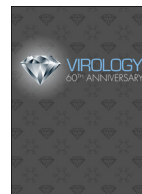Contents lists available at ScienceDirect

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Review

Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms

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ABSTRACT

The natural outcome of some plant–virus interactions is symptom recovery, which is characterized by the emergence of asymptomatic leaves following a systemic symptomatic infection. Symptom recovery is generally accompanied with reduced virus titers and sequence-specific resistance to secondary infection and has been linked with the induction of antiviral RNA silencing. Recent studies have revealed an unsuspected diversity of silencing mechanisms associated with symptom recovery in various host-virus interactions, including degradation or translation repression of viral RNAs and in the case of DNA viruses, transcriptional arrest of viral minichromosomes. RNA silencing may also contribute to symptom alleviation by regulating plant gene expression. In this review, we discuss the evidence supporting the role of various RNA silencing mechanisms in symptom recovery. We also discuss how a delicate equilibrium between RNA silencing and virus counter-defense responses in recovered leaves may help maintain virus titers at levels below the threshold required for symptom induction.

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The outcome of plant–virus interactions depends on the effectiveness of plant defense mechanisms and on the ability of the virus to counteract these defense responses and to usurp host factors that are beneficial for its replication cycle (Fraile and Garcia-Arenal, 2010; Palukaitis et al., 2013). Incompatible interactions between viruses and plant dominant resistance genes result in localized infections that are usually restricted to the inoculated leaves (Carr et al., 2010; Eitas and Dangl, 2010; Moffett, 2009; Padmanabhan and Dinesh-Kumar, 2014). On the other hand, compatible interactions are characterized by the establishment of a systemic infection in the plant. Although many systemic plant virus infections are asymptomatic, others result in the manifestation of symptoms that can vary greatly. These range from mosaic patterns, yellowing or mottling of the leaves, to dwarfing or developmental abnormalities of the plant and even systemic necrotic symptoms, sometimes leading to the death of the plant (Roossinck, 2010). The causes of symptom induction are varied but

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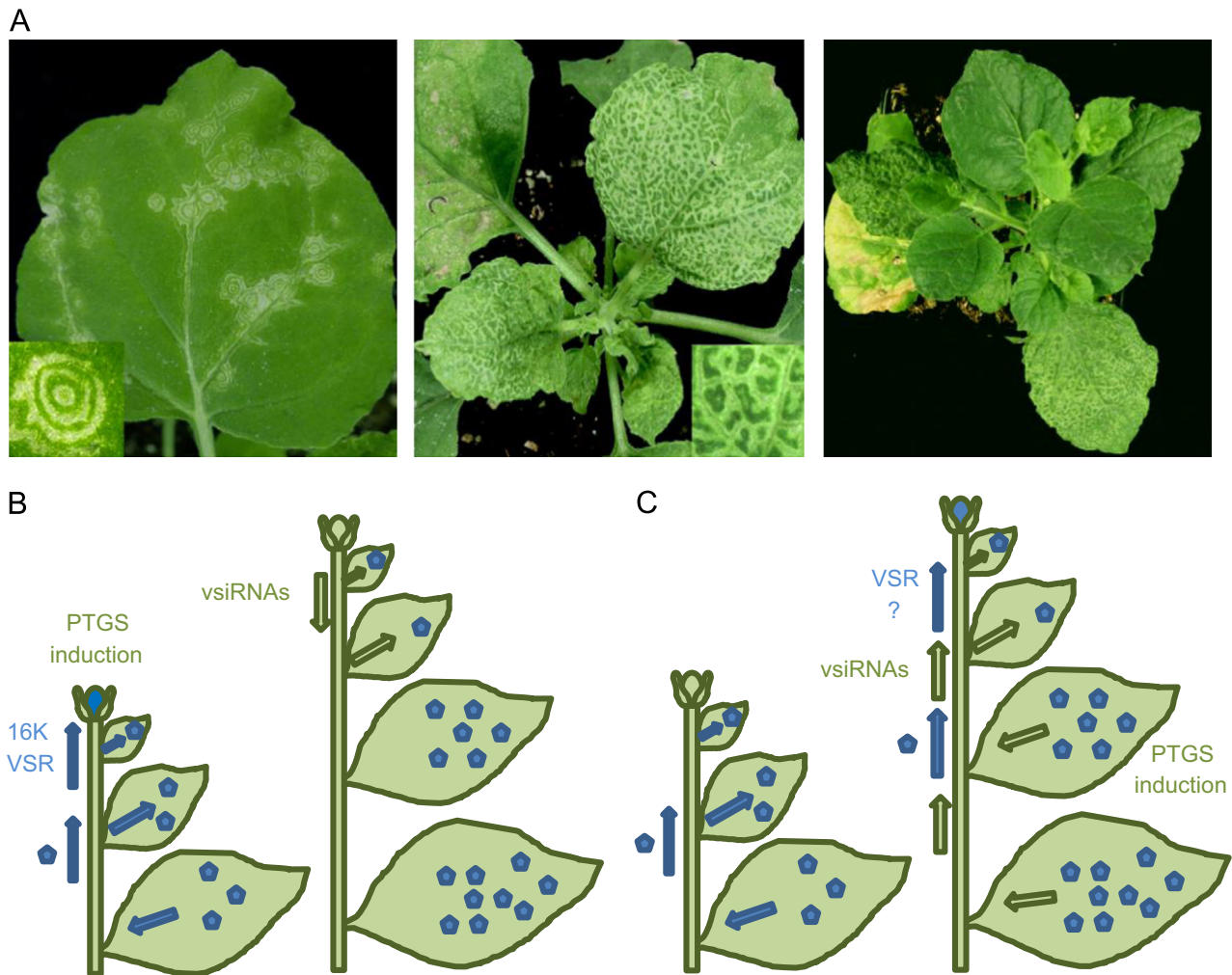E-mail address: helene.sanfacon@agr.gc.ca (H. Sanfaçon).

Fig. 1. Symptom recovery, PTGS induction and meristem invasion. (A) Symptom development in ToRSV-infected *N. benthamiana* plants. Pictures show symptoms on inoculated leaves (left), systemically infected leaves (centre) and the absence of symptoms on recovered leaves (right). Reproduced with permission from Jovel et al. (2007). (B) Model for meristem invasion and PTGS induction in TRV-infected plants. Transient invasion of the meristem (depicted in blue) is facilitated by the 16K VSR and triggers antiviral PTGS. Movement of vsiRNAs follows and this leads to recovery. (C) Model for PTGS induction in AlLV-infected plants. PTGS is induced by a threshold of viral RNA accumulation in infected leaves.

always depend on the accumulation of viral nucleic acids or proteins that interfere with the normal function of the plant and/or induce a symptomatic defense response (Culver and Padmanabhan, 2007; Pallas and Garcia, 2011). In this review, we focus on the phenomenon of symptom recovery that occurs in some compatible interactions. Symptom recovery is defined by the emergence of asymptomatic leaves following a systemic symptomatic infection (Fig. 1A). In some cases, the entire plant appears fully recovered at late stages of infection.

Symptom recovery was first reported in tobacco plants infected with tobacco ringspot virus (genus *Nepovirus*, family *Secoviridae*) (Wingard, 1928). Because symptoms are induced upon accumulation of viral products, it is logical to assume that symptom recovery should be accompanied with a reduction in virus titers. Indeed, in the initial Wingard study, virus titers were shown to be reduced in recovered leaves, although the virus was still present since sap extracted from recovered leaves could reinitiate a symptomatic infection in naïve plants. Recovered leaves were also reported to be resistant to reinfection by the cognate virus, suggesting that a specific plant defense response was induced. Similar symptom recovery phenotypes have been observed in plants infected not only with other nepoviruses but also with taxonomically unrelated positive-sense ((+)-strand) RNA viruses (Cadman and Harrison, 1959; Ross, 1941; Xin and Ding, 2003), pararetroviruses (Covey et al., 1997) and single-stranded DNA (ssDNA) viruses (Chellappan et al., 2004). While these

viruses use very different strategies to replicate their RNA or DNA genomes, in all cases documented so far, symptom recovery is concomitant with the induction of RNA silencing. As will be discussed below, RNA silencing is a well-recognized plant antiviral response that provides sequence-specific resistance to secondary infection. Although the presence of antiviral RNA silencing in recovered leaves seems to be universal, recent studies have unraveled an unsuspected diversity of silencing mechanisms that lead to symptom recovery in various plant–virus interactions. This review aims to discuss these mechanisms and highlight unanswered questions and potential areas for future research.

