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Review

# RNA silencing suppressors encoded by viruses of the family *Tombusviridae*

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**Abstract** RNA silencing is a sequence-specific gene-inactivation mechanism conserved among eukaryotes that functions as an antiviral defense in plants and animals. To counteract this defense, viruses encode RNA silencing suppressors. Over 30 RNA silencing suppressors have been identified, but the mechanisms by which they suppress RNA silencing is unclear for most of them. The best-characterized suppressor is P19, encoded by viruses of the genus *Tombusvirus*, which belongs to the family *Tombusviridae*. Three suppressors have also been identified in the genera *Carmovirus*, *Aureusvirus*, and *Dianthovirus* in the family *Tombusviridae*. In this review, we summarize recent findings regarding these four RNA silencing suppressors, focusing on their modes of action in RNA silencing suppression and their roles in virus infection.

**Key words:** RNA silencing, *Tombusviridae*, suppressor.

## RNA silencing in plants

RNA silencing is small RNA-guided sequence-specific gene inactivation mechanism in eukaryote that is involved in diverse biological phenomena (e.g. development, heterochromatin formation, and defense against molecular parasites such as transposons and viruses) (reviewed by Baulcombe 2004; Vastenhouw and Plasterk 2004; Voinnet 2005). RNA silencing is induced by double-stranded RNAs (dsRNAs) that are the precursors of small RNAs. In this review, we do not address heterochromatin formation or repeat-associated small-interfering RNA (rasiRNA), but we refer to other reviews of these topics (Sontheimer and Carthew 2005; Wassenegger 2005). RNA silencing pathways in plants other than those involved in heterochromatin formation and in rasiRNA are illustrated in Figure 1.

In plants, the small RNAs involved in RNA silencing are 21–24 nucleotides (nt) (Hamilton et al. 2002) and are classified into three distinct RNA groups depending on their biogenesis. First, small-interfering RNA (siRNA) is processed from dsRNAs that are mainly derived from molecular parasites, including transposons, viruses, transgenes, and exogeneously supplied dsRNAs (Hamilton and Baulcombe 1999). siRNA is involved in defense against molecular parasites. siRNA-mediated gene inactivation is now widely used in molecular biology. Second, microRNA (miRNA) (reviewed by Bartel 2004) is processed from short endogenous RNA hairpins that are derived from miRNA precursors transcribed by RNA polymerase II. miRNA regulates the expression of genes from mRNA with nucleotide sequences complementary to the miRNA by mRNA cleavage or translation repression, to control diverse developmental processes. Interestingly, miRNA is involved in antiviral defenses in animals (Lecellier et al. 2005). It is unclear whether miRNA is involved in antiviral defenses in plants. Third, *trans*-acting siRNA (ta-siRNA) is a recently identified endogenous siRNA (Peragine et al. 2004; Vazquez et al. 2004). The generation of ta-siRNA requires miRNA-mediated mRNA cleavage and subsequent dsRNA synthesis by an RNA-dependent RNA polymerase (RdRP), RDR6 (Allen et al. 2005). ta-siRNA regulates gene expression in a way similar to that of miRNA.

The key molecules in RNA silencing in plants are the Dicer-like enzymes (DCLs), Argonaute proteins (AGOs), and RdRPs (Reviewed by Baulcombe 2004). The *Arabidopsis thaliana* genome encodes four DCLs, ten AGOs, and six RdRPs. DCL is an RNase III enzyme that

Abbreviations: AGO, Argonaute protein; CP, coat protein; Cym19stop, P19-defective CymRSV; CymRSV, *Cymbidium ringspot virus*; DCL, Dicer-like enzyme; DI RNA, defective-interfering RNA; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; miRNA, microRNA; nt, nucleotide; PoLV, *Pothos latent virus*; RCNMV, *Red clover necrotic mosaic virus*; rasiRNA, repeat-associated small-interfering RNA; RdRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; satRNA, satellite RNA; siRNA, small-interfering RNA; ta-siRNA, *trans*-acting siRNA; TCV, *Turnip crinkle virus.*

