View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Virology 344 (2006) 158 – 168

VIROLOGY

www.elsevier.com/locate/yviro

Silencing suppression by geminivirus proteins

David M. Bisaro

Department of Molecular Genetics and Plant Biotechnology Center, The Ohio State University, Columbus, OH 43210, USA

Received 16 September 2005; accepted 19 September 2005

Abstract

RNA silencing is an RNA-directed gene regulatory system that is present in a wide range of eukaryotes, and which functions as an antiviral defense in plants. Silencing pathways are complex and partially overlapping, but at least three basic classes can be distinguished: cytoplasmic RNA silencing (or post-transcriptional gene silencing; PTGS) mediated by small interfering RNAs (siRNAs), silencing mediated by microRNAs (miRNAs), and transcriptional gene silencing (TGS) mediated by siRNA-directed methylation of DNA and histone proteins. Recent advances in our understanding of different geminivirus silencing suppressors indicate that they can affect all three pathways, suggesting that multiple aspects of silencing impact geminivirus replication.
© 2005 Elsevier Inc. All rights reserved.

Contents

The Geminiviridae 158
RNA silencing pathways 159
Plant virus silencing suppressors 160
Geminiviruses are inducers and targets of RNA silencing 161
Transcription-dependent and -independent silencing suppression by AL2/AC2 and L2 proteins 162
Silencing suppression by AC4 proteins and AC4–AC2 synergy in virus disease 164
Silencing suppression by βC1 protein 165
The miRNA pathway and antiviral defense. 165
Summary 166
Acknowledgments. 166
References 166

The Geminiviridae

Members of the family Geminiviridae are single-stranded DNA (ssDNA) viruses that infect a wide range of plant species and are responsible for considerable losses of food and fiber crops. The family takes its name from the unique twin icosahedral capsid structure of its members. Each paired particle encapsidates a single ssDNA circle which varies in size from ~2.5 to 3.0 kb, depending on the virus. Genome replication occurs in the nucleus by a rolling circle mechanism that employs circular double-stranded DNA (dsDNA) replicative form (RF) intermediates, although some recombination-mediated replication can also occur. The dsDNA RF molecules, which serve as replication and transcription templates, associate with histone proteins and are assembled into minichromosomes. Geminiviruses do not encode DNA or RNA polymerases and so depend on the cellular replication and transcription machinery to express their genes and amplify their genomes (Gutierrez, 1999; Hanley-Bowdoin et al., 1999). Thus, they are excellent models for the study of host replication and transcription and how these processes can be affected by epigenetic modification.

The geminiviruses are classified into four genera, *Begomovirus*, *Curtovirus*, *Mastrevirus*, and *Topocuvirus*, depending on

E-mail address: bisaro.1@osu.edu.

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.09.041

genome organization, host range, and type of insect vector (Fauquet et al., 2003). The begomoviruses infect dicotyledonous plants and are whitefly-transmitted. Most, such as *Tomato golden mosaic virus* (TGMV), *African cassava mosaic virus* (ACMV), and *Cabbage leaf curl virus* (CaLCuV), have genomes consisting of two components. These bipartite viruses can be further divided into those originating in the Old World (e.g. ACMV) or the New World (e.g. TGMV, CaLCuV). The two separately encapsidated genome components, called A and B, are similar in size but differ in sequence except for a common region (CR) of 200 to 250 bp that is nearly identical in the two components of a given virus, but differs between viruses. The CR is part of a larger intergenic region (IR) that contains the origin of replication and divergent RNA polymerase II promoters. All geminiviruses, regardless of genus, have a similar IR, which occupies a nucleosome-free region in the minichromosome (Pilartz and Jeske, 2003). Other begomoviruses, for example *Tomato yellow leaf curl virus* (TYLCV), have only a single genome component that is similar to the A component of bipartite viruses in its organization. The monopartite begomoviruses are confined to the Old World and some are associated with a ssDNA satellite known as DNAβ, which is required for the induction of characteristic disease symptoms. The curtoviruses, exemplified by the type member *Beet curly top virus* (BCTV), also infect dicots and have monopartite genomes that are similar to the A component. However, they can be distinguished from monopartite begomoviruses by several criteria, including some unique genes, transmission by leafhoppers, and an extremely broad host range. The curtoviruses and the begomoviruses, including their satellites, have recently been shown to encode proteins capable of suppressing RNA silencing. What is known about the mechanism of action of these proteins, and what this tells us about silencing and related mechanisms, is the subject of this review. To date, there has been no report of a silencing suppressor encoded by a mastrevirus (e.g. *Maize streak virus*). These monopartite agents infect primarily monocotyledonous plants and have a genome organization that differs in significant ways from the typical A component. Of relevance here is that they lack homologues of the begomovirus and curtovirus silencing suppressors. Few details are available concerning the replication of the only known topocuvirus, *Tomato pseudo curly top virus*. Mastreviruses and topocuviruses will not be further discussed.

RNA silencing pathways

RNA silencing is a term often used to refer to related mechanisms also known as post-transcriptional gene silencing (PTGS) in plants, quelling in fungi, and RNA interference (RNAi) in animals. RNA silencing pathways are involved in a number of fundamental processes, including cellular defense against viruses, control of transposon mobility, gene regulation via microRNAs (miRNAs), de novo histone and DNA methylation, and the establishment of heterochromatin (Baulcombe, 2004; Carrington and Ambros, 2003; Lippman and Martienssen, 2004; Voinnet, 2005). Key players in the RNA silencing machinery include the ribonuclease Dicer, RNA-dependent RNA polymerase (RDR), and Argonaute (AGO). The machinery in plants appears to be more elaborate than in fungal or animal systems. The *Arabidopsis* genome encodes four Dicer-like (DCL) enzymes, six RDRs, and 10 AGO proteins. Genetic studies indicate that these factors functionally partner in specific ways to effect distinct but partially overlapping pathways that are commonly triggered by double-stranded RNA (dsRNA).