Plant antiviral post-transcriptional silencing mechanisms

RNA silencing is a ubiquitous eukaryotic gene regulation mechanism. In plants, it can act transcriptionally (TGS for transcriptional gene silencing) by cytosine methylation of DNA targets and histone methylation or it can act post-transcriptionally (PTGS) by cleavage or translation repression of RNA targets (reviewed in Brodersen and Voinnet (2006), Ghildiyal and Zamore (2009), Meins et al. (2005), Neilson and Sharp (2008) and Parent et al. (2012)). RNA silencing pathways regulate the plant physiology, growth and development, repress the proliferation of transposable elements and have been implicated in the plant response to environmental changes (Baulcombe and Dean, 2014). RNA silencing

is also a well-established plant antiviral response (Ding and Voinnet, 2007; Pumplin and Voinnet, 2013; Wang et al., 2012).

PTGS is a cytoplasmic mechanism that is initiated by the recognition of double-stranded RNA (dsRNA) structures and targets sequence-related single-stranded RNAs. Most plant viruses are (+)-strand RNA viruses that replicate in the cytoplasm through the action of viral RNA-dependent RNA polymerases, creating dsRNA replication intermediates. DNA viruses replicate their genomes in the nucleus (see below) but their mRNAs are exported to the cytoplasm for translation (ssDNA viruses and pararetroviruses) or reverse transcription (pararetroviruses only) and are therefore also potential targets of PTGS mechanisms.

The sequence specificity of PTGS is conferred by base-pair complementarity between the RNA target and small RNAs (sRNAs) incorporated into the RNA-induced silencing complex (RISC). sRNA duplexes are produced after cleavage of dsRNAs by a class of endoribonuclease III enzymes known as DICER (commonly called DICER-like or DCL in plants) (Liu et al., 2009). The activity of DCLs can be influenced by cofactors, such as DOUBLE-STRANDED-RNA-BINDING PROTEINS (DRBs). The model plant *Arabidopsis thaliana* encodes four DCLs and five DRBs. Newly formed sRNAs are protected against degradation by 2′-O-methylation of the terminal nucleotide by HUA ENHANCER 1 (HEN1) (Li et al., 2005). DCL2, DCL4 and DRB4 have been implicated in the production of 21–22 nt viral-derived small interfering RNAs (vsiRNAs) that originate from the dsRNA replication intermediates of (+)-strand RNA viruses or from structured regions of viral genomic RNAs or mRNAs (Deleris et al., 2006; Donaire et al., 2009; Molnar et al., 2005). In the case of DNA viruses, complementarity of overlapping mRNAs synthesized by convergent bidirectional transcription can also create regions of dsRNAs that serve as templates for vsiRNAs production (Aregger et al., 2012; Blevins et al., 2011; Chellappan et al., 2004). All four DCLs contribute to the production of 21–24 nt vsiRNAs in the interactions between *A. thaliana* and cauliflower mosaic virus (CaMV, genus *Caulimovirus*, family *Caulimoviridae*, a pararetrovirus) or cabbage leaf curl virus (CaLCuV, genus *Begomovirus*, family *Geminiviridae*, a ssDNA virus) (Aregger et al., 2012; Blevins et al., 2011; Moissiard and Voinnet, 2006). MicroRNAs (miRNAs), a class of DCL1-dependent small RNAs, are derived from plant-encoded stem-loop precursor RNAs and may participate in antiviral mechanisms either directly by targeting viral RNAs (Ramesh et al., 2014) or indirectly by regulating the expression of plant genes implicated in RNA silencing (Allen et al., 2005; Mallory and Vaucheret, 2009) or in other defense responses (Boccara et al., 2014; Li et al., 2012, 2010; Shivaprasad et al., 2012; Yi and Richards, 2007).

Once sRNA duplexes are generated, one of the two strands associates with a member of the ARGONAUTE (AGO) protein family, the core component of the RISC (Ender and Meister, 2010; Mallory and Vaucheret, 2010; Poulsen et al., 2013; Pumplin and Voinnet, 2013). AGO proteins contain a C-terminal RNAse H-like PIWI domain that directs RNA slicing, the best characterized PTGS mechanism in plants, although some AGOs are deficient in RNA slicing due to posttranslational modifications or mutations in the catalytic triad of the PIWI domain. *A. thaliana* encodes 10 AGOs (AtAGO1–AtAGO10). AtAGO1 and AtAGO2, which are both implicated in siRNA-directed silencing of RNA viruses, are active in RNA slicing (Carbonell et al., 2012; Harvey et al., 2011; Jaubert et al., 2011; Morel et al., 2002; Qu et al., 2008; Scholthof et al., 2011; Wang et al., 2011). Co-factors are recruited to the RISC by interacting with AGO proteins through their Gly-Trp/Trp-Gly (GW/WG) motifs. In plants, interaction of GW proteins with AGOs has been suggested to alter the conformation of AGOs and inhibit their slicing activity (Poulsen et al., 2013). Translation repression has recently emerged as an alternative PTGS mechanism in plants that can be directed by siRNAs or miRNAs (Brodersen et al., 2008; Iwakawa and Tomari, 2013; Karran and Sanfacon, 2014; Lanet et al., 2009; Li et al., 2013; Yang et al., 2012) and may also function against plant viruses (Bhattacharjee et al., 2009; Ghoshal and Sanfacon, 2014;

Jakubiec et al., 2012; Ma et al., in press). In animal cells, the GW182 protein is a scaffold protein that interacts stably with AGO2 and also binds translation factors, such as the poly(A)-binding protein and cellular deadenylases (Pfaff and Meister, 2013; Tritschler et al., 2010). These interactions direct the decapping and translation repression of mRNA targets. The evidence for similar GW proteins acting in translation repression in plants is scarce (Poulsen et al., 2013). SUO, a GW-containing protein, was shown to be necessary for miRNA-directed translation repression, although its interaction with an AGO protein has not been confirmed (Yang et al., 2012).

RNA-dependent RNA polymerases (RDRs, six in *A. thaliana*) orchestrate the amplification phase of PTGS mechanisms (Qu, 2010). They use aberrant RNAs, e.g., RNAs cleaved by the RISC, as templates to synthesize new dsRNAs that are recognized by DCLs, allowing the production of secondary siRNAs. RDR1 and RDR6 have emerged as the two main RDRs implicated in PTGS against (+)-strand RNA viruses (Garcia-Ruiz et al., 2010; Qu et al., 2005, 2008; Schwach et al., 2005; Wang et al., 2010; Xie et al., 2001; Yang et al., 2004). SUPPRESSOR OF GENE SILENCING 3 (SGS3) interacts with RDR6 and facilitates dsRNA synthesis (Kumakura et al., 2009; Mourrain et al., 2000). Systemic movement of primary and secondary siRNAs through the vascular system provides non-cell autonomous activation of RNA silencing (Brosnan and Voinnet, 2011; Melnyk et al., 2011). Potentially, this could allow movement of the silencing signal and the antiviral mechanism ahead of the virus invasion front.