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processes dsRNAs and RNA hairpins into 21–24 nt RNA duplexes. Although there is functional redundancy among the four DCLs, it has been suggested that DCL1 is involved in the biogenesis of miRNA and 21 nt siRNA, DCL2 in viral siRNA biogenesis, DCL3 in 24 nt siRNA biogenesis, and DCL4 in ta-siRNA biogenesis (Park et al. 2002; Reinhart et al. 2002; Kurihara and Watanabe 2004; Xie et al. 2004; Gasciolli et al. 2005; Qi et al. 2005). AGO is a "slicer" in the RNA-induced silencing complex, RISC (Liu et al. 2004). *Arabidopsis* AGO1 has slicer activity (Baumberger and Baulcombe 2005; Qi et al. 2005). AGO1 recruits miRNA, ta-siRNA, and transgene-derived siRNA, but not 24 nt siRNA or virus-derived siRNA (Baumberger and Baulcombe 2005). AGO4 is involved in DNA and histone methylation (Zilberman et al. 2003), and AGO7 and AGO10 in development (Moussian et al. 1998; Lynn et al. 1999; Hunter et al. 2003). The function of other AGOs remain unknown, and an AGO involved in virus-induced RNA silencing has not been identified. RdRP generates dsRNA from aberrant RNAs, including uncapped mRNAs and mRNAs lacking a poly-A tail, which are generated by RISC-mediated mRNA cleavage (Gazzani et al. 2004; Allen et al. 2005). RDR6 is involved in transgene-induced RNA silencing, virus resistance, the generation of silencing signals, and the biogenesis of ta-siRNA (Dalmay et al. 2000; Mourrain et al. 2000; Himber et al. 2003; Peragine et al. 2004; Vazquez et al. 2004; Allen et al. 2005), together with SDE3 or SGS3. Thus, plant RNA silencing pathways are complex, and the molecular mechanisms of RNA silencing in plants are largely unknown.

## RNA silencing suppressors encoded by viruses

Viruses express suppressors to counteract RNA silencing-mediated antiviral defenses. Over 30 suppressors are identified (reviewed by Voinnet 2005; Reavy et al. 2004; Vanitharani et al. 2004; Bennasser et al. 2005; Kreuze et al. 2005; Lecellier et al. 2005; Merai et al. 2005; Takeda et al. 2005). Most of these probably use different suppression strategies, because the suppressors have no obvious sequence similarities and cause distinct phenotypes in various suppression assays. However, in plant RNA silencing, the target molecules of most RNA silencing suppressors and their roles in virus infection are largely unknown, primarily because plant RNA silencing is complex and the RNA silencing pathways have not yet been elucidated. Of the more than 30 suppressors, one of the best characterized is tombusviral P19. In the following sections, we review the suppressors encoded by viruses belonging to the family *Tombusviridae*, focusing on recent findings that show the molecular mechanisms of RNA silencing suppression and the roles of these suppressors in viral infection.

## Viruses of the family *Tombusviridae*

The family *Tombusviridae* includes eight genera (Lommel et al. 2000; Stuart et al. 2004). RNA silencing suppressors have been identified in four of these genera: *Aureusvirus*, *Carmovirus*, *Dianthovirus*, and *Tombusvirus* (Voinnet et al. 1999; Qu et al. 2003; Mérai et al. 2005; Takeda et al. 2005). The putative modes of action of the RNA silencing suppressors encoded by viruses in these genera are illustrated in Figure 1. The genome structures of the viruses in these genera are shown in Figure 2. The genomes of viruses in this family are positive-sense single-stranded RNAs. The viral genome is monopartite, except for viruses in the genus *Dianthovirus*, which have bipartite genomes. The viruses of the family *Tombusviridae* studied to date, including dianthovirus (Mizumoto et al. 2003), contain neither 5′ cap structures nor 3′ poly-A tails on their genomic RNAs (Lommel et al. 2000). These viruses do not encode proteins containing the helicase motif that is conserved in eukaryotes (Koonin and Dolja 1993). Some viruses in this family are associated with subviral RNAs, which modulate the symptoms induced by helper viruses, including defective-interfering RNA (DI RNA) and satellite RNA (satRNA) (reviewed by Simon et al. 2004).