Plants have at least three silencing pathways (Baulcombe, 2004). PTGS, or cytoplasmic RNA silencing, was the first identified. This mechanism results in the degradation of target mRNA (or the genome of an RNA virus), and a defining feature is the appearance of 21–22 nucleotide short interfering RNA species (siRNA) which are generated from inducing dsRNA (Hamilton and Baulcombe, 1999) (Fig. 1). Although some functional redundancy is apparent among the DCL proteins, this small class of siRNA is possibly generated by DCL-2 and DCL-4 (Gasciolli et al., 2005; Xie et al., 2004). The inducing dsRNA may come from exogenous or endogenous sources, such as RNA virus replication intermediates synthesized by viral RDRs (vRDR), structured ssRNA or annealed overlapping transcripts of opposite polarity that can serve as Dicer substrates, or products of RDR acting on certain transcripts or on aberrant or over-expressed mRNAs (Gazzani et al., 2004; Molnar et al., 2005; Szittya et al., 2002). Duplex siRNA is subsequently unwound and one strand is incorporated into an RNase-containing effector complex known as RISC (RNA-induced silencing complex), which contains at least one AGO protein (Hammond et al., 2000; Hannon, 2002). Cleavage specificity is a consequence of complementary base-pairing between the siRNA and the target mRNA. By analogy to the mammalian system, an AGO protein in RISC is most likely the "slicer" that carries out transcript cleavage (Liu et al., 2004). RDR can have multiple roles in the pathway. In addition to initial generation or processing of the dsRNA trigger, RDR can also mediate the amplification and transitive spreading of siRNAs (Himber et al., 2003; Vaistij et al., 2002). Another remarkable feature of RNA silencing is its ability to spread from cell-to-cell and systemically throughout the plant (Palaqui et al., 1997; Voinnet and Baulcombe, 1997). The nature of the mobile systemic silencing signal is unknown but its sequence specificity strongly suggests that it is nucleic acid, and most likely a small RNA (Hamilton et al., 2002). A second silencing pathway is dedicated to the endogenous, 21–22 nucleotide miRNAs that are processed by DCL-1 from larger miRNA precursors specified by non-protein-coding genes (Bartel, 2004; Qi et al., 2005; Xie et al., 2004). The miRNAs negatively regulate their target mRNAs, either by inhibiting translation (primarily in animal systems) or by degradation. In plants, miRNAs are usually perfectly complementary to their target mRNAs and direct RISC cleavage in essentially the same manner as siRNAs (Llave et al., 2002). The third pathway leads to siRNA-directed transcriptional gene silencing (TGS) and heterochromatic silencing (Lippman and Martienssen, 2004). It can be triggered by transcription of inverted repeats or tandemly repeated sequences, and

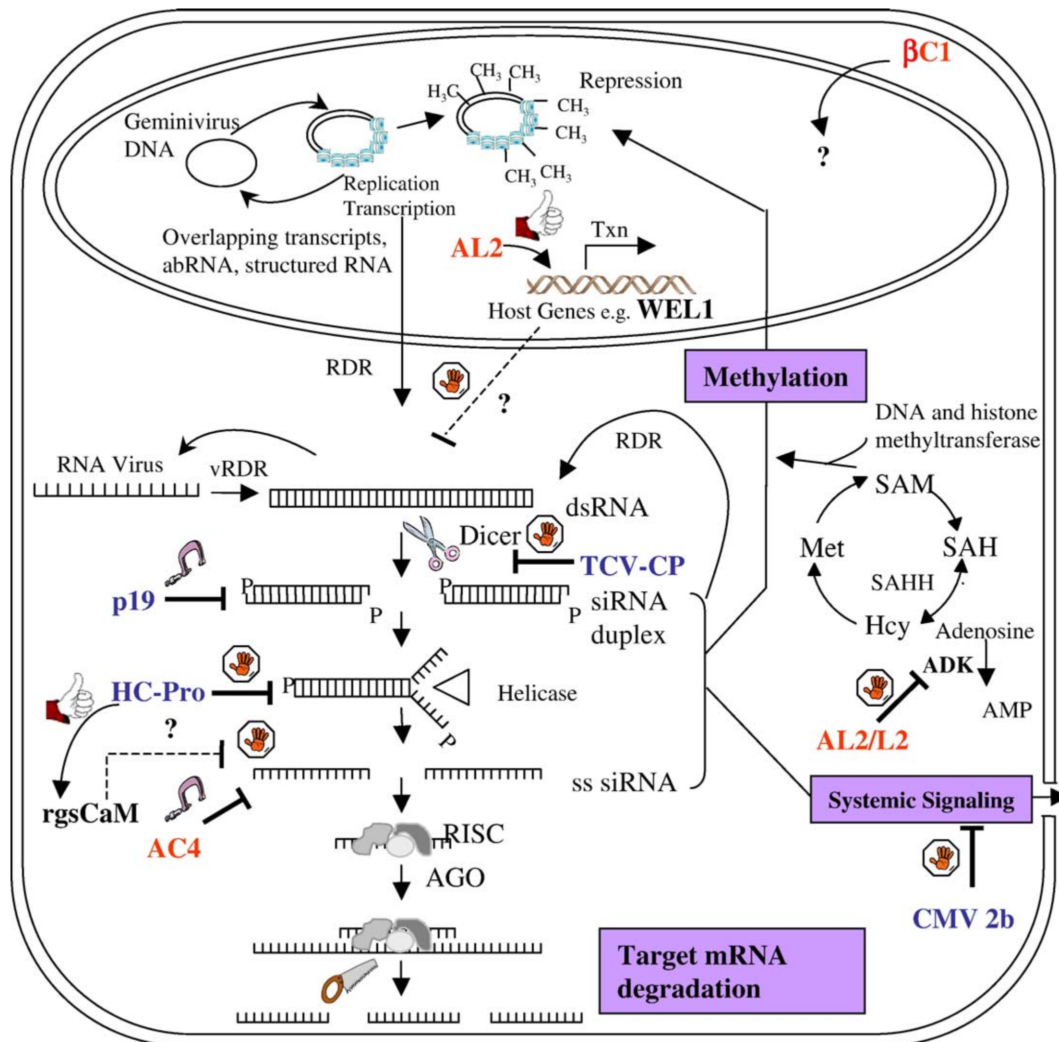

Fig. 1. Antiviral RNA silencing pathways in plants and the action of geminivirus suppressors. The scheme depicts two silencing pathways: cytoplasmic RNA silencing (PTGS) leading to target mRNA degradation, and siRNA-directed methylation leading to transcriptional gene silencing (TGS) (see text for details). Geminivirus replication occurs in the nucleus, and the dsRF is a potential target of methyltransferases that modify DNA and histone proteins (blue rectangles) in viral minichromosomes. Geminivirus silencing suppressors (red) interfere with silencing pathways at multiple steps. In a transcription-independent mechanism, begomovirus AL2/AC2 (indicated AL2) and curtovirus L2 proteins interfere with the methyl cycle by inhibiting ADK and thus impede transmethylation. In the nucleus, begomovirus AL2/AC2 activates transcription of host genes including *WEL1*, which suppresses silencing by an unknown mechanism. AC4 binds single-stranded forms of siRNA (and miRNA, not shown) and prevents RISC programming. The βC1 protein suppresses silencing by acting in the nucleus in an unknown manner. The steps at which suppressors from RNA viruses (blue) are believed to act are also indicated.

experimentally by ectopic expression of RNA corresponding to promoter regions (Jones et al., 1999, 2001; Mette et al., 2000). The slightly larger siRNAs (24–26 nt) associated with this system are generated by DCL-3 which acts in conjunction with AGO4 and RDR2 (Hamilton et al., 2002; Qi et al., 2005; Xie et al., 2004; Zilberman et al., 2003). Suppression of gene activity is usually coincident with methylation of cytosine residues in DNA (RNA-directed DNA methylation; RdDM) and specific post-translational modifications of histone proteins, including methylation of histone H3 at lysine 9 (H3mK9) (Bender, 2004). Multiple links between siRNA and these two epigenetic marks have been established. This pathway is believed to maintain genome integrity by preventing rearrangement in centromeric and telomeric repeats and by suppressing transposons and other invasive DNAs.