Plant antiviral TGS mechanisms

Pararetroviruses, including caulimoviruses, and ssDNA viruses such as geminiviruses, form histone-associated minichromosomes during their replication (reviewed in Pooggin (2013) and Raja et al. (2010)). The pararetrovirus encapsidated genome is a discontinuous viral dsDNA that has one or several gaps generated during reverse transcription of the viral pregenomic RNA. The gaps are repaired by the host machinery to form supercoiled dsDNA (SC DNA) that assembles into minichromosomes in the nucleus. Geminiviruses replicate using either rolling-circle or recombination-dependent mechanisms. Both mechanisms are orchestrated by host DNA polymerases. As for pararetroviruses, geminivirus dsDNA replication intermediates lead to the formation of the minichromosomes. Because of their location in the nucleus and dependence on host RNA polymerases for transcription to synthesize viral mRNAs, pararetrovirus and geminivirus viral minichromosomes are potentially susceptible to TGS.

RNA-dependent DNA methylation (RdDM) of plant genes, the hallmark of TGS, requires the coordinated action of RNA polymerase (Pol) IV and V (previously named Pol IVa and IVb, respectively) (reviewed in Haag and Pikaard (2011), Law and Jacobsen (2010), Pooggin (2013) and Pumplin and Voinnet (2013)). dsRNAs generated from Pol IV RNA transcripts by RDR2 are cleaved by DCL3 to produce 24 nt siRNAs, which are loaded onto AGO4. A scaffold transcript generated by Pol V is recognized by the AGO4–siRNA complex in Cajal bodies in the nucleolus (Li et al., 2006). *De novo* cytosine methylation requires the recruitment of the methyltransferase DRM2 and other co-factors to the Pol V–AGO4–siRNA complex. Maintenance of methylation is orchestrated by a variety of enzymes including methyltransferases MET1, CMT3 and CMT2. S-adenosyl-ʟ-methionine (SAM) is the donor of the methyl group and enzymes implicated in the methyl cycle leading to the synthesis of SAM are also essential for RdDM and maintenance methylation. DECREASE IN DNA METHYLATION 1 enables methylation by remodeling the chromatin. DNA methylation is normally accompanied by histone H3 methylation. Methylated DNA is

packed in dense heterochromatin which is inaccessible to Pol II, resulting in transcription repression.

That DLC3-dependent 24 nt vsiRNAs accumulate in plants infected with caulimoviruses and geminiviruses suggests that the RdDM pathway functions as an antiviral mechanism against DNA viruses (Blevins et al., 2011; Moissiard and Voinnet, 2006). Although methylation of geminivirus genomes is well documented, methylation of caulimovirus genomes seems to be inefficient (reviewed in Pooggin (2013) and Raja et al. (2010)). DRB3, AGO4, Pol IV, Pol V, DECREASE IN DNA METHYLATION 1 and the methyltransferases DRM1 and DRM2 have been implicated in the antiviral mechanism against two geminiviruses: CaLCuV and beet curly top virus (BCTV, genus *Curtovirus*) and *A. thaliana* mutants deficient in these enzymes showed increased susceptibility to these viruses (Raja et al., 2014, 2008). However, a separate study did not find a significant effect of AGO4, Pol IV or Pol V on the accumulation of CalCuV or CaMV (Blevins et al., 2011). Thus, our understanding of the specific requirements for antiviral RdDM is still incomplete.

Viral counter-silencing responses

Viruses have evolved multiple ways to evade or counteract RNA silencing mechanisms targeting their genome. Evasion tactics include the deployment of decoy RNAs (Blevins et al., 2011), specialized replication mechanisms (Pooggin, 2013) and sheltering of viral RNAs in large protein/membrane complexes such as replication or movement complexes. In addition, the majority of plant viruses encode viral suppressors of RNA silencing (VSR) that function by sequestering small RNAs away from the RISC, by interacting with RNA silencing enzymes and causing their destabilization or inactivation or by interfering with the expression of plant silencing enzymes (reviewed in Burgyan and Havelda (2011), Incarbone and Dunoyer (2013), Omarov and Scholthof (2012), Pumplin and Voinnet (2013) and Wu et al. (2010b)). The tombusvirus p19 protein is the archetypal example of a VSR that binds siRNAs in a size-specific manner (Silhavy et al., 2002; Vargason et al., 2003; Ye et al., 2003). Well-characterized VSRs that inactivate and/or destabilize AGO proteins include the cucumber mosaic virus (CMV) 2b protein (Zhang et al., 2006), the turnip crinkle virus coat protein (CP) (Azevedo et al., 2010), the polerovirus P0 protein (Baumberger et al., 2007; Bortolamiol et al., 2007; Csorba et al., 2010; Derrien et al., 2012), the potato virus X (PVX) p25 protein (Chiu et al., 2010), the ipomovirus P1 protein (Giner et al., 2010) and the tomato ringspot virus (ToRSV) CP (Karran and Sanfacon, 2014). Some of these VSRs contain GW/WG motifs that are necessary for their interaction with AGO proteins, thereby mimicking and possibly displacing plant AGO interactors (Azevedo et al., 2010; Giner et al., 2010; Karran and Sanfacon, 2014). There are relatively few examples of VSRs that target other silencing enzymes or co-factors, but they include the CaMV P6 protein that interacts with DRB4 (Haas et al., 2008), the tomato yellow leaf curl virus (genus *Begomovirus*) V2 protein that interacts with SGS3 (Glick et al., 2008) and the geminivirus AL2/L2 protein that interacts with adenosine kinase (ADK), an enzyme contributing to the methyl cycle that produces SAM (Wang et al., 2005, 2003). Several examples of VSRs that alter the expression of plant silencing enzymes have been documented. The tombusvirus p19 protein and other VSRs induce miR168 accumulation in order to reduce AGO1 expression (Varallyay and Havelda, 2013). The geminivirus Rep protein down-regulates the methyltransferases MET1 and CMT3 to suppress TGS (Rodriguez-Negrete et al., 2013) and the geminivirus AC2 protein, a transcriptional activator, was proposed to suppress silencing by enhancing the expression of an endogenous silencing suppressor (Trinks et al., 2005).

RNA silencing during symptom recovery and the delicate balance between silencing and silencing suppression

The first evidence that RNA silencing is involved in symptom recovery came from experiments using transgenic plants expressing the tobacco etch virus CP (Lindbo et al., 1993). Instead of showing immunity to virus challenge, the plants recovered from infection after an initial systemic symptomatic phase, a phenotype very similar to the symptom recovery observed in some natural viral infections. The recovered plants were resistant to secondary infection against related viruses but not against more distant viruses, revealing the sequence-specificity of the antiviral mechanism. Symptom recovery was also observed in transgenic lines expressing untranslatable transgene mRNAs, confirming that the resistance is RNA-based (Dougherty et al., 1994). The degree of resistance in various transgenic lines was inversely correlated with the level of expression of transgene mRNA prior to virus challenge. While plants with the lowest mRNA steady-state levels were completely resistant, plants with higher initial mRNA levels displayed the recovery phenotype. After recovery, accumulation of both viral RNA and transgene mRNA dropped in spite of continued transcription of the transgene mRNA, suggesting that a post-transcriptional RNA degradation mechanism initiated by the presence of the transgene mRNA is reinforced after virus challenge.

A link between a sequence-specific RNA degradation mechanism and symptom recovery was also revealed in the interaction between wild-type *Nicotiana clevelandii* plants and tomato black ring virus (TBRV, a nepovirus) (Ratcliff et al., 1997). Recovered leaves showed significantly reduced levels of viral RNAs compared to symptomatic leaves and were resistant to reinfection by the cognate virus but fully susceptible to unrelated viruses. The sequence specificity of the resistance was further demonstrated using a PVX vector in which a segment of TBRV was inserted. A correlation between reduced accumulation of viral RNAs in recovered leaves, sequence-specific resistance to reinoculation and symptom recovery was later described not only for other plant–nepovirus interactions (Santovito et al., 2014; Siddiqui et al., 2008), but also in plants infected with tobacco rattle virus (TRV, genus *Tobravirus*, a taxonomically unrelated RNA virus) (Ratcliff et al., 1999). PTGS and TGS have also been implicated in the symptom recovery associated with DNA viruses, such as CaMV (Covey et al., 1997) and geminiviruses (Chellappan et al., 2004).