## Tombusviral P19

After potyviral HC-Pro and cucumoviral 2b were identified as RNA silencing suppressors, tombusviral P19 was identified as RNA silencing suppressor (Voinnet et al. 1999). P19 is the first suppressor, of which the target molecules in RNA silencing suppression pathway was identified; P19 forms homodimers and specifically binds siRNA duplexes through recognition of 5′ ends (Silhavy et al. 2002; Vargason et al. 2003; Ye et al. 2003; Park et al. 2004). Consequently, to suppress RNA silencing, P19 probably interferes with the loading of a strand of the siRNA duplex into the RISC.

P19 also binds miRNA duplexes (Chapman et al. 2004; Dunoyer et al. 2004). As mentioned above, miRNA is involved in a diverse range of developmental processes. Therefore, symptoms observed in tombusvirus-infected plants may be partly caused by the P19-mediated inhibition of miRNA functions. Supporting this proposition, P19-expressing transgenic *A. thaliana* exhibits developmental abnormalities (Chapman et al. 2004; Dunoyer et al. 2004).

P19 functions as an RNA silencing suppressor in heterologous organisms, including *Drosophila* in an *in vitro* system and human HeLa cells (Dunoyer et al. 2004; Lakatos et al. 2004), because small RNA duplexes are

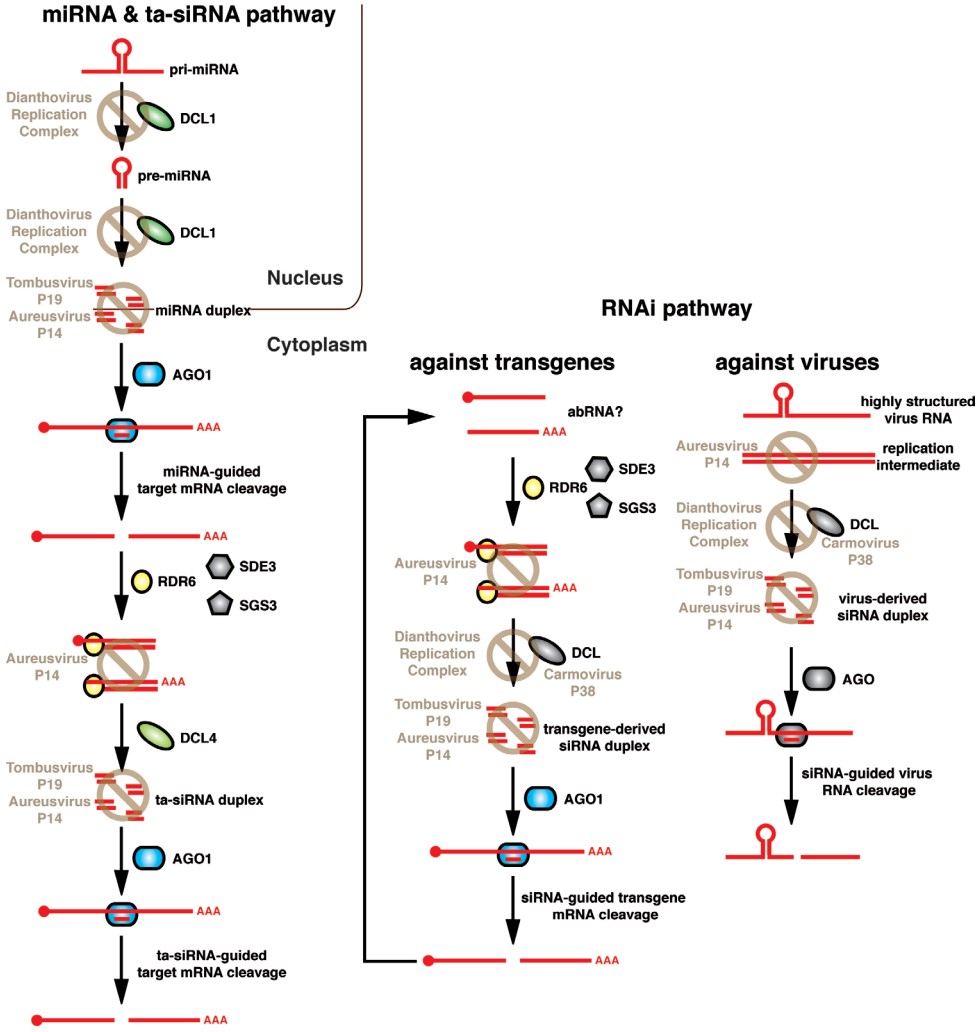


Figure 1. RNA silencing pathways in plants and the steps putatively inhibited by RNA silencing suppressors encoded by the viruses of the family *Tombusviridae*. See text for a detailed explanation of this figure.

common in the RNA silencing mechanisms of these organisms. Using this feature, Lecellier et al. (2005) showed that P19 enhances the accumulation of viral RNA in mammalian cells and demonstrated that cellular miRNA, as well as siRNA, functions to limit the replication of the virus.