Plant virus silencing suppressors

The strongest evidence for the now widely accepted idea that RNA silencing acts as an adaptive defense is the existence of viral suppressors (Ding et al., 2004; Li and Ding, 2001; Roth et al., 2004). Viruses from different families have acquired a variety of unrelated suppressors that affect different, and perhaps multiple, steps in the silencing pathway. To illustrate this point, some examples from RNA viruses are briefly discussed here (Fig. 1). First, HC-Pro encoded by *Tobacco etch virus* and other potyviruses is able to reverse established silencing in plants and block local silencing in transient assays (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Llave et al., 2000). In addition, it interacts with the cellular protein rgsCaM that is itself a silencing suppressor, suggesting that HC-Pro stimulates an

endogenous mechanism that negatively regulates RNA silencing (Anandalakshmi et al., 2000). HC-Pro also partially inhibits dsRNA processing by Dicer and interferes with the unwinding of duplex siRNA (and miRNA/miRNA*), thereby preventing the incorporation of targeting information into RISC (Chapman et al., 2004; Dunoyer et al., 2004). In contrast, the p19 protein of *Cymbidium ringspot virus* and other tombusviruses cannot reverse established silencing, although it can suppress local silencing and block production of the systemic silencing signal. The suppression activity of p19 is attributable to its ability to bind and sequester siRNAs (and miRNA/miRNA*), preventing their incorporation into RISC (Lakatos et al., 2004; Silhavy et al., 2002; Vargason et al., 2003). That p19 and HC-Pro impact both siRNA and miRNA metabolism underscores the similar and overlapping nature of these pathways (Chapman et al., 2004; Dunoyer et al., 2004). In contrast, *Turnip crinkle virus* coat protein (TCV-CP) does not significantly affect the miRNA pathway. This protein blocks local RNA silencing and prevents systemic spread by interfering with the activity of DCL-2, which does not play a major role in processing miRNA precursors (Xie et al., 2004). The 2b protein of *Cucumber mosaic virus* (CMV), on the other hand, cannot inhibit the initiation of silencing but effectively prevents its systemic spread to naïve tissues (Brigneti et al., 1998; Guo and Ding, 2002). Thus, RNA viruses have adopted many different counter-defense strategies aimed at different aspects of RNA silencing. From this perspective, the molecular basis for synergistic diseases that can result from mixed infections with viruses carrying unrelated suppressors becomes clear (Pruss et al., 1997). It is also clear that viral suppressors can be powerful tools for the analysis of RNA silencing mechanisms and the relationships between different silencing pathways.

Geminiviruses are inducers and targets of RNA silencing

Because geminiviruses have DNA genomes that replicate in the nucleus, they lack a particular vulnerability of RNA viruses, whose RNA genomes can be degraded by cytoplasmic RNA silencing (PTGS). Geminivirus transcripts, however, should be exposed to this pathway. The first evidence that virus-specific siRNAs are induced by natural geminivirus infection came from an analysis of RNA extracts from TYLCV-infected tomato plants (Lucioli et al., 2003). In this study, hallmark siRNAs of both sense and antisense polarity were detected using a probe corresponding to the viral *Rep* gene (replication initiator protein). The susceptibility of geminiviruses to suppression by cytoplasmic RNA silencing was subsequently confirmed by the demonstration that synthetic siRNA designed to target the coding region of ACMV *Rep*, the only viral gene essential for replication, significantly interfered with *Rep* mRNA accumulation and to a lesser extent reduced viral DNA replication in cultured cells (Vanitharani et al., 2003).

Transgenic plants expressing various *Rep* sequences have been constructed for the purpose of producing virus-resistant plants. Most attempts have met with at least moderate success, and some resistance has been achieved to different geminiviruses in several species (e.g. Asad et al., 2003; Chellappan et al., 2004a). This illustrates the potential power of RNA silencing to control virus infection: cells primed for an RNA silencing response to a specific virus are able to at least partially resist infection by that virus, and sometimes also others that are very closely related with respect to the target sequence (Lindbo et al., 1993). However, there are cases where *Rep* transgene silencing has been overcome, presumably by the action of viral suppressors (Lucioli et al., 2003; Noris et al., 2004).

A further demonstration of the exposure of geminivirus transcripts to cytoplasmic RNA silencing has come from studies of DNA-VIGS (virus-induced gene silencing; Ruiz et al., 1998). Using the A components of bipartite viruses as episomal replicons, TGMV and CaLCuV vectors carrying sequences corresponding to transgenes or endogenous genes were shown to efficiently induce silencing of the corresponding genes in *Nicotiana benthamiana* and *Arabidopsis*, even in tissues such as the meristem where virus is normally excluded (Kjemtrup et al., 1998; Muangsan et al., 2004; Peele et al., 2001). Vectors based on other geminiviruses and even a TYLCV DNAβ satellite have also been used to successfully suppress the expression of target genes (e.g. Atkinson et al., 1998; Tao and Zhou, 2004). An analysis of the genetic requirements for DNA-VIGS in *Arabidopsis* has been particularly informative (Muangsan et al., 2004). Of the genes known to be necessary for PTGS of sense transgenes, this study showed that effective target gene suppression requires *SGS2/SDE1* (*suppressor of gene silencing 2/silencing defective 1*, or *RDR6*) and *SGS3*, but not *SGS1* or *AGO1*. The requirements also differ from RNA-VIGS and inverted repeat-induced silencing, which do not need RDR6 to generate an initial dsRNA trigger (Beclin et al., 2002; Dalmay et al., 2000). Genes known to be involved in TGS maintenance, including *DDM1* (*defective DNA methylation 1*), *MOM1* (*maintenance of methylation 1*), and *MET1* (*methyltransferase 1*) were not required. These results indicate that silencing directed against sequences carried in a geminivirus vector, and hence against geminivirus transcripts themselves, can be mediated by cytoplasmic RNA silencing. However, DNA-VIGS appears to use a somewhat different branch of the pathway. Interestingly, *sgs2/sde1* and *sgs3* mutants are only slightly more susceptible to geminivirus infection than wild-type plants (Muangsan et al., 2004), suggesting either that silencing is not a major factor in defense against geminiviruses, or more likely that cytoplasmic RNA silencing (PTGS) is only one component of the silencing response to geminivirus infection.

