the repression of replicase protein production, but not *via* competition with the replicase proteins *in vitro* (Iwakawa et al., 2008). SR1f seems to play important roles in virus infection and survival, although its precise roles in RCNMV infection in nature remain to be resolved.

The stable decay products of viral noncoding RNAs generated by a mechanism similar to that for SR1f have been reported in flaviviruses (Moon et al., 2012; Pijlman et al., 2008) and *Beet necrotic yellow vein virus* (Peltier et al., 2012). The *Flavivirus* noncoding RNA play important roles in viral pathogenicity by affecting host mRNA stability (Moon et al., 2012).

4.12. Cell-to-cell and systemic movement

A 35-kDa MP encoded by RNA2 is essential for the cell-to-cell and systemic movement of RCNMV (Osman & Buck, 1987; Paje-Manalo & Lommel, 1989; Xiong, Kim, Giesman-Cookmeyer, et al., 1993). RCNMV MP has the ability to bind single-stranded nucleic acids cooperatively (Giesman-Cookmeyer & Lommel, 1993; Osman, Hayes, & Buck, 1992; Osman, Thömmes, & Buck, 1993), localizes in the cell wall (Osman & Buck, 1991; Tremblay, Vaewhongs, Turner, Sit, & Lommel, 2005), and increases the size exclusion limit (SEL) of the plasmodesmata (PD) (Fujiwara, Giesman-Cookmeyer, Ding, Lommel, & Lucas, 1993). RCNMV MP fused with GFP (RCNMV MP:GFP) can form a homopolymer and is targeted to the cell wall, and this targeting is required for viral cell-to-cell movement (Tremblay et al., 2005).

Further analysis of the subcellular localization of RCNMV MP:GFP expressed from a recombinant virus in *N. benthamiana* epidermal cells and protoplasts showed that MP:GFP first appeared in the cell wall and was subsequently observed as punctate spots in the cortical ER (Kaido, Tsuno, Mise, & Okuno, 2009). The ER-localization of RCNMV MP:GFP was associated with the replication of RNA1, but not with that of RNA2 or the viral replicase component proteins (p27 and p88) *per se* (Kaido et al., 2009). This characteristic of MP:GFP is dependent on the 70 amino acids in the C-terminal region of MP and is required for the efficient cell-to-cell movement of the recombinant virus (Kaido, Funatsu, Tsuno, Mise, & Okuno, 2011). Interestingly, the deletion of the C-terminal 70 amino acids of MP had no deleterious effects on its localization to the cell wall, or its ability

to increase the PD SEL, to bind to single-stranded RNA, or to interact with MP *in vivo* (Kaido et al., 2011). These findings show that the recruitment of MP to the viral replication complexes is required for viral cell–to–cell movement. The mechanism that targets RCNMV MP:GFP to the ER, dependent on the replication of RNA1, which does not encode MP, may reflect a strategy to retain MP close to RNA1, thereby achieving its intracellular movement, followed by the intercellular movement of the viral genome.

Transgenic *N. benthamiana* expressing RCNMV MP supported both the cell–to–cell and systemic movement of movement–defective TMV and CMV (Giesman-Cookmeyer et al., 1995; Rao et al., 1998). A chimeric TMV with the MP gene replaced with the RCNMV MP gene systemically infected *N. benthamiana* (Giesman-Cookmeyer et al., 1995). Therefore, the MPs of tobamoviruses and dianthoviruses are functionally homologous.

In contrast, transgenic *N. benthamiana* expressing RCNMV MP supported the cell–to–cell movement, but not the systemic movement, of movement–defective *Cowpea chlorotic mottle bromovirus* (Rao et al., 1988). A chimeric *Barley stripe mosaic virus*, in which the triple gene block was replaced with the RCNMV MP gene, accumulated in the inoculated leaves of *N. benthamiana*, but not in the upper uninoculated leaves (Solovyev et al., 1997). Immunocytochemical studies by Wang, Wang, Giesman-Cookmeyer, Lommel, and Lucas (1998) showed that point mutations in RCNMV MP that do not affect the cell–to–cell movement of the virus prevent systemic viral movement, presumably by inhibiting RCNMV loading into the companion cell–sieve element complex. These findings suggest that the roles of RCNMV MP in the two processes are genetically distinct.