Ectopic expression of VSRs, e.g., the potyvirus HC-Pro protein, prevented symptom recovery in plants infected with two distinct nepoviruses and enhanced viral RNA accumulation (Santovito et al., 2014; Siddiqui et al., 2008). The PVX p25 protein had a similar effect, although other VSRs did not (Siddiqui et al., 2008). The increased severity of nepovirus infection in the presence of some VSRs supports the notion that recovery-type RNA viruses, such as nepoviruses and tobraviruses, do not counteract the RNA silencing mechanism efficiently. Consistent with this idea, inactivation of VSRs in non-recovery-type viruses can lead to reduced virus accumulation and symptom recovery phenotypes. This is exemplified by geminiviruses lacking the AL2/AC2 protein (Hormuzdi and Bisaro, 1995; Wang et al., 2003), potyviruses with mutations of HC-Pro (Wu et al., 2010a), cucumoviruses with deletions of 2b (Lewsey et al., 2009) and tombusviruses with mutations or deletions of p19 (Chu et al., 2000; Omarov et al., 2006; Silhavy et al., 2002). However, symptom recovery does not imply that the virus is unable to suppress silencing. In fact, many recovery-type viruses encode weak VSRs. Although most plant viruses are excluded from the meristem, recovery-type viruses can invade the meristem and are generally seed transmitted. Meristem exclusion has been attributed to RNA silencing mechanisms

(Schwach et al., 2005). A TRV mutant deficient in the 16K protein, a weak suppressor of silencing, caused enhanced symptomatology on systemically infected leaves but failed to enter the meristem (Martin-Hernandez and Baulcombe, 2008). It was suggested that meristem entry facilitated by the weak VSR is necessary to trigger antiviral systemic silencing, leading to symptom recovery (see Fig. 1B) (Martin-Hernandez and Baulcombe, 2008). A similar role in transient meristem invasion was attributed to the CMV (Pepo strain) 2b VSR and this was also linked to the induction of symptom recovery (Mochizuki and Ohki, 2004; Sunpapao et al., 2009). However, a detailed kinetic study established that the induction of RNA silencing in systemically infected leaves occurred prior to meristem entry in plants infected with artichoke Italian latent virus (AILV, a nepovirus) and suggested that the induction of RNA silencing was triggered by a threshold of virus accumulation in the leaves rather than by meristem invasion (Fig. 1C) (Santovito et al., 2014). Nevertheless, it seems clear that the weak VSRs encoded by many recovery-type viruses allow these viruses to persist in modest levels in recovered plant tissues, thereby remaining below the threshold necessary for the induction/maintenance of damaging symptoms. Recovery has been compared to persistent or latent infections in animal cells and depends on establishing an adequate equilibrium between the host RNA silencing defense response and the virus counter-defense response (Baulcombe, 2005; Saumet and Lecellier, 2006).

RISC-dependent degradation of viral RNAs as the first PTGS mechanism implicated in the recovery from (+)-strand RNA virus infections

That symptom recovery is accompanied by the induction of RNA silencing and a concomitant reduction of viral RNA concentration suggests the induction of an RNA degradation mechanism. Although this may be the result of an AGO-directed RNA slicing mechanism, a translation repression mechanism could also result in reduced viral RNA accumulation by either destabilizing the mRNAs as shown in mammalian cells (Djuranovic et al., 2012; Huntzinger et al., 2013) or by preventing the synthesis of viral replication proteins responsible for the synthesis of the viral RNA. Two studies provided a direct link between RISC-directed slicing of viral RNA and symptom recovery in plants infected with p19-deficient tombusviruses (Omarov et al., 2007; Pantaleo et al., 2007). In the first study, sensor constructs were transiently expressed in recovered leaves of plants infected with a p19-deletion mutant of cymbidium ringspot virus (CymRSV, genus *Tombusvirus*) (Pantaleo et al., 2007). A detailed kinetic study confirmed sequence-specific degradation of the sensor mRNA that contained a CymRSV segment. Cloning of the cleaved CymRSV RNAs allowed mapping of the cleavage sites and confirmed that they are produced by the specific action of an endonucleolytic RNase. This was concomitant with the purification of large protein complexes containing vsiRNAs, likely the RISCs, from the recovered leaves. In the second study, similar large vsiRNAs-containing complexes were extracted from recovered leaves of plants infected with a tomato bushy stunt virus (TBSV) p19 mutant (Omarov et al., 2007). These complexes could direct in vitro cleavage of TBSV transcripts at specific sites. Similar extracts derived from non-recovered plants infected with wild-type TBSV did not have RNase activity, consistent with the notion that sequestering of siRNAs by p19 prevents the formation of the RISC. NbAGO2 was shown to be required for the slicing of TBSV p19 mutant genomic RNA and for symptom recovery (Fig. 2A) (Scholthof et al., 2011).

More recently, RISCs were also purified from *Nicotiana benthamiana* plants infected with wild-type TRV, a recovery-type tobravirus (Ciomperlik et al., 2011). The sequence-specific RNase

activity associated with the RISC was dependent on the presence of the vsiRNAs, as it was lost after salt treatments that dissociated the vsiRNA, but could be reinstated by addition of new vsiRNAs. Interestingly, the purified complexes could also be reprogrammed with vsiRNAs from a distinct virus (Ciomperlik et al., 2011), confirming the well-accepted notion that RISC-directed RNA slicing is a widespread RNA silencing mechanism that targets a variety of positive-strand RNA viruses.

Translation repression of viral RNAs as an alternate PTGS mechanism associated with symptom recovery from a (+)-strand RNA virus

Although the evidence supporting a link between RNA slicing and symptom recovery is compelling, some plant–virus interactions do not show a clear correlation between viral RNA clearance and symptom recovery. In *N. benthamiana* plants infected with ToRSV, a nepovirus, symptom recovery is concomitant with accumulation of vsiRNAs and sequence-specific repression of sensor constructs, but not with viral RNA clearance (Jovel et al., 2007). Symptom recovery in ToRSV-infected plants is temperature-dependent but the recovery associated with higher temperatures is accompanied by a faster and more robust accumulation of viral RNAs (Ghoshal and Sanfacon, 2014). Similarly, the temperature-dependent survival of turnip crinkle virus-infected *A. thaliana* is dependent on RNA silencing enzymes (DLC2, AGO2 and HEN1) but is correlated with increased accumulation of viral RNAs (Zhang et al., 2012). Thus, RNA silencing mechanisms other than RNA slicing may operate in these recovery phenotypes.

In vivo labeling experiments demonstrated that the temperature-dependent recovery of ToRSV-infected *N. benthamiana* plants is concomitant with reduced translation of viral RNA2, resulting in decreased accumulation of RNA2-encoded proteins, e.g., the CP (Ghoshal and Sanfacon, 2014). Plants deficient in AGO1 did not recover and translation of viral RNA2 remained active until late stages of infection (Ghoshal and Sanfacon, 2014). AGO1 has been implicated in miRNA and siRNA-directed translation repression of plant genes (Brodersen et al., 2008), raising the possibility that an AGO1-dependent mechanism represses the translation of ToRSV RNA2. In support of this suggestion, the ToRSV CP was shown to act as a weak VSR that suppresses the translation repression of a transiently expressed green fluorescent protein (GFP) reporter gene (Karran and Sanfacon, 2014). The CP enhanced fractionation of GFP mRNAs with polysomes and reduced the association of GFP-derived siRNAs with monosomes. The CP also interacted with AGO1 in a manner dependent on a WG motif, suggesting that it may be displacing a WG-cellular factor implicated in translation repression. The interaction between the CP and AGO1 led to the degradation of a sub-population of AGO1 (probably the sub-population involved in translation repression since the RNA slicing mechanism, which is also directed by AGO1 was not affected by the CP) (Karran and Sanfacon, 2014). Taken together, these results indicate that the CP counteracts an AGO1-directed siRNA-dependent translation repression mechanism that targets a transiently expressed GFP reporter gene and by extension, probably also ToRSV RNAs in infected plants. In mammalian cells, translation repression is correlated with imperfect sequence complementarity between miRNAs and their targets (Doench et al., 2003; Doench and Sharp, 2004; Huntzinger and Izaurralde, 2011). However, plant siRNAs with perfect complementarity to their target mRNAs have been shown to direct translation repression (Brodersen et al., 2008; Iwakawa and Tomari, 2013). Thus, vsiRNAs or other cellular sRNAs with sequence identity to ToRSV may be loaded on AGO1 to repress the translation of ToRSV RNA2.