The roles of P19 in tombusviral infection at various temperatures have been well documantated for *Cymbidium ringspot virus* (CymRSV) (Szittya et al. 2003). P19-defective CymRSV (Cym19stop) accumulates similarly to wild-type CymRSV in the protoplasts, indicating that P19 is not required for the multiplication of CymRSV at the single cell level (Szittya et al. 2003). RNA silencing-mediated antiviral defense is a temperature-dependent mechanism. RNA silencing is inhibited at low temperatures (15°C) and is gradually activated with increasing temperature. The accumulation of CymRSV-derived siRNAs increases with increasing temperature. At 15°C, at which antiviral RNA silencing is inhibited, Cym19stop-infected plants display strong viral symptoms, similar to those of wild-type CymRSV-infected plants, and Cym19stop invades entire systemic leaves. At 24°C, Cym19stop-infected plants display the recovery phenotype (Szittya et al. 2003) and Cym19stop infection is restricted in and around the vasculature in the systemic leaves (Havelda et al. 2003). These results indicate that P19 is involved in the efficient spread of infection to the systemic leaves at 24°C by preventing the onset of systemic RNA silencing ahead of the CymRSV infection. At 27°C, at which antiviral RNA silencing is activated, wild-type-CymRSV-infected plants display the recovery phenotype (Szittya et al. 2003). In CymRSV-infected plants, CymRSV-derived siRNAs are present in complexes with P19 (Lakatos et al. 2004). Collectively, these results suggest that the efficient spread of viral infection to systemic leaves and the phenotype of tombusvirus-infected plants depend on a balance between the amount of tombusvirus-derived siRNA and the amount of P19.