Unlike their transcripts, geminivirus genomes are not sensitive to cytoplasmic RNA silencing, although they are potential targets of siRNA-directed epigenetic modification, a complication not faced by RNA viruses. This could have the effect of reducing virus transcription. Evidence that geminiviruses are susceptible to this nuclear pathway is so far indirect but nonetheless provocative. First, it has been demonstrated that transgenes driven by geminivirus promoters can be transcriptionally silenced following infection of transgenic plants with the homologous virus. Silencing is associated with

hypermethylation of promoter sequences and does not occur with heterologous geminivirus infection (Seemanpillai et al., 2003). These findings suggest that signals capable of directing TGS are produced during infection and can negatively regulate homologous promoter sequences in chromatin (in this case in a host chromosome). The second piece of evidence is complementary to the first. It has been reported that geminivirus-infected plants can recover from infection following the later introduction, by particle bombardment, of a construct designed to express dsRNA specific for the IR, which contains the origin of replication and divergent promoters (Pooggin and Hohn, 2003). This suggests that TGS signals generated by the dsRNA construct can negatively regulate the homologous promoter sequences of replicating viral genomes. However, a direct effect on replication is also possible.

Since geminiviruses lack a dsRNA phase, the question arises as to how the initial inducing dsRNA is produced during natural infection. The possibilities include Dicer-catalyzed processing of structured regions in viral mRNA, the action of host RDR on aberrant or over-expressed viral transcripts, overlapping read-through transcription from the divergent promoters in the IR, or some combination of these mechanisms. A study of siRNA accumulation following infection of *N. benthamiana* and cassava with distinct bipartite geminiviruses inducing symptoms of varying severity has been revealing (Chellappan et al., 2004b). Not surprisingly, an inverse correlation was found between the severity of disease and the amount of virus-specific siRNA accumulated in infected plants, and recovery from infection (characterized by significant reductions in disease symptoms and virus in newly emerging tissues) was associated with the highest levels of siRNA accumulation. While evidence for overlapping, complementary transcription was also found, the labeled siRNA generated during infection hybridized to DNA probes corresponding to all regions of the genome, including the IR. Transcript overlap regions were at best weak siRNA hotspots. Thus, while transcript overlap might contribute to the production of initiating dsRNA, the role of host RDR activity, possibly acting on over-expressed viral mRNAs, is likely to be highly significant. This is consistent with the requirement of RDR6 for DNA-VIGS (Muangsan et al., 2004). However, siRNA was preferentially directed against different genome components. In the case of a mild recovery-type virus, more siRNA was directed against DNA A (which provides genes required for replication) than DNA B (which encodes genes required for virus spread), while the reverse was true for a more severe, non-recovery type virus. The generality of this interesting observation needs to be determined. In addition, hotspots corresponding to the *Rep* gene (*AC1*) and the *BC1* movement gene were apparent in the more highly targeted A and B components, respectively (Chellappan et al., 2004b). Why these particular regions might be preferred for siRNA production is not clear, although transcript secondary structure might play a role. In any event, it can be said that virus–host interactions involved in RNA silencing are complex and the outcome of infection likely depends on a balance between host recognition of features specific to a particular virus (or possibly even a specific viral gene) and the efficiency of viral silencing suppressors in a particular host.

Transcription-dependent and -independent silencing suppression by AL2/AC2 and L2 proteins

The 15 kDa AL2 protein found in all begomoviruses is also known in the literature as AC2, C2, or TrAP (transcriptional activator protein). AL2 is more commonly used with New World viruses such as TGMV, while AC2 and C2 (collectively AC2 in this review) usually refer to Old World bipartite and monopartite virus homologues, respectively. The AL2/AC2 proteins from New World and Old World viruses share extensive homology although they can be distinguished in sequence alignments, primarily by differences in the C-terminal activation domain. In contrast, the related L2 protein (also known as C2) from the curtovirus BCTV shows little direct sequence homology with its begomovirus counterparts, except for a central zinc finger-like region.

AL2/AC2 is a transcription factor that was initially found to be required for the expression of late viral genes (Sunter and Bisaro, 1992, 1997, 2003). This function is not virus-specific among the begomoviruses, and the proteins from several other New and Old World begomoviruses have been shown to complement the transcriptional activation defect of a TGMV *al2* mutant (Sunter et al., 1994). BCTV L2 does not complement this same mutant. AL2/AC2 has a C-terminal, acidic-type activation domain that is functional in plant, yeast, and mammalian cells (Hartitz et al., 1999). However, it binds ssDNA and weakly binds dsDNA in a sequence non-specific manner, suggesting that it is directed to responsive promoters primarily through interactions with cellular proteins rather than direct recognition of specific promoter sequences (Hartitz et al., 1999; Noris et al., 1996). DNA binding is promoted by zinc binding, which occurs through the conserved cysteine and histidine residues that comprise the zinc finger (Hartitz et al., 1999; van Wezel et al., 2003). Consistent with its ability to activate transcription, studies with GFP (green fluorescent protein) fusion proteins have shown that AL2/AC2 localizes to the nucleus (van Wezel et al., 2001). Four consecutive arginine residues located in the N-terminus comprise part of the nuclear localization signal (NLS), which appears to be bipartite (Dong et al., 2003; Trinks et al., 2005). Interestingly, however, fluorescence microscopy indicates that AL2 is located in both the nucleus and the cytoplasm of TGMV-infected *N. benthamiana* cells (Wang et al., 2003). Phosphorylation appears to influence its subcellular localization. Following expression in insect cells, non-phosphorylated AL2 is present in both the nucleus and the cytoplasm whereas the phosphorylated form preferentially accumulates in the nucleus (Wang et al., 2003). Thus, cellular kinases may in part control its distribution and functions. The less-studied BCTV L2 protein does not appear to be a transcription factor. As noted above, it cannot complement a begomovirus *al2* mutant in this regard, and unlike AL2, it is not required for the expression of late viral genes (Hormuzdi and Bisaro, 1995; Stanley et al., 1992; Sunter et al., 1994). In addition, it lacks a recognizable activation

domain and is at best a weak and inconsistent self-activator in the yeast two-hybrid system. However, both TGMV AL2 and BCTV L2 condition an enhanced susceptibility phenotype when expressed in transgenic *N. benthamiana* or tobacco, indicating that they share functions in viral pathogenesis (Sunter et al., 2001).