The persistent steady-state levels of ToRSV RNA2 after the onset of symptom recovery could be explained by encapsidation, which would protect the viral RNA against degradation and sequester it away from
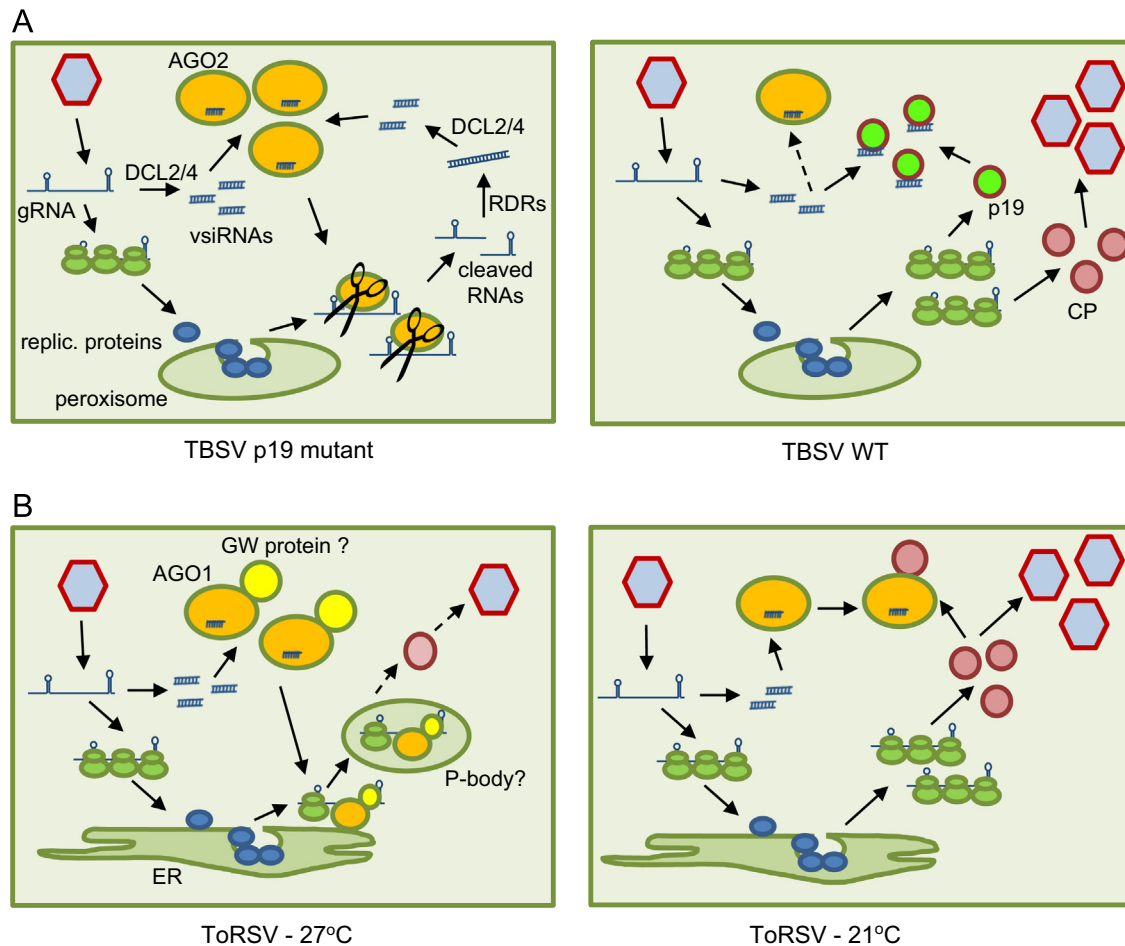
Fig. 2. Antiviral PTGS mechanisms in recovered leaves of plants infected with (+)-strand RNA viruses. (A) Simplified model of the RNA slicing mechanism in recovered leaves of *N. benthamiana* plants infected with a TBSV p19 mutant and viral counter-silencing mechanism in symptomatic leaves of plants infected with wild-type (WT) TBSV. In plants infected with TBSV-p19 mutants, stem-loop structures on the genomic RNA (gRNA) are recognized by DCL2/4 to form vsiRNAs, which are incorporated into AGO2-RISCs. Limited translation and replication (in modified peroxisome membrane compartments) may initially occur but the AGO2-RISCs will eventually cleave the viral RNAs. These cleaved RNAs are recognized by RDR enzymes to form dsRNAs that serve as templates for a new round of DCL-directed vsiRNA formation. In plants infected with TBSV WT, the TBSV p19 protein, which is translated from subgenomic RNAs produced during the replication/transcription step, binds the vsiRNAs, preventing their incorporation into AGO2-RISCs. Please note that although all events are depicted in a single cell for simplicity, steps may occur sequentially in different cells or leaves. For example, in recovered leaves (left panel) the vsiRNAs may move ahead of the virus front and prevent the unloading of virus from the vascular system as has been suggested for the related CymRSV (Havelda et al., 2003). Thus, preloaded AGO2-RISCs may lead to degradation of viral RNA before any replication can occur. (B) Simplified model for the temperature-dependent translation repression mechanism associated with symptom recovery in *N. benthamiana* plants infected with ToRSV. At 27 °C, vsiRNAs presumably produced by DCL2/4 cleavage of gRNAs are incorporated into AGO1-RISCs that may be associated with an unidentified GW protein. Initial translation/replication of the gRNA in modified ER membranous compartments releases the newly synthesized viral RNAs in proximity to ER-associated AGO1-RISCs, which repress further translation of these RNAs. The viral RNA–RISC complex may subsequently be incorporated into P-bodies or other cytoplasmic granules and could potentially be recycled for limited translation. At 21 °C, the silencing mechanism is less active, which leads to slower accumulation of vsiRNAs, allowing continued translation of viral RNAs and accumulation of viral proteins. In addition, the CP, which accumulates following the regulated cleavage of the RNA2-encoded polyprotein by the mature form of the protease (Chisholm et al., 2001), interacts with AGO1, possibly displacing an interacting GW factor. The AGO1-CP interaction also leads to the degradation of AGO1. It should be noted however that suppression of the AGO1-directed translation repression mechanism may also occur to some extent at the higher temperature, at least initially (not shown in the figure).

the translation machinery. However, viral RNA encapsidation would also occur for other viruses, including nepoviruses such as TBRV, tobacco ringspot virus or AILV that show drastically reduced RNA levels after symptom recovery (Ratcliff et al., 1997; Santovito et al., 2014; Siddiqui et al., 2008). An alternative model, presented in Fig. 2B, is that ToRSV evades RISC-directed RNA slicing as a consequence of the translation repression mechanism. In this model, incorporation of ToRSV RNAs in AGO1-RISCs active in translation repression may protect them against degradation, as shown in vitro for cellular mRNAs (Iwakawa and Tomari, 2013). In plants, AGO1 is a peripheral membrane protein (Brodersen et al., 2012). MiRNA-mediated translation repression is associated with the endoplasmic reticulum (ER) and requires AMP1, an integral membrane protein (Li et al., 2013). Given that the ER is the site of ToRSV replication (Chisholm et al., 2007; Han and Sanfacon, 2003), it is possible that ToRSV RNAs are recruited to an ER-associated AGO1/AMP1 RISC complex immediately after their release from the replication complex. The translationally repressed RNAs may later be stored in P-bodies or other cytoplasmic granules, as has been shown for animal cells, which would also allow their recycling for renewed translation under changing environmental conditions (Bhattacharyya et al., 2006a, 2006b). Indeed, the translation repression mechanism was shown to be partially reversible after a shift of the ToRSV-infected recovered plants to lower temperature (Ghoshal and Sanfacon, 2014). Similarly, recruitment of translationally repressed flock house virus RNAs to cytoplasmic granules may protect them against degradation (Petrillo et al., 2013). Localization of ToRSV RNAs in plant cells before or after the onset of translation repression may provide some insights into the mechanisms regulating their stability.