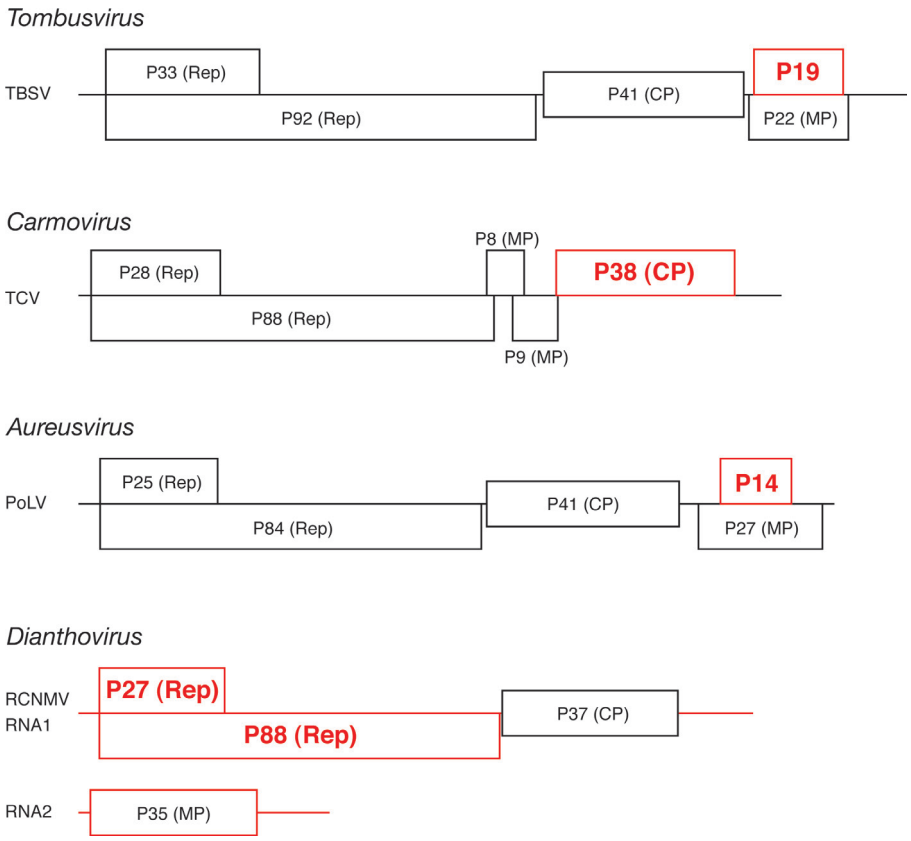


Figure 2. Schematic representation of the genome structures of four viruses of the family *Tombusviridae*. Lines, boxes, and gene names in red represent the RNA silencing suppressors of each virus. TBSV, *Tomato bushy stunt virus*; TCV, *Turnip crinkle virus*; PoLV, *Pothos latent virus*; RCNMV, *Red clover necrotic mosaic virus*. Rep, replicase; CP, coat protein; MP, movement protein.

## Aureusviral P14

Aureusviral P14 is a recently identified RNA silencing suppressor (Mérai et al. 2005). The genomic positions of P14 and tombusviral P19 are identical, in that both are nested in the movement protein gene. However, these proteins do not have sequence homology (Rubino and Russo 1997). P14 binds dsRNA in a manner distinct from that of tombusviral P19. P14 binds not only siRNA duplexes but also long dsRNA (Mérai et al. 2005), whereas P19 selectively binds siRNA duplexes. It is suggested that P14 does not recognize the 5′ ends of siRNAs (Mérai et al. 2005). When RNA silencing is induced by long hairpin dsRNA in agroinfiltration assays, P14 prevents the accumulation of siRNAs, whereas P19 does not (Mérai et al. 2005). This suggests that P14 interferes with RNA silencing mainly by binding to long dsRNA but not to siRNA.

## Carmoviral P38

Carmoviral P38 is the first example of a viral structural protein with suppressor activity (Qu et al. 2003). P38 is the coat protein (CP) and its initial role is to package viral genomic RNA into virions. Expression of P38 alone suppresses RNA silencing, but expression of P38 during viral multiplication does not suppress RNA silencing (Thomas et al. 2003). This can be explained by the fact that only free CP, but not CP assembled into virions, is an active suppressor of RNA silencing (Thomas et al. 2003, Zhang and Simon 2003). It has been suggested that P38 interferes with DCL-mediated dsRNA cleavage, but the mechanism is unclear (Qu et al. 2003; Chapman et al. 2004). Although the amino acid sequences of CPs are conserved among the genera *Tombusvirus*, *Aureusvirus*, *Carmovirus*, and *Dianthovirus*, dianthoviral CP at least does not suppress RNA silencing (Takeda et al. 2005). Determining whether tombusviral and aureusviral CPs suppress RNA silencing may clarify how these CPs have evolved.