Using a *Potato virus* X (PVX) vector to infect *N. benthamiana* plants carrying a GFP transgene (line 16c), the Baulcombe lab first showed that ACMV AC2 expressed from the vector was capable of reversing established silencing (Voinnet et al., 1999). This was followed by similar studies with wild-type and mutant TYLCV protein, which confirmed suppressor activity and suggested that it depended on an intact NLS as well as cysteine and histidine residues in the zinc finger, and thus on the zinc and non-specific DNA binding activities (Dong et al., 2003; van Wezel et al., 2002). However, neither the TGMV nor the ACMV protein binds siRNA or miRNA, ruling out the possibility that they might act by a mechanism similar to p19 (Chellappan et al., 2005; Wang et al., 2005). Studies with AC2 from *Mung bean yellow mosaic virus* (MYMV) have confirmed the requirement for an intact zinc finger and NLS, and further demonstrated a requirement for the activation domain (Trinks et al., 2005). Taken together, these observations suggest that AC2 from Old World viruses (ACMV, MYMV, and TYLCV) acts in the nucleus by a mechanism that depends on interaction with DNA and transcriptional activation activity. In fact, evidence for AC2-mediated modification of the host transcriptome has been obtained. Transcriptional profiling in *Arabidopsis* protoplasts following transient expression of ACMV and MYMV AC2 showed that these proteins induced the expression of about 30 genes, including *WEL1* (*Werner exonuclease-like 1*). Subsequent analysis of WEL1 indicated that it is capable of suppressing RNA silencing in *N. benthamiana* line 16c (Trinks et al., 2005). These remarkable findings suggest that AC2 suppresses silencing indirectly by activating the expression of a cellular protein that may function as an endogenous negative regulator of the system. The mechanism by which suppression occurs is not yet clear. Since genes encoding other Werner-like exonucleases have been implicated as positive regulators of silencing, it is possible that WEL1 might exert a dominant-negative effect (Trinks et al., 2005). It should be pointed out, however, that the relevance of *WEL1* activation to virus infection needs to be confirmed. TGMV AL2, for example, cannot suppress silencing in *N. benthamiana* protoplasts (Qi et al., 2004), and the ability of the ACMV and MYMV proteins to suppress silencing in protoplasts has not been tested. Further, increased expression of *WEL1* has yet to be demonstrated in virus-infected plants. It will also be interesting to see if TGMV AL2 and other New World virus homologues can activate *WEL1* in their hosts.

Evidence for transcription-independent silencing suppression has also been obtained. In a yeast two-hybrid screen, TGMV AL2 (lacking the activation domain) and BCTV L2 were found to specifically interact with adenosine kinase (ADK), a nucleoside kinase that catalyzes the synthesis of 5′-AMP from adenosine and ATP (Wang et al., 2003). Further, AL2 and L2 inactivate ADK in vitro and following co-expression in *E. coli* and yeast. ADK activity is reduced in transgenic plants expressing AL2 and L2, and is also significantly reduced in virus-infected tissue in an L2-dependent manner. Interestingly, plants infected with BCTV *l2* mutants and unrelated RNA viruses actually show enhanced ADK activity, suggesting that increased activity of this enzyme is part of the response to virus infection (Wang et al., 2003). A link between ADK, silencing, and viral pathogenesis comes from the observation that ADK plays a key role in sustaining the methyl cycle and *S*-adenosyl-methionine (SAM)-dependent methyltransferase activity (Lecoq et al., 2001; Moffatt et al., 2002; Weretilnyk et al., 2001). Methyl group transfer from SAM to a methyl acceptor produces *S*-adenosyl-homocysteine (SAH), which is hydrolyzed to homocysteine (Hcy) and adenosine by *S*-adenosyl-homocysteine hydrolase (SAHH) (Fig. 1). However, the equilibrium lies strongly toward synthesis and the reaction is driven in the direction of hydrolysis only by the metabolism of both the products (Hcy and adenosine). Thus, adenosine phosphorylation by ADK is important for the removal of SAH because it promotes flux through the methyl cycle which regenerates SAM. In addition, SAHH can act as a competitive inhibitor of SAM due to its greater affinity for methyltransferases. That ADK-deficient plants display silencing defects implies an indirect role for the methyl cycle in silencing (Moffatt et al., 2002; Wang et al., 2003).

To test the ability of TGMV AL2 and BCTV L2 proteins to suppress silencing, and to determine if ADK is involved in supporting silencing pathways, a three-component transient *Agrobacterium*-based system was used in conjunction with wild-type or line 16c *N. benthamiana* plants. In this system, mixed *Agrobacterium* cultures are used to simultaneously deliver constructs expressing GFP, inverted repeat GFP RNA (dsGFP, a strong silencing inducer), and a test construct (Johansen and Carrington, 2001). This study demonstrated that TGMV AL2, TGMV $AL2_{1-100}$ (lacking the activation domain), and BCTV L2 were able to suppress silencing directed against GFP, with increased GFP mRNA accumulation and reduced accumulation of GFP-specific siRNAs of both the small and large size classes (Wang et al., 2005). Silencing was also suppressed by an ADK inverted repeat construct (dsADK) and A-134974, an adenosine analogue that inhibits ADK. ADK activity was shown to be reduced in tissues showing silencing suppression and infiltrated with GFP-dsGFP and AL2, L2, dsADK, or A-134974, but not control constructs. These findings indicate that AL2 and L2 can suppress silencing in a transcription-independent manner, and that ADK activity is needed for silencing. AL2 and L2 thus suppress silencing indirectly by inhibiting ADK, which is needed to sustain the methyl cycle (Wang et al., 2005). In support of this conclusion, transgenic tobacco lines expressing antisense RNA to SAHH, a dedicated methyl cycle enzyme, show DNA hypomethylation of HRS60 repeats (Tanaka and Masuta, 1997). In addition, the *HOG1* locus (*homology-dependent gene silencing 1*) required for TGS and DNA methylation-dependent silencing was recently demonstrated to

encode this same enzyme (Rocha et al., 2005). Preliminary studies indicate that, like dsADK, an inverted repeat dsSAHH construct can also suppress silencing in the *Agrobacterium*-based transient system (R.C. Buchmann and D.M. Bisaro, unpublished). Thus, the evidence suggests that AL2 and L2 participate in an indirect suppression mechanism involving metabolic inhibition of siRNA-directed transmethylation, which could interfere with epigenetic modification of the viral genome. Methylation would be important in the transient system if T-DNA templates were subject to epigenetic modification. Although the structure of transforming DNA is not known at this time, a role for histone proteins in transformation has been established (Gelvin, 2003; HoChul et al., 2002). The inability of TGMV AL2 to function as a silencing suppressor in protoplasts might then be explained by the "naked" nature of transfected plasmid templates used to express the GFP reporter in these experiments (Qi et al., 2004).