Translation repression was also implicated in the reduction of viral RNAs and viral-encoded proteins observed at late stages of infection in *A. thaliana* plants infected with TRV-GFP, a TRV modified to encode GFP (Ma et al., in press). This drop in TRV-GFP accumulation was

termed virus recovery and may be related to symptom recovery, although plants remain asymptomatic throughout the course of infection. TRV RNAs showed decreased association with ribosomes after virus recovery, suggesting reduced translation. In addition, the average number of P-bodies was significantly increased in cells from recovered leaves (Ma et al., in press). However, whether viral RNAs accumulate in these P-bodies remains to be tested. Surprisingly, virus recovery was still observed in plants deficient for various AGO proteins and in a triple *dcl* mutant, suggesting either redundant action of silencing enzymes or an RNA silencing-independent mechanism. As RNA slicing was shown to target TRV in *N. benthamiana* (Ciomperlik et al., 2011), both RNA slicing and translation repression may operate concurrently. Alternatively, the selection for translation repression or RNA slicing mechanisms may be influenced by the host-virus isolate combination and/or by environmental conditions.

Although AGO1 can direct slicing and translation repression of the same RNA target (Brodersen et al., 2008; Iwakawa and Tomari, 2013), how it coordinates these two functions is not well understood. In a first model, the activity of AGO1 may be regulated by post-translational modifications. In human cells, phosphorylation of AGO2 by the AKT3 kinase facilitates its interaction with the GW182 protein, providing a molecular switch between its RNA slicing and translation repression activities (Horman et al., 2013). The plant AGO1 may be similarly modified by phosphorylation or other post-translational modifications during virus infection. In a second model, the activity of AGO1 may be regulated by its subcellular localization. It has been suggested that several pools of AGO1 exist that are loaded with different classes of sRNAs and may have distinct subcellular localizations (Schott et al., 2012). AGO1 was found to partition both in soluble and membrane-enriched fractions (Brodersen et al., 2012). Although the ER-associated AGO1 translation repression activity is hindered in *amp1* mutants, its RNA slicing activity is not affected (Li et al., 2013). The subcellular localization of the AGO1 RNA slicing activity is however not known. Further study of translation repression mechanisms associated with symptom recovery may provide further insights into the regulation of AGO activities and the mechanisms by which viruses are targeted by RNA slicing and/or translation repression.

Contribution of PTGS and TGS in symptom recovery from geminiviruses and caulimoviruses

As discussed above, the replication cycle of plant DNA viruses (geminiviruses and pararetroviruses) includes nuclear and cytoplasmic phases making them potentially susceptible to both PTGS and TGS mechanisms (Fig. 3) (reviewed in Pooggin (2013) and Raja et al. (2010)). Depending on the specific host-virus combination, symptom recovery can be observed in plants infected with wild-type geminiviruses. Recovery is accompanied with a reduction of viral DNA and mRNA accumulation, sequence-specific virus resistance and vsiRNA accumulation (Carrillo-Tripp et al., 2007; Chellappan et al., 2005, 2004; Hagen et al., 2008). Both 21–22 nt vsiRNAs (the hallmark of PTGS) and 24 nt vsiRNAs (characteristic of TGS) accumulate in recovered leaves (Blevins et al., 2006; Rodriguez-Negrete et al., 2009). DCL4, RDR6 and SGS3 are required for the induction and spread of silencing signals induced by a CalCuV-derived vector corroborating the activity of PTGS against geminiviruses (Blevins et al., 2006; Muangsan et al., 2004). However, *A. thaliana dcl4* or *rdr6* mutants were only modestly more susceptible to wild-type CalCuV or BCTV and symptom recovery from a VSR-deficient BCTV was not prevented in single *dcl4* or *dcl2* mutants (Raja et al., 2014, 2008). Although these results could be attributed to functional redundancy between

DCL2 and DCL4, which are both implicated in antiviral PTGS, additional studies will be required to examine the contribution of PTGS to symptom recovery in geminivirus-infected plants. The involvement of TGS in symptom recovery from geminivirus infection is better documented. In recovered leaves of pepper plants infected with pepper golden mosaic virus, the 21–22 nt vsiRNAs are primarily derived from the coding regions, while the 24 nt vsiRNAs predominantly map to the intergenic region, a region which is preferentially methylated (Rodriguez-Negrete et al., 2009). Symptom recovery is associated with enhanced methylation of geminivirus DNA, especially in the intergenic region (Hagen et al., 2008; Raja et al., 2008; Rodriguez-Negrete et al., 2009). Although the extent of methylation in geminivirus DNA has been disputed (Paprotka et al., 2011; Pooggin, 2013), recent analyses have confirmed the increase in viral DNA methylation in recovered leaves from geminivirus-infected plants (Raja et al., 2014). Direct evidence that TGS plays a role in recovery from geminivirus infection came from the study of BCTV L2-deficient (L2$^-$) mutants (Fig. 3A). L2 is a VSR that inhibits TGS by inactivating ADK (Wang et al., 2005, 2003). *A. thaliana* plants inoculated with BCTV L2$^-$ recover from infection, while plants infected with wild-type BCTV do not (Buchmann et al., 2009; Hormuzdi and Bisaro, 1995). Interestingly, *ago4*, *drb3* and *dcl3* mutants of *A. thaliana* did not recover from BCTV L2$^-$ infection, and this was accompanied with reduced methylation of the viral DNA compared to that observed in recovered leaves of wild-type plants (Raja et al., 2014, 2008). Although highly methylated viral minichromosomes examined in BCTV L2$^-$ recovered leaves were associated with histone 3 lysine 9 dimethylation, as observed for repressed plant heterochromatin, some viral DNAs remained hypomethylated and were associated with active acetylated histone 3 (Raja et al., 2014). This would allow the maintenance of a sub-population of transcriptionally active viral minichromosomes in recovered leaves. Unfortunately, genetic determinants affecting symptom recovery from infections with wild-type geminiviruses in other host plants has not been studied, due to the unavailability of similar *dcl*, *drb* or *ago* mutants.