## Dianthoviral replication complex (P27+P88 +viral RNA)

Dianthovirus uses a unique strategy to suppress RNA silencing. The dianthoviral suppressor consists of multiple components including P27, P88, and replicable viral RNA, which form dianthoviral replication complex (Takeda et al. 2005). This is the first example that multiple viral components coordinately function to

suppress RNA silencing, and that viral genomic RNA itself is a component of RNA silencing suppressor. Dianthoviral replication complex interferes with DCL-mediated siRNA biogenesis as well as with miRNA biogenesis (Takeda et al. 2005). Interestingly, *Red clover necrotic mosaic virus* (RCNMV), a member of the genus *Dianthovirus*, requires DCL1 as host factor for efficient viral infection (Takeda et al. 2005). The roles of DCL1 in RCNMV infection are unclear. An attractive hypothesis is that RCNMV uses DCL1 as a helicase in virus replication because RCNMV, like other viruses of the family *Tombusviridae*, does not encode proteins with the helicase motif that is conserved in eukaryotes (Koonin and Dolja 1993). The replicase component proteins of RCNMV localize at the endoplasmic reticulum (ER) (Turner et al. 2004). In *Arabidopsis*, miRNA is processed in the nucleus (Park et al. 2005), where most DCL1 is localized (Papp et al. 2003). RCNMV may alter the intracellular localization of DCL1 by recruiting and using it as a host factor, resulting in the inhibition of miRNA biogenesis. A recent study by Qi et al. (2005) using *Arabidopsis* extracts showed that DCL1 processes long dsRNA into 21 nt siRNA *in vitro*. This suggests that the DCL1 localized in the cytoplasm produces 21 nt siRNAs. RCNMV suppresses transgene-induced RNA silencing, probably through the deprivation of DCL1 localized in the cytoplasm. RCNMV also inhibits the generation of 24 nt siRNA (Takeda et al. 2005), which is processed by DCL3 (Qi et al. 2005). This suggests that RCNMV infection may affect DCL3 function. A direct interaction between the RCNMV replication complex and DCLs has not yet been confirmed. Further study of RCNMV replication complexes, including biochemical and immunological analyses, is required to confirm our model of the RNA silencing suppression mechanism of RCNMV, and to examine how RCNMV suppresses RNA silencing.

## Effects of subviral RNAs on RNA silencing suppression

A characteristic of the viruses of the family *Tombusviridae* is that some are accompanied by subviral RNAs that enhance or attenuate symptom severity (reviewed by Simon et al. 2004). Recent studies have shown that this symptom modulation is closely related to RNA silencing-mediated antiviral defenses and the suppression of RNA silencing.

Some viruses of the genus *Tombusvirus* are associated with DI RNAs, which are small RNAs derived from a parental viral genome by extensive deletion and are dependent on helper viruses for replication. Tombusviral infection together with DI RNAs displays attenuated symptom severity. DI RNAs are efficient inducers of RNA silencing but DI RNAs themselves are poor target molecules for RNA silencing (Szittya et al. 2002). In other words, DI RNAs (including their replication intermediates) are efficiently cleaved by DCLs to supply siRNAs, but DI RNAs are not targeted by RISC-mediated cleavage. This is probably because DI RNAs are highly structured and therefore the RISC cannot access DI RNAs to degrade them. In the presence of DI RNA, siRNAs complementary to viral RNA accumulate to high levels during helper virus infection, leading to the saturation of P19 and the presence of viral-siRNA-programmed RISCs (Havelda et al. 2005). As a result, tombusvirus accumulation is reduced by RNA silencing-mediated antiviral defenses in the presence of DI RNAs, resulting in symptom attenuation in plants.

In *Carmovirus*, symptom severity is enhanced by satRNA, which is a small RNA with little or no sequence homology to the helper viruses and which is dependent on helper viruses for its replication. SatC is a satRNA associated with *Turnip crinkle virus* (TCV) of the genus *Carmovirus*. SatC interferes with TCV virion formation, resulting in an increase in free P38 (Zhang and Simon 2003). As mentioned above, free P38 but not P38 assembled into virions suppresses RNA silencing. Therefore, the presence of satC enhances the RNA silencing suppression activity of P38 in TCV-infected plants. Consequently, plants infected with TCV together with satC display more severe symptoms than those of plants infected with TCV alone.

## Control of suppressor expression in viral infection

The initial roles of RNA silencing suppressors during viral infection are to suppress RNA silencing-mediated antiviral defenses. Because RNA silencing pathways in plants are complex and several components are shared among those pathways, viral RNA silencing suppressors inevitably affect some pathways that are not involved in antiviral defenses, resulting in the induction of developmental abnormalities and, in some cases, killing the host plants. To reduce the damage to the host plant and to avoid these situations, viruses are equipped with strategies to control the activity of RNA silencing suppressors in their infection processes, either quantitatively or qualitatively.