RCNMV CP is not essential for the cell–to–cell movement of the virus but virion formation is important for its systemic movement in *N. benthamiana* (Vaewhongs & Lommel, 1995; Xiong, Kim, Giesman-Cookmeyer, et al., 1993). The N-terminal lysine-rich motif of CP is involved in the systemic viral movement, virion accumulation, and symptomatology in *N. benthamiana* (Park, Sit, Kim, & Lommel, 2012). A sobemovirus CP gene complemented the long-distance movement of a CP-null RCNMV (Callaway, George, & Lommel, 2004).

4.13. Virions

The virions of *Dianthovirus* contain 180 copies of the 37-kDa CP subunit, forming an icosahedral particle with $T = 3$ symmetry and a diameter of about 36 nm (Hiruki, 1987; Sherman et al., 2006). Analysis of the RCNMV

virions with heat treatment and UV cross–linking suggested that RCNMV is composed of two virion populations (Basnayake et al., 2006): one type contains RNA1 and RNA2, and the other type contains multiple copies (possibly four copies) of RNA2. The origin of assembly sequence (OAS) was identified in RNA2 using the TBSV-vector-based expression of RCNMV CP (Basnayake, Sit, & Lommel, 2009). RNA1 is copackaged with RNA2 via an OAS–RNA1 interaction. Interestingly, the OAS is the TA that is required for the transcription of CPsgRNA from RNA1 via an RNA–RNA interaction (Sit et al., 1998). The N-terminal lysine-rich motif of CP is involved in the formation of the virions in *N. benthamiana* (Park et al., 2012). The heterologous combinations of RNA1 and RNA2 between RCNMV and SCNMV were infectious and produced progeny virions in their host plants (Chen, Hiruki, & Okuno, 1984; Okuno et al., 1983). A mixture of RNA1 from CRSV and RNA2 from RCNMV was also infectious and produced progeny virions in the host plants (Callaway, Giesman-Cookmeyer, Gillock, Sit, & Lommel, 2001). However, unilateral compatibility of RNA1 and RNA2 was reported using the different strains of RCNMV (Rao & Hiruki, 1987).

The electrophoretic mobility of purified virions differs among the dianthoviruses, even in the different strains of the same virus (Pappu & Hiruki, 1989).

Analysis of the RCNMV virions by cryoelectron microscopy and three-dimensional image reconstruction showed that the structures of the CP subunits and the entire virion are similar to those observed in the other viruses of the *Tombusviridae*, such as TBSV and TCV (Sherman et al., 2006). Preliminary diffraction data are available for the X-ray structure of RCNMV virion crystals at higher than 4 Å resolution (Martin et al., 2010). Atomic absorption spectroscopic analysis of RCNMV virions showed that the virions contain significant amounts of Ca^{2+} and Mg^{2+} ions (Sherman et al., 2006). Removal of these ions from the virions with a chelator, followed by exposure to ribonucleases, reduces viral infectivity, suggesting a role for these ions in virion stabilization (Sherman et al., 2006).

Studies of the use of RCNMV virions as the viral nanoparticles that function as drug carriers in human cells are in progress (Lockney et al., 2011).

4.14. Suppression of RNA silencing

RNA silencing is an RNA-mediated plant defense mechanism against viral infection (Baulcombe, 2004). To counteract RNA silencing, viruses have

developed a variety of strategies and evolved to encode a viral suppressor of RNA (VSR) silencing (Csorba, Pantaleo, & Burgyan, 2009; Ding & Voinnet, 2007; Li & Ding, 2006). Many plant viruses encode at least one VSR and some viruses encode more than one. Viral proteins that function as VSRs include CPs, replicase proteins, MPs, and other nonstructural proteins, and their targets in RNA silencing are diverse (Csorba et al., 2009; Ding & Voinnet, 2007; Li & Ding, 2006).