Susceptibility of *Brassica* species to a pararetrovirus (CaMV) infection varies from highly symptomatic systemic infections in *Brassica rapa* to mild or asymptomatic infections in *Brassica oleracea* (Covey et al., 2000). Early studies established that highly susceptible hosts contain high virus titers, high levels of viral mRNAs and reverse transcription products, but low levels of CaMV minichromosomes (SC DNA). In contrast, mild infections are associated with low virus titers and low levels of viral mRNAs, but an abundance of SC DNA (Fig. 3B) (Covey et al., 1990; Sanfacon and Wieczorek, 1992; Saunders et al., 1990). In CaMV-infected *B. oleracea gongylodes*, symptom recovery was preceded by a sudden increase in SC DNA level and a concomitant decrease in reverse transcription products and mRNAs (Covey et al., 1997). Run-on experiments revealed constant transcription levels after recovery in spite of a 50-fold increase in the concentration of viral minichromosomes and a drastic decline in viral mRNA levels. This implies that viral mRNAs are degraded post-transcriptionally and also that the majority of the viral minichromosomes are transcriptionally inactive, suggesting the activation of both PTGS and TGS mechanisms (Covey et al., 1997). CaMV infection causes transcriptional arrest of transgene mRNAs driven by the CaMV 35S promoter, confirming the activation of sequence-specific TGS in response to CaMV infection (Al-Kaff et al., 1998, 2000). In contrast to geminiviruses, the CaMV genome is apparently not differentially methylated following symptom recovery, although changes in populations of SC DNA topoisomers were noted (Covey et al., 1997), which may reflect changes in histone modification and chromatin compacting. Thus, the mechanisms leading to the transcriptional arrest of CaMV minichromosomes and the
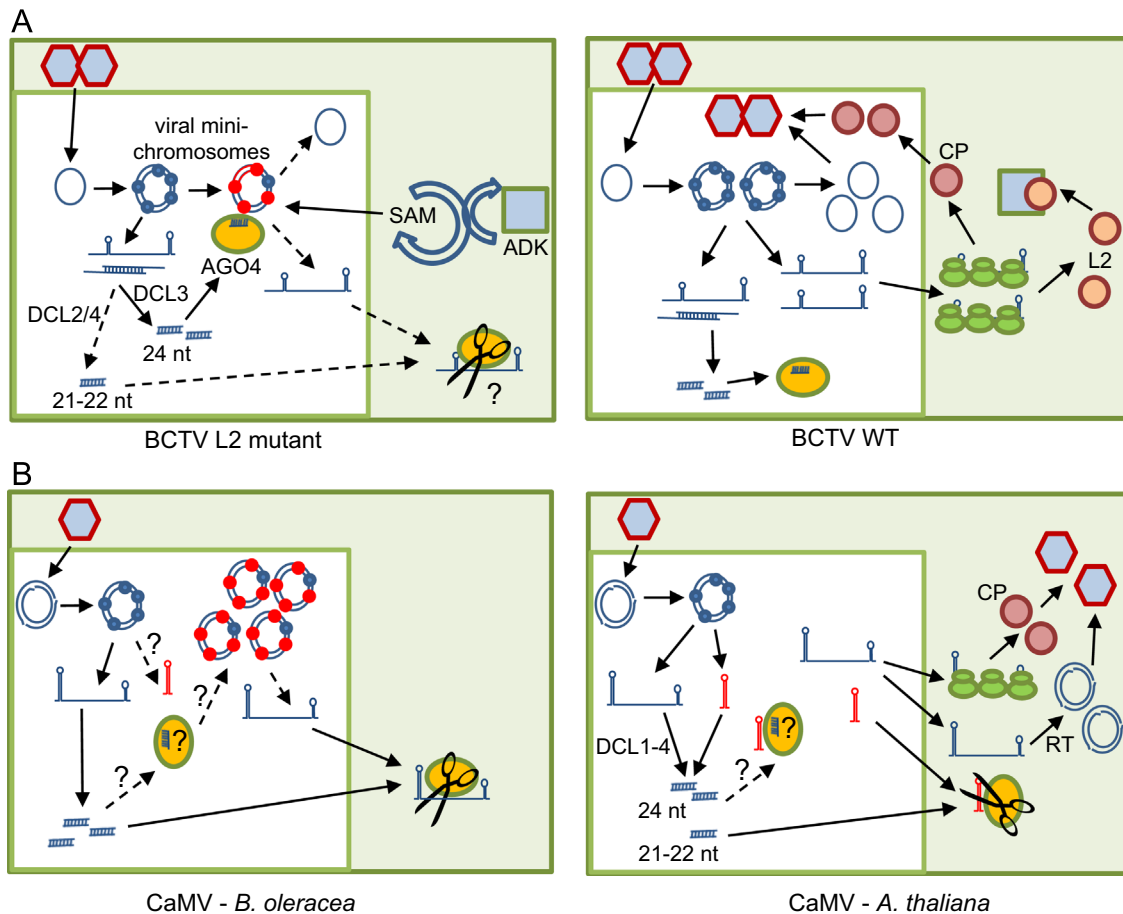
Fig. 3. Antiviral TGS and PTGS mechanisms in recovered leaves of plants infected with DNA viruses. (A) Simplified model of TGS and PTGS in recovered leaves of *A. thaliana* plants infected with a BCTV L2 mutant and viral counter-silencing mechanisms in symptomatic leaves of plants infected with wild-type (WT) BCTV. Events are shown that occur in the nucleus (white background) or cytoplasm (green background). In plants infected with the L2 mutant, the viral genomic ssDNA replicates through a rolling circle mechanism. The dsDNA replication intermediates associate with plant histones to form viral mini-chromosomes which are transcribed by host polymerases. Stem-loop structures on the viral mRNAs or convergent overlapping transcription, which produces region of dsRNA, are recognized by DCL enzymes to create the 21–22 nt and 24 nt vsiRNAs. The 24 nt vsiRNAs are loaded on AGO4-RISCs and together with host methylation enzymes (not shown) orchestrate the methylation of the viral dsDNA and associated histones (in red). The methylated minichromosomes are poor templates for transcription and replication. SAM is the methyl donor and is produced in the cytoplasm. ADK is an enzyme associated with the methyl cycle producing SAM. The 21–22 nt vsiRNAs may also be loaded on AGO-RISCs in the cytoplasm to degrade viral RNAs, although the contribution of this PTGS mechanism to recovery needs to be further investigated. In plants infected with BCTV WT, the L2 VSR interacts with and inactivates ADK, thereby inhibiting SAM production and preventing methylation of the viral mini-chromosomes. Transcription, replication and translation can occur, which allows the virus to complete its replication cycle. (B) Simplified models of PTGS and TGS mechanisms associated with host-dependent recovery in CaMV-infected plants. The nicked dsDNA circular genome is repaired by cellular enzymes and associates with plant histones in the nucleus to form viral minichromosomes, which are transcribed by plant polymerases. The viral full-length RNA (35S) as well as the 19S RNA (not shown) and 8S decoy RNA (in red) are transported to the cytoplasm. In *B. oleracea*, symptom recovery is associated with increased accumulation of transcriptionally inactive chromosomes. The mechanisms governing the accumulation and transcriptional inactivation of the minichromosomes and the potential role of an AGO-RISC in this process are not well understood. The viral DNA is apparently not methylated, although histones may be modified (shown in red). Viral RNAs are degraded in the cytoplasm, presumably by RISCs associated with the 21–22 nt vsiRNAs, although the nature of vsiRNAs accumulating in this host has not been examined. In *A. thaliana*, although 21–22 nt and 24 nt vsiRNAs accumulate, viral RNAs are protected from degradation, likely because of the accumulation of the decoy RNA that binds to RISCs in the cytoplasm and possibly also in the nucleus. The protected viral RNAs proceed to translation and reverse transcription and complete the replication cycle.

potential role of the 24 nt vsiRNAs in this process are not well understood. Antiviral CaMV silencing mechanisms have been studied in *A. thaliana*, which is a highly susceptible host. The vast majority of vsiRNAs map to the leader region of the large 35S RNA, and it was suggested that a small RNA corresponding to this region acts as a decoy to protect the viral RNAs from degradation (Blevins et al., 2011). Indeed, quadruple *dcl A. thaliana* mutants did not accumulate higher levels of CaMV RNAs, even though the generation of vsiRNAs was prevented (Blevins et al., 2011). The decoy RNA probably corresponds to an 8S RNA detected in CaMV-infected *B. rapa* (Guilley et al., 1982). Unfortunately, the presence or activity of the decoy RNA, the accumulation of vsiRNAs or the role of various silencing enzymes has not been studied in tolerant hosts that display symptom recovery.

Does illegitimate integration of viral sequences into plant genomes play a role in symptom recovery?