Tombusviral P19 specifically binds 21 nt small RNA duplexes, thus precluding their interference in other pathways involving long dsRNAs or 24 nt small RNAs. Tombusviruses also sometimes control P19 function through the generation of DI RNA. Re-examination of the conditions under which DI RNAs are generated, in terms of RNA silencing activity at various temperatures, may help us understand the natural roles of DI RNA generation in tombusviral infection. Aureusvirus controls the expression of P14 by controlling the

expression of the subgenomic RNA from which P14 is translated (Rubino and Russo 1997; Mérai et al. 2005). Carmovirus controls the suppressor activity of P38 by forming virions, in which P38 does not suppress RNA silencing (Zhang and Simon 2003). The suppressor activity of dianthoviral replication complex is transient (Takeda, Tsukuda and Okuno, unpublished), probably because viral replication does not last long and is completed at a late stage of the viral infection process.

## Concluding remarks

RNA silencing pathways in plants and the modes of action of RNA silencing suppressors of some viruses have been identified in the past few years.

Although viruses of the family *Tombusviridae* have conserved replicases and CPs, and their genomic RNAs have common features including the lack of a 5′ cap and a 3′ poly-A tail, the strategies with which they suppress RNA silencing are quite different. As shown in a closterovirus (Lu et al. 2004) and geminiviruses (Vanitharani et al. 2004), viruses can have multiple suppressors. It will be interesting to investigate whether tombusviruses, aureusviruses, and carmoviruses express additional suppressors. Do tombusviral and aureusviral CPs suppress RNA silencing? Do tombusviral, carmoviral, and aureusviral replication complexes suppress RNA silencing? It will also be interesting to identify RNA silencing suppressors in the other four genera in the family *Tombusviridae*. These results may clarify the evolutionary progression of the viruses of the family *Tombusviridae*.

Further investigation of the roles of tombusviral P19, aureusviral P14, carmoviral P38 and the dianthoviral replication complex may clarify RNA silencing mechanisms in plants. For example, how does P19 bind to siRNA *in vivo* and how does P14 bind to long dsRNA *in vivo*? P19 and P14 probably have mechanisms to sequester siRNAs from DCLs before they are loaded into RISCs, and P14 may have a mechanism to sequester long dsRNA from RdRP before its digestion by DCLs into siRNAs. What DCLs are inhibited by P38 and the dianthoviral replication complexes? Interestingly, P38 inhibits siRNA biogenesis but not miRNA biogenesis, and P38 may not bind long dsRNAs (Dunoyer et al. 2003). The DCLs or DCL-associated molecules that are inhibited by P38 should be determined. The mechanism by which dianthoviral replication complexes suppress RNA silencing remains unknown. Does the dianthoviral replication complex bind DCL1 and other DCLs directly? If so, which component of the replication complex (P27, P88, or viral RNA) binds DCLs? Does dianthovirus use the helicase activity of DCL1 in viral replication? Does the dianthoviral replication complex affect the intracellular localization of DCL1? Further experiments are required to answer these questions. It will be exciting to determine how the viruses of the family *Tombusviridae* protect their genomes and subgenomic RNAs from recognition by RNA silencing-associated RdRP, because their RNAs lack 5′ cap structures and 3′ poly-A tail (Lommel et al. 1999; Mizumoto et al. 2003), which are recognized as templates for RDR6 (Gazzani et al. 2004; Allen et al. 2005).

Recently, some important new concepts have been demonstrated, including miRNA-mediated antiviral defenses (Lecellier et al. 2005), miRNA-directed ta-siRNA biogenesis (Allen et al. 2005), and qualitative differences between virus- and transgene-induced RNA silencing (Baumberger and Baulcombe 2005). Future challenges will include the full elucidation of the RNA silencing pathways and the clarification of the effects of RNA silencing suppressors on these pathways.

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