RCNMV has at least two strategies for suppressing RNA silencing. One strategy requires p27, p88, and viral RNAs (Takeda et al., 2005). In a sense-transgene-mediated posttranscriptional gene silencing (S-PTGS) assay involving the *Agrobacterium*-mediated transient expression of viral components and GFP in a GFP-expressing transgenic plant, the RNA-silencing-suppression activity of RCNMV was linked to the ability of viral factors to initiate negative-strand RNA synthesis (Takeda et al., 2005), and correlated with the formation of the 480-kDa replicase complex (Mine, Hyodo, et al., 2010). A close relationship between negative-strand RNA synthesis and the suppression of RNA silencing implies a possible scenario, in which RCNMV sequesters the host factors required for RNA silencing and reduces the antiviral silencing response. RCNMV replication also inhibits microRNA biogenesis, in which DCL1 plays an essential role, and dcl1 mutant plants show reduced susceptibility to RCNMV infection (Takeda et al., 2005). Therefore, it has been suggested that DCL1 or its homologues are recruited by the viral RNA replication complex (Takeda et al., 2005). Alternatively, the reduced susceptibility of a *dcl1* mutant to RCNMV infection can also be explained by the observations that the disruption of the DCL1 function leads to the higher expression of DCL4 and DCL3 in *Arabidopsis* leaves (Qu, Ye, & Morris, 2008) and that the destabilization of the miRNA pathway, including the disruption of DCL1, leads to the increased accumulation of AGO1 and positively influences S-PTGS (Martinez de Alba, Jauvion, Mallory, Bouteiller, & Vaucheret, 2011). Given that RCNMV infection inhibits miRNA biogenesis, reduced accumulations of miRNAs, including miR168 that is a regulator of AGO1 mRNA, could increase the accumulation levels of AGO1, which is the core component of the RNA-induced silencing complex.

MP has been identified as the second VSR of RCNMV in another assay system (Powers, Sit, Heinsohn, et al., 2008; Powers, Sit, Qu, et al., 2008). This assay relies on TCV that contains a reporter GFP gene in place of the CP ORF (TCV-sGFP) (Powers, Sit, Qu, et al., 2008). The TCV CP is a VSR (Qu, Ren, & Morris, 2003), and TCV requires CP for its cell-to-cell

movement in *N. benthamiana* (Cohen, Gisel, & Zambryski, 2000). TCV–sGFP moved from cell to cell in leaves infiltrated with *Agrobacterium* expressing RCNMV MP, but did not in leaves infiltrated with *Agrobacterium* expressing RCNMV p27, p88, and CP (Powers, Sit, Heinsohn, et al., 2008). The functional domains in RCNMV MP required for RNA silencing (Powers, Sit, Heinsohn, et al., 2008) differ from those required for the cell-to-cell movement of the virus and SEL modification (Fujiwara et al., 1993; Tremblay et al., 2005). Thus, RCNMV has evolved two strategies for suppressing RNA silencing and counteracting the host defenses.

5. TRANSMISSION STUDIES AND EPIDEMIOLOGY

Dianthoviruses multiply in infected leaves to high concentrations and are very stable *in vitro* (Hiruki, 1987). Soil transmission has been reported to be a general feature of dianthoviruses (Brunt et al., 1996).

The transmission of CRSV by nematodes such as *L. macrosoma* and *X. diversicaudatum* (Fritzsche & Schmelzer, 1967; Kegler et al, 1977) and *L. elongates* (Fritzsche et al., 1979) has been reported but not confirmed (Brunt et al., 1996).

The nematode transmission of FNSV is suspected but not demonstrated (Morales et al., 1992). No other vectors have been investigated in terms of the soil transmission of this virus.

The possible transmission of RCNMV by soil-inhabiting fungi was suggested (Bowen & Plumb, 1979). A Swedish isolate of RCNMV was transmitted to *N. clevelandii* when the seedlings were planted into the soil that had been infested by growing RCNMV-infected plants, or by adding virus suspension to the soil prior to planting. The concomitant presence of *O. brassicae* only increased the rate of transmission, but not essential as an intrinsic vector for the soil transmission of the virus (Gerhardson & Insunza, 1979). Independent tests by different groups did not demonstrate that *O. brassicae* serves as a vector of RCNMV (Lynes, Teakle, & Smith, 1981). Hiruki (1986b) reported that the presence of a tobacco strain of *O. brassicae* was not required for the transmission of SCNMV in sand culture.