That plants might use methylation as a defense against geminiviruses implies that the viral genome is a target for DNA and/or histone methyltransferases. Support for this comes from experiments which showed that in vitro methylation of TGMV impairs its ability to replicate in tobacco protoplasts (Brough et al., 1992). While this early study found no evidence for in vivo methylation of wild-type viral DNA in the few sites examined, a reevaluation suggests that at least a portion of TGMV RF molecules are methylated in infected plants (P. Raja and D.M. Bisaro, unpublished). The relevance of the siRNA-directed methylation pathway to defense against geminiviruses must also be established. To this end, an analysis of the susceptibility of selected *Arabidopsis* mutants to geminivirus infection is clearly warranted. Of particular interest are mutants defective in upstream pathway components (e.g. *dcl-3*, *ago4*, and *rdr2*) and downstream effectors such as de novo DNA and histone methyltransferases. In addition, HEN1, which is involved in PTGS and required for miRNA accumulation, is a SAM-dependent methyltransferase that can methylate the 3′-ends of both miRNAs and siRNAs (Boutet et al., 2003; Li et al., 2005; Yu et al., 2005). Thus, the susceptibility of *hen1* mutants relative to wild-type plants is also of interest.

At this time, it is not clear whether the very similar Old World virus AC2 proteins and the New World virus proteins typified by TGMV AL2 exclusively possess either transcription-dependent or transcription-independent suppression activity, or if this apparent difference is due to the different assays used to examine them. The BCTV L2 protein is unlikely to have the former activity, but it would certainly be exciting to discover that AL2/AC2 proteins could suppress silencing by two independent mechanisms, one of which targets cytoplasmic RNA silencing and the other siRNA-directed methylation. In this regard, it is interesting to note that ACMV AC2 also interacts with ADK, although its ability to inhibit the enzyme has not yet been examined (Wang et al., 2003).

In addition to ADK, AL2 and L2 proteins also interact with and inhibit SNF1-related kinase in vitro and in vivo. Inhibition of SNF1 activity in transgenic *N. benthamiana* and tobacco plants results in a novel enhanced susceptibility phenotype, characterized by a reduction in the concentration of virus required to elicit infection (Hao et al., 2003). This suggests that SNF1-mediated responses constitute a novel defense pathway in plants, which is inhibited by AL2 and L2. AL2/L2 suppression of SNF1 does not appear to be involved in silencing suppression (Wang et al., 2005). However, that SNF1 can be activated by AMP suggests a second function for AL2/L2 inactivation of ADK. It is possible that AL2/L2 interaction with this nucleoside kinase serves the dual purpose of attenuating SNF1-mediated responses (by limiting cellular AMP levels) and inhibiting the methyl cycle, which is required for RNA silencing. Further work is required to unravel the link between these quite different defense pathways.

Silencing suppression by AC4 proteins and AC4–AC2 synergy in virus disease

The *AC4* gene (known as *C4* in monopartite begomoviruses and curtoviruses; here collectively referred to as *AC4*) lies entirely within the *Rep* coding region, but in a different reading frame. Yet, despite the conservation of *Rep*, *AC4* is the one of the least conserved of all geminivirus genes. Functional analysis has proved enigmatic. Mutagenesis and/or transgenic expression of some *AC4* genes results in no phenotype, while others produce phenotypes consistent with a movement protein or a symptom determinant (Jupin et al., 1994; Krake et al., 1998; Latham et al., 1997). A measure of clarification has been achieved by the discovery that *AC4* can suppress RNA silencing, allowing it to enhance disease and promote viral invasiveness.

The suppression activity of AC4 from four different cassava-infecting geminiviruses was tested in the *Agrobacterium*-based transient assay in *N. benthamiana* 16c plants (Vanitharani et al., 2004). Two of the AC4 proteins, from viruses associated with recovery-type symptoms in cassava, showed suppressor activity with increased accumulation of GFP mRNA and inhibition of GFP-specific siRNAs. Two other AC4 proteins from non-recovery-type viruses showed little or no activity in this assay. Conversely, the AC2 proteins of the non-recovery viruses were effective silencing suppressors, while those from recovery-type viruses were less effective. Besides revealing a new function for AC4, these experiments provide some insight into the molecular basis for synergistic disease that can result from mixed infection. Specifically, mixed infection of cassava by ACMV (recovery-type, with a relatively strong AC4 suppressor) and *East African cassava mosaic virus* (EACMV; non-recovery-type, with a relatively strong AC2 suppressor) causes an unusually severe disease in the field (Vanitharani et al., 2005). It is important to note that synergy is made possible by the fact that not all AC2 and AC4 proteins are alike with respect to their ability to suppress silencing. While the molecular basis for this is not yet clear, variable activities could reflect adaptations to natural host reservoirs or differences in the preferred mode of action of individual suppressors (e.g. transcription-dependent vs. -independent suppression by AL2/AC2). The different phenotypes of these viruses further suggest that AC2 and AC4 act at discrete steps in the silencing pathway and that the effect of AC4 is more transient and can be overcome by some hosts.

Transgenic expression of *AC4*/*C4* leads to severe developmental defects which might be explained by effects on the miRNA pathway (Chellappan et al., 2005; Latham et al., 1997). Developmental effects which resemble virus disease symptoms have been associated with RNA virus suppressors, such as p19 and HC-Pro, that interfere with miRNA metabolism (Dunoyer et al., 2004; Kasschau et al., 2003; Xie et al., 2004). These effects are believed to result from "collateral damage" caused by suppression of overlapping steps in the siRNA and miRNA pathways. Indeed, AC4 from ACMV, but not EACMV, causes developmental defects when expressed as a transgene in *Arabidopsis*. The defects are associated with reduced accumulation of specific miRNAs and a parallel over-accumulation of their target mRNAs. Surprisingly, ACMV AC4, but not EACMV AC4, binds single-stranded miRNA and siRNA in vitro but does not bind the corresponding duplex forms. Further, a single-stranded, complementary miRNA oligonucleotide (miR159*) acted as bait to pull down ACMV AC4 from protoplast extracts, and the cognate miR159 co-purified with AC4 immunoprecipitates (Chellappan et al., 2005). Thus, AC4 appears to block cytoplasmic RNA silencing, and coincidentally the miRNA pathway, by a novel mechanism that involves binding single-stranded siRNA and miRNA. This suggests that silencing-active AC4 proteins interfere with RISC loading by acting downstream of small RNA biogenesis and duplex unwinding, possibly by facilitating the degradation of single-stranded miRNAs and siRNAs. This in turn implies that the single-stranded forms are accessible at some point between the unwinding and RISC loading steps. The function, if any, of silencing-inactive AC4 remains to be determined.