Although pararetroviruses and geminiviruses normally form episomal minichromosomes, illegitimate integration of these viruses in the plant genome is well documented (Hohn et al., 2008; Staginnus and Richert-Poggeler, 2006). In addition, integrated cDNA sequences of RNA viruses have also been found in plant genomes (Chiba et al., 2011; Hohn et al., 2008; Tanne and Sela, 2005). Integrated viral sequences are usually present in multiple copies and are rearranged, frequently defective versions of the virus. In addition, they are often inserted in the vicinity of retrotransposon sequences, possibly borrowing their reverse transcriptase activity for integration of cDNAs from RNA viruses. Endogenous viral sequences may contribute to
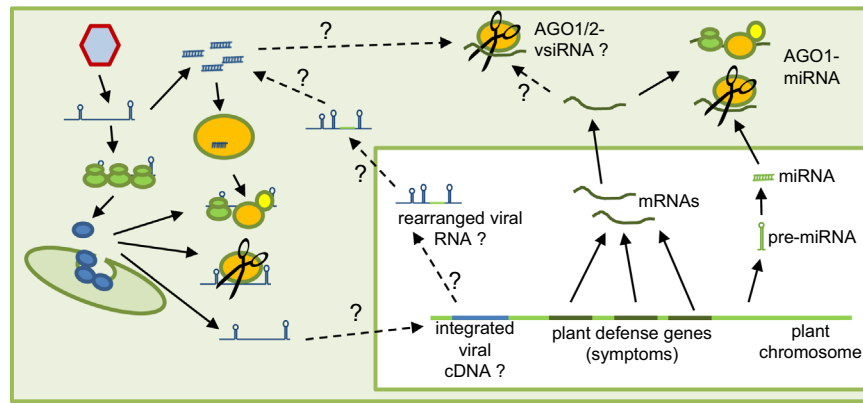
Fig. 4. Characterized and tentative RNA silencing mechanisms operating in association with symptom recovery. For simplicity, we only depict mechanisms related to infection with (+)-strand RNA viruses. Antiviral PTGS mechanisms detailed in Fig. 2 are shown on the left of the figure. Symptoms are often caused by the activation of plant defense genes, which are regulated by plant miRNAs as shown by the cytoplasmic AGO1–miRNA RISCs. These complexes may direct RNA slicing or translation repression of the plant mRNAs. VsiRNAs may also target plant mRNAs as shown. Finally, the possibility that a cDNA copy of the viral genome is integrated in the plant genome with the help of a retrotransposon reverse transcriptase is illustrated. A putative rearranged viral RNA may be transcribed from an integrated viral cDNA resulting in a second round of vsiRNA production.

resistance to infection with exogenous viruses of related sequences, much like transgenic plants with viral transgenes (Covey and Al-Kaff, 2000; Teycheney and Tepper, 2007). Indeed, 24 nt sRNAs derived from an endogenous pararetrovirus sequence were found to accumulate to high levels in *Fritillaria imperialis L.* plants (Becher et al., 2014). Although illegitimate integration of viral sequences in plant genomes is generally considered a rare event, somatic endogenization may in fact occur frequently but would remain undetected because it is not passed on to the next generation (Covey and Al-Kaff, 2000). Recently, the establishment of a persistent infection of an RNA virus in insect cells was found to be associated with reverse transcription of the virus genome and embedding of rearranged viral sequences in retrotransposon sequences (Goic et al., 2013). Reverse transcriptase inhibitors or down-regulation of RNA silencing enzymes prevented the establishment of persistent infection, suggesting that transcripts derived from the integrated sequences contribute to the production of siRNAs that target the virus. Surprisingly, reverse transcription of RNA virus fragments occurred early, reproducibly and efficiently following the initial infection of naïve insect cells (Goic et al., 2013). Could similar illegitimate integration events play a role in the establishment of symptom recovery in plants (see Fig. 4)? Early studies with CaMV-infected *B. oleracea* plants noted the presence of high-molecular weight DNA in Southern blots that used DNA extracted from recovered leaves and suggested that these could correspond to viral sequences integrated in the plant genome (Covey and Al-Kaff, 2000). However, further research will be required to investigate a possible correlation between endogenization of viral sequences and the induction of symptom recovery. Similarly, it is not known whether cDNA forms of RNA viruses are synthesized prior to the onset of symptom recovery.

Concluding remarks

In this review, we have discussed antiviral RNA silencing mechanisms that target recovery-type viruses in order to control the accumulation of their genomic DNAs, genomic RNAs, mRNAs and/or proteins. However, in the case of tobacco plants infected with tobacco streak virus (TSV, genus *Ilarvirus*, family *Bromoviridae*), symptom recovery is not accompanied by a significant reduction in viral RNAs or CP levels in spite of the presence of vsiRNAs (Xin and Ding, 2003). More surprisingly, a naturally-occurring mutant of TSV defective in recovery accumulated to lower levels compared to other TSV isolates. It was suggested that mechanisms other than RNA silencing may

cause symptom recovery in this interaction, e.g., a viral protein may actively suppress the symptomatic necrotic response (Xin and Ding, 2003). Similarly, CMV 2b mutants with increased nucleus-cytoplasm partitioning had decreased VSR activity but increased virulence resulting in the loss of symptom recovery (Du et al., 2014). This uncoupled two functions of 2b in symptom virulence and silencing suppression, and suggested that the nuclear/nucleolus localization of 2b may alter the transcription of genes implicated in plant cell death The nucleus has also been implicated in the recovery of tobacco plants from TRV and TBRV infection (Shaw et al., 2014). Knock-down of coilin, a scaffold protein of nucleolus-located Cajal bodies, prevented symptom recovery and enhanced virus accumulation in late systemic leaves but did not interfere with vsiRNA accumulation. It is not clear whether coilin plays a role in an as-yet unknown RNA silencing mechanism or whether it triggers a separate defense response that restricts virus accumulation concomitantly with RNA silencing. Thus, the interplay of RNA silencing and other plant defense responses and/or virus counterdefense mechanisms may regulate the establishment of symptom recovery in some plant–virus interactions.

Finally, RNA silencing may mitigate symptoms not only by controlling virus accumulation but also by targeting host genes involved in symptomatic defense responses (Fig. 4). Several plant miRNAs down-regulate the expression of plant defense genes involved in salicylic acid-dependent defense responses (Boccara et al., 2014; Li et al., 2012; Li et al., 2010; Shivaprasad et al., 2012; Yi and Richards, 2007). These defense responses can be deregulated in acute virus infection or by the expression of strong VSRs, such as the potyvirus HC-Pro, resulting in enhanced pathogen resistance and increased symptomatology (Jovel et al., 2011; Pruss et al., 2004). Similarly, deregulation of the miRNA pathway may contribute to the enhanced symptomatology and lack of symptom recovery observed in plants defective for AGO1 (Ghoshal and Sanfacon, 2014). There are few documented examples of vsiRNAs with plant gene targets. One example is the targeting of a plant chlorophyll synthesis gene by an siRNA derived from a CMV satellite RNA, which produces a yellow mosaic (Shimura et al., 2011; Smith et al., 2011). Future research may well identify vsiRNAs that direct symptom remission using plant gene targets. Finally, a recently identified new class of virus-induced plant siRNAs could also participate in symptom alleviation (Cao et al., 2014). Thus, deep-sequencing of the plant transcriptome and of plant and viral sRNAs in symptomatic or recovered leaves may unravel new layers of RNA silencing-directed regulation of symptom expression. Transcriptome analysis of pepper golden mosaic virus-infected peppers before or after symptom recovery identified many differentially

expressed genes including defense genes and a set of histone modification genes that may direct the transcriptional repression of geminivirus minichromosomes (Gongora-Castillo et al., 2012). However, concurrent analysis of sRNAs would be necessary to determine whether RNA silencing directed by miRNAs, vsiRNAs or other classes of siRNAs induced by viruses are implicated in these differential expression patterns. In conclusion, symptom recovery is a complex phenomenon that likely requires simultaneous control of virus accumulation and symptom remission through a variety of RNA silencing pathways, some of which are waiting to be unraveled, and possibly other as yet unidentified mechanisms.

Acknowledgments

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