Compared with leguminous forage crops such as alfalfa, alsike clover, red clover, crown vetch, and white clover, sweet clover is highly susceptible to SCNMV, while the others remain practically resistant to the virus. It is very interesting to note that the most prevalent area of SCNMV occurrence in Alberta coincides with the area where beekeeping industry is most active

and where sweet clover is commonly used for seed production. Hiruki, Kudo, and Figueiredo (1989) reported that the pollen from SCNMV-infected sweet clover is highly contaminated with the virus and that the virus, detectable by enzyme-linked immunosorbent assays, could be recovered by washing.

In 2007, an area of approximately a quarter section of land, consisting of 50% arable land and 50% popular forest, was cleared for residential development in a suburb of Edmonton. The topsoil, about 10 cm in depth, was completely removed and piled aside. However, due to unexpected economic downturn, the construction was halted for 3 years. To prevent erosion, sweet clover was seeded over the whole area (Fig. 2.3), offering a rare opportunity to observe the natural development of plant virus epidemic.

Regular visual inspections and samplings were conducted, and followed by infectivity tests on the indicator plants in the greenhouse. The results indicated that SCNMV infection occurred at a rate of about 6%. The source of the natural infection remains unknown. In the past, the natural infection of a solitary sweet clover plant in the forest, roadside, or riverbanks was observed frequently and shown to test positive (C. Hiruki, unpublished data). When infected sweet clover is not disturbed, abundant seeds are shed around the plant, and the young seedlings are subsequently infected by the soil transmission of SCNMV.

Figure 2.3 A general view of the site where sweet clover plants were grown to prevent soil erosion. This site was used to observe the natural spread of SCNMV.

6. EFFECTS ON ENVIRONMENT

Although considerable attention has been directed toward the pollution of environmental waters with human and animal viruses, there have been only sporadic attempts to shed light on the contamination of rivers, lakes, and sea with plant viruses (Buttner, Jacobi, & Koenig, 1987; Koenig & Lesemann, 1985; Tomlinson, Faithfull, Flewett, & Beards, 1982; Tomlinson, Faithfull, & Fraser, 1983; Tomlinson, Faithfull, & Seeley, 1982).

6.1. Release of dianthoviruses from intact roots, decaying plant materials, and pollen grains

Generally, dianthoviruses occur at high concentrations in infected cells and are very stable *in vitro*, thus serve as potentially highly infective inoculum sources. The release of CRSV from infected roots to drainage water was reported in Germany (Kegler and Kegler, 1981). Actively growing plants of *N. clevelandii* released much larger amounts of RCNMV into drainage water than did plants from which the aerial parts had been removed or plants which were senescent or dead (Gerhardson & Insunza, 1979). CRSV was isolated from the water samples collected from a canal near a sewage plant in Germany (Koenig, An, Lesemann, & Burgermeister, 1988).

While sweet clover is a biennial crop, successive infections with SCNMV can occur from the soil around an infected plant to young germinating plants in the second year, and from the soil around the dead infected plants to young plants emerging from fallen seeds in the third year (Hiruki, unpublished data).

Another strong possibility is pollen transmission in the case of SCVMV, although its direct experimental evidence is lacking at present. However, significantly high degrees of SCNMV occurrence and release of the virus particles from pollen grains were detected serologically (Hiruki et al., 1989). Moreover, in systematic field surveys over many years, high-specific incidences of SCNMV coincide with the zones 3 (black soil) and 4 (gray-wooded soil) in the Agriculture map of Alberta (Hiruki, 1987) where legume seed industry and bee keeping industries are concentrated (Hiruki, unpublished data). There are three main species of bees to pollinate legume crops such as honey bees (*Apis mellifera*), leafcutting bees (*Megachile rotundata*), and American bumble bees (*Bombus fervidus*). Honey bees are effective pollinators of a number of legume crops including sweet clover. However, honey bees are of little value as pollinator of alfalfa (Fairey,

2003). This fact may explain a minor incidence of SCNMV in alfalfa while frequent incidence of the same virus is observed in sweet clover in the same area (Hiruki, 1986a, 1986b; Hiruki et al., 1984). The occurrence of SCNMV in sweet clover in the forests, river banks, roadsides, and abandoned field may be explained by pollen transmission mediated mostly by honey bees and to lesser degrees by other bees.