Silencing suppression by βC1 protein

The cloned DNA genomes of most monopartite begomoviruses are sufficient to produce symptomatic infections in their hosts. However, the genomes of some others are not. In these cases, elegant studies have recently demonstrated the existence of disease complexes consisting of the geminivirus and a satellite known as DNAβ (Briddon et al., 2001; Mansoor et al., 2003; Saunders et al., 2000). DNAβ is about half the size (1.3 to 1.4 kb) of the helper virus on which it depends for replication, encapsidation, and systemic spread. Mutagenesis has shown that its single open reading frame encodes the essential pathogenicity determinant βC1, and transgenic expression of the ~14 kDa βC1, or expression from a PVX vector, results in severe developmental abnormalities (Cui et al., 2004; Saeed et al., 2005; Saunders et al., 2004; Zhou et al., 2003). The molecular basis of βC1 pathogenicity can be explained by silencing suppression activity.

The βC1 protein of *Tomato yellow leaf curl China virus*-Y10 (TYLCCV) has been shown to behave as a silencing suppressor in *N. benthamiana* 16c plants (Cui et al., 2005). Infection of plants silenced for GFP expression showed that TYLCCV plus DNAβ, but not TYLCCV alone, could prevent silencing in newly emerging leaves of infected plants. Expression of βC1 also interfered with local silencing in transient *Agrobacterium*-based assays. The recombinant protein binds ssDNA and dsDNA in vitro in a sequence nonspecific fashion, and βC1 fusion proteins are primarily localized in the nucleus in insect and plant cells. The putative NLS is required for silencing suppression activity (Cui et al., 2005). Although reminiscent of AL2/AC2 with respect to size, DNA binding properties, and nuclear localization, βC1 lacks a zinc finger and shares little or no homology with the begomovirus protein. In addition, AL2/AC2 and BCTV L2 do not generate developmental defects when expressed in transgenic plants (Chellappan et al., 2005; Sunter et al., 2001). Thus, the developmental defects observed with βC1 expression suggest that it targets a different step in the silencing process and most likely one that overlaps the miRNA pathway. However, there is insufficient information at present to allow the separation of AC4 and βC1 activities in this regard. Again, since related monopartite begomoviruses, including TYLCV and even a different strain of TYLCCV (Dong et al., 2003), can cause disease on their own and encode functional silencing suppressors, it is logical to assume that a requirement of βC1 for pathogenicity reflects attenuated function of other suppressors in viruses associated with DNAβ.

The miRNA pathway and antiviral defense

Several recent studies with mammalian viruses suggest roles for the miRNA pathway in regulating viral replication and antiviral defense. For example, an analysis of Epstein–Barr virus (EBV), a member of the Herpesviridae, points to a possible role for virus-encoded miRNA genes in regulating the expression of viral and cellular genes, presumably to the benefit of the virus (Pfeffer et al., 2004). Evidence that a small inverted repeat RNA, similar to a miRNA precursor, encoded in the genome of human immunodeficiency virus (HIV-1) is targeted by silencing is perhaps a more straight forward example of a defense elicited by a specific viral sequence. Silencing is suppressed by the viral Tat protein, which interferes with Dicer activity (Bennasser et al., 2005). Recent work with primate foamy virus (PFV-1, a retrovirus) has also implicated the miRNA pathway in antiviral defense, in this case by fortuitous homology between an endogenous cellular miRNA and the viral genome. PFV-1 encodes a protein that can suppress the miRNA pathway in mammalian cells, and both the miRNA and siRNA pathways in *Arabidopsis*, most likely by affecting a shared step (Lecellier et al., 2005). That such fortuitous targeting might also occur in plants is predicted by homology between *Arabidopsis* small RNAs and several viral genomes, which raises the possibility that it might be a relatively common occurrence (Llave, 2004). If this is so, then one might expect plant virus suppressors that impinge on both the miRNA and siRNA pathways to be common as well. Indeed, two of the three geminiviruses suppressors (AC4 and possibly βC1) and several from RNA viruses affect both pathways (Chapman et al., 2004; Dunoyer et al., 2004). In this context, it is noteworthy that most animal virus proteins that have been implicated as silencing suppressors bind dsRNA or inhibit the single Dicer present in mammalian cells (Bennasser et al., 2005; Li et al., 2004; Lichner et al., 2003). However, in

instances where no fortuitous miRNA homology exists, disruption of the miRNA pathway by plant viral suppressors would appear to be incidental.

Summary

This brief review of geminiviruses and their ability to counter RNA silencing illustrates that they encode or can be associated with as many as three distinct silencing suppressors, underscoring the importance of silencing as a host defense against DNA viruses. However, not all these suppressors are equally functional in different viruses or in different hosts, giving rise to a rich potential for synergism in mixed infections (Vanitharani et al., 2005). In addition, one of the suppressors might operate by alternative mechanisms that target different aspects of the silencing response: AL2/AC2 appears to target both cytoplasmic RNA silencing (PTGS) and siRNA-directed DNA methylation (Fig. 1). The others, AC4 and possibly βC1, suppress cytoplasmic RNA silencing and the miRNA pathway by interfering with a step common to both. Thus, exciting recent developments in our understanding of geminivirus silencing suppressors combined with new insights from animal virus systems demand a reconsideration of the role of RNA silencing pathways in plant defense. Once thought to be the exclusive province of cytoplasmic RNA silencing (PTGS), the miRNA and siRNA-directed TGS pathways also warrant serious consideration as antiviral defense mechanisms.

Acknowledgments

Research in the author's laboratory is supported by grants from the USDA National Research Initiative and by the NIH. The author is grateful to present and past lab members for helpful discussions and to D.S. Parris for critical reading of the manuscript.

References

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Smith, T.H., Vance, V.B., 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. U.S.A. 95, 13079–13084.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., Vance, V.B., 2000. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. Science 290, 142–144.

Asad, S., Haris, W.A., Bashir, A., Zafar, Y., Malik, K.A., Malik, N.N., Lichtenstein, C.P., 2003. Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. Arch. Virol. 148, 2341–2352.