6.2. Sewage

CRSV was isolated from river water that was fed by the outlets of a city's sewage plant in Germany (Koenig et al., 1988). The virus was identified by means of nucleic acid hybridization analysis and serology. CRSV and other viruses can also be released into streams or rivers. It is known that several plant viruses are capable of surviving a passage through the alimentary tracts of animals (Kegler et al., 1987; Tomlinson, Faithfull, Flewett, et al., 1982; Tomlinson, Faithfull, & Seeley, 1982). SCNMV was not transmitted by *Sitona cylindricollus* from sweet clover to sweet clover but remained infectious in feces after a passage through the alimentary tract (Hiruki, 1986a, 1986b).

At any rate, these observations on the occurrence of plant viruses in streams, rivers, and lakes illustrate the potential danger of using polluted water in horticultural and agricultural practices (Buttner et al., 1987; Koenig, 1986; Koenig & Lesemann, 1985; Koenig et al., 1988).

7. CONCLUDING REMARKS AND PERSPECTIVES

The dianthoviruses have moderately broad natural host ranges, restricted to dicot plant species, including legume plants (Hiruki, 1986a, 1986b; Descriptions lists from the VIDE Database; Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses, 2012), and the viruses and many of their strains have been reported frequently worldwide except for the tropical and subtropical areas of the world. However, a natural host of FNSV, an apparent member of the dianthoviruses, is *Furcraea* spp., which are xerophytic monocots native to the tropical regions of Mexico, the Caribbean, Central America, and northern South America. Dianthovirus RNA1-like RNA has also been reported in grassy stunt-diseased rice plants coinfected with *Rice grassy stunt virus* in the Philippines (Miranda et al., 2001). These reports imply that other unidentified *Dianthovirus* species or viruses related to the dianthoviruses might exist in tropical and subtropical areas. Therefore, a systematic search for dianthoviruses worldwide, including in wild plants in fields, forests, and

areas of water such as river, ponds, and seas, should open the way to finding additional members of *Dianthovirus*.

Dianthoviruses are known to release large numbers of infective viral particles from infected roots into soil and drainage waters in the surrounding area (Gerhardson & Insunza, 1979; Hiruki, 1986a, 1986b; Kegler and Kegler, 1981). FNSV is detected consistently in the roots of infected fique plants (Morales et al., 1992). In nature, soil is a complex medium and acts as a heterogeneous environment. Its properties can be altered by seasonal climatic changes and agricultural practices in a particular area. This implies that any infective agents, such as viruses, introduced into the soil and drainage water may be affected by a variety of environmental factors or combination of several factors that are influenced by the interplay between the climate, vegetation, and soil type. It is also expected that the application of sewage and sludge to agricultural land may alter some of the soil's physical and chemical properties that affect viral survival.

Our understanding of the molecular mechanisms underlying the infection processes of the dianthoviruses has advanced greatly in recent years, as described in this review. These advances have also given rise to many interesting questions that are yet to be addressed. These questions include the differences in the gene expression strategies of RNA1 and RNA2. For example, RNA1 contains strong translation enhancer elements (3′TE-DR1 and ARS), whereas RNA2 has no such elements: instead, the translation of MP is linked to the replication of RNA2 (Mizumoto et al., 2006). What do these differences in translation strategies mean? RNA1 and RNA2 might use distinct translation factors. The recruitment of MP to viral RNAs, a process essential for viral cell-to-cell movement, also differs between RNA1 and RNA2. The replication of RNA1, but not RNA2, is associated with MP recruitment to the ER–associated viral replication complexes (Kaido et al., 2009). This strategy might assist the cell-to-cell movement of RNA1, which does not encode MP. These findings also suggest that the replication complexes formed on RNA1 might differ from those on RNA2, and may localize to different sites on the ER. Indeed, RNA2 has an RNA element (YRE) that recruits p27 and the replicase complex (An et al., 2010; Hyodo et al., 2011), whereas RNA1 has no such recruiting element. The replication of RNA1 is linked to the translation of the replicase proteins (Iwakawa et al., 2011; Okamoto et al., 2008).

The TA of RNA2 is a key regulator of translation, transcription, replication, virion formation, and cell-to-cell movement. The structural requirements of TA seem to differ depending on its role, because nucleotide

substitutions in RNA2 that disrupt TA-mediated base pairing have no effect on the copackaging of RNA1 and RNA2 (Basnayake et al., 2009). Further analyses of the temporal changes in the TA structure and the host and viral factors that interact with TA are necessary to clarify the complex regulatory mechanisms of the *Dianthovirus* infection processes.

The auxiliary replication protein p27 plays multiple roles in RNA replication. These roles include the recruitment of the viral RNA to the ER membranes, the assembly of the replication complexes, and the formation of the viral replication factory. p27 performs these roles by interacting with p27 itself, p88, and the viral RNA, and also by interacting with host proteins, such as Hsp70, Hsp90, and Arf1. How can p27 perform these roles properly? A model proposed by Nagy, Barajas, & Pogany (2012) for the *Tombusvirus* auxiliary replication protein p33 might help to answer this question. These researchers propose that "depending on the given interacting partners, the abundant p33 molecules are divided into many groups that perform different functions at different subcellular locations." Further identification and analysis of the host proteins associated with the different functions of RCNMV p27 are required to answer this question.

Another interesting question to be addressed is the mechanism that delivers the translation initiation factors from the 3′-UTR of RCNMV RNA1 to the 5′-UTR, where the ribosome starts to scan the template (Sarawaneeyaruk et al., 2009). In 3′TE-DR1-mediated cap-independent translation, PABP plays an essential role in the binding of eIF4F/eIFiso4F to 3′TE-DR1 (Iwakawa et al., 2012). It is generally believed that the binding of eIF4F and eIFiso4F to viral 3′CITEs facilitates ribosome recruitment to the 5′ end of the RNA via 5′–3′ communication, which is mediated by a long-distance RNA–RNA interaction or a protein factor (Miller & White, 2006; Nicholson & White, 2011). However, the factors other than eIF4F/eIFiso4F or PABP that are required for 3′CITE-mediated translation remain unclear. How the ribosome recognizes the uncapped 5´ end, even when eIF4F/iso4F is delivered to the vicinity of the 5´ end of RNA1 by 5′–3′ communication is also unclear. Although we do not know how RCNMV RNA1 achieves the communication between its 5′- and 3′-ends in 3′TE-DR1-mediated cap-independent translation, our recent data suggest that the binding of eIF4F/iso4F and PABP to the 3′-UTR of RCNMV RNA1 is important for delivering cap-binding factors other than eIF4F/iso4F and PABP to its nonfunctional G-capped 5′ end, and in enhancing the recruitment of the 40S ribosomal subunit to the viral mRNA (H. Iwakawa & T. Okuno, unpublished data). Identification of the proteins

associated with the 5′ end of RNA1 will help to clarify these unknown mechanisms.

As described in this review, recent advances in our knowledge of the dianthoviruses, especially of their molecular biology, have relied on the development and availability of several experimental systems, such as an *in vitro* translation/replication system (BYL) (Komoda et al., 2004), and the *Agrobacterium*-mediated expression of viral and host components in combination with a protein-mediated or RNA-aptamer-mediated pull-down assay and mass spectrometric analysis. Genome-wide screens of the host factors that affect viral replication have been performed using yeast as the model host. The lists of host proteins obtained from these studies in yeast (Kushner et al., 2003; Panavas, Serviene, Brasher, & Nagy, 2005; Serviene et al., 2005), together with those obtained with other approaches, including those involving RCNMV, will enhance our understanding of the molecular mechanisms underlying viral infection. This knowledge will contribute to the development of antiviral measures that are more efficient, which will reduce the disease-mediated damage caused by the virus infections. However, we must consider that both the viral and host factors that affect viral infection might differ in different combinations of viruses and hosts (Li & Wong, 2007; Sarawaneeyaruk et al., 2009).

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