Atkinson, R.G., Bieleski, L.R.F., Gleave, A.P., Janssen, B.-J., Morris, B.A.M., 1998. Post-transcriptional silencing of chalcone synthase in petunia using a geminivirus-based episomal vector. Plant J. 15, 593–604.

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Baulcombe, D.C., 2004. RNA silencing in plants. Nature 431, 356–363.

Beclin, C., Boutet, S., Waterhouse, P., Vaucheret, H., 2002. A branched pathway for transgene-induced RNA silencing in plants. Curr. Biol. 12, 684–688.

Bender, J., 2004. DNA methylation and epigenetics. Annu. Rev. Plant Biol. 55, 41–68.

Bennasser, Y., Le, S.-Y., Benkirane, M., Jeang, K.-T., 2005. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity 22, 607–619.

Boutet, S., Vazquez, F., Liu, J., Beclin, C., Fagard, M., Gratias, A., Morel, J.B., Crete, P., Chen, X., Vaucheret, H., 2003. *Arabidopsis* HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr. Biol. 13, 843–848.

Briddon, R.W., Mansoor, S., Bedford, I.D., Pinner, M.S., Saunders, K., Stanley, J., Zafar, Y., Malik, K.A., Markham, P.G., 2001. Identification of DNA components required for induction of cotton leaf curl disease. Virology 285, 234–243.

Brigneti, G., Voinnet, O., Li, W.-X., Ji, L.-H., Ding, S.-W., Baulcombe, D.C., 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. 17, 6739–6746.

Brough, C.L., Gardiner, W.E., Inamdar, N., Zhang, X.Y., Ehrlich, M., Bisaro, D.M., 1992. DNA methylation inhibits propagation of tomato golden mosaic virus DNA in transfected protoplasts. Plant Mol. Biol. 18, 703–712.

Carrington, J.C., Ambros, V., 2003. Role of miRNAs in plant and animal development. Science 301, 336–338.

Chapman, E.J., Prokhnevsky, A.I., Gopinath, K., Dolja, V.V., Carrington, J.C., 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev. 18, 1179–1186.

Chellappan, P., Masona, M.V., Vanitharani, R., Taylor, N.J., Fauquet, C.M., 2004a. Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. Plant Mol. Biol. 56, 601–611.

Chellappan, P., Vanitharani, R., Fauquet, C.M., 2004b. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. J. Virol. 78, 7465–7477.

Chellappan, P., Vanitharani, R., Fauquet, C.M., 2005. MicroRNA-binding viral protein interferes with *Arabidopsis* development. Proc. Natl. Acad. Sci. U.S.A. 102, 10381–10386.

Cui, X., Tao, X., Yie, Y., Fauquet, C.M., Zhou, X., 2004. A DNAβ associated with *Tomato yellow leaf curl* China virus is required for symptom induction. J. Virol. 78, 13966–13974.

Cui, X., Li, G., Wang, D., Hu, D., Zhou, X., 2005. A begomovirus DNAβ-encoded protein binds DNA, functions as a suppressor of RNA silencing, and targets the cell nucleus. J. Virol. 79, 10764–10775.

Dalmay, T., Hamilton, A.J., Rudd, S., Angell, S., Baulcombe, D.C., 2000. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell 101, 543–553.

Ding, S.-W., Li, H., Lu, R., Li, F., Li, W.-X., 2004. RNA silencing: a conserved antiviral immunity of plants and animals. Virus Res. 102, 109–115.

Dong, X., van Wezel, R., Stanley, J., Hong, Y., 2003. Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. J. Virol. 77, 7026–7033.

Dunoyer, P., Lecellier, C.-H., Parizotto, E.A., Himber, C., Voinnet, O., 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell 16, 1235–1250.

Fauquet, C.M., Bisaro, D.M., Briddon, R.W., Brown, J.K., Harrison, B.D., Rybicki, E.P., Stenger, D.C., Stanley, J., 2003. Revision of taxonomic criteria for species demarcation in the family Geminiviridae, and an updated list of begomovirus species. Arch. Virol. 148, 405–421.

Gasciolli, V., Mallory, A.C., Bartel, D.P., Vaucheret, H., 2005. Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing *trans*-acting siRNAs. Curr. Biol. 15, 1494–1500.

Gazzani, S., Lawerson, T., Woodward, D., Headon, R., Sablowski, R., 2004. A link between mRNA turnover and RNA interference in *Arabisopsis*. Science 306, 1046–1048.

Gelvin, S.B., 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. Rev. 67, 16–37.

Guo, H.S., Ding, S.W., 2002. A viral protein inhibits the long range signaling activity of the gene silencing signal. EMBO J. 21, 398–407.

Gutierrez, C., 1999. Geminivirus DNA replication. Cell. Mol. Life Sci. 56, 313–329.

Hamilton, A., Baulcombe, D.C., 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286, 950–952.

Hamilton, A., Voinnet, O., Chappell, L., Baulcombe, D.C., 2002. Two classes of short interfering RNA in RNA silencing. EMBO J. 21, 4671–4679.

Hammond, S., Bernstein, E., Beach, D., Hannon, G., 2000. An RNA-directed nuclease mediates PTGS in *Drosophila* cells. Nature 404, 293–296.

Hanley-Bowdoin, L., Settlage, S., Orozco, B.M., Nagar, S., Robertson, D., 1999. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit. Rev. Plant Sci. 18, 71–106.

Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.

Hao, L., Wang, H., Sunter, G., Bisaro, D.M., 2003. Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. Plant Cell 15, 1034–1048.

Hartitz, M.D., Sunter, G., Bisaro, D.M., 1999. The geminivirus transactivator (TrAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain. Virology 263, 1–14.

Himber, C., Dunoyer, P., Moissard, G., Ritzenthaler, C., Voinnet, O., 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J. 22, 4523–4533.

HoChul, Y., Mysore, K.S., Gelvin, S.B., 2002. Expression of the *Arabidopsis* histone H2A-1 gene correlates with susceptibility to *Agrobacterium* transformation. Plant J. 32, 285–298.

Hormuzdi, S.G., Bisaro, D.M., 1995. Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis. Virology 206, 1044–1054.

Johansen, L.K., Carrington, J.C., 2001. Silencing on the spot: induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. Plant Physiol. 126, 930–938.

Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J., Baulcombe, D.C., 1999. RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell 11, 2291–2302.

Jones, L., Ratcliff, F., Baulcombe, D.C., 2001. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr. Biol. 11, 747–757.

Jupin, I., de Kouchkovsky, F., Jouanneau, F., Gronenborn, B., 1994. Movement of tomato yellow leaf curl geminivirus (TYLCV): involvement of the protein encoded by ORF C4. Virology 204, 82–90.

Kasschau, K.D., Carrington, J.C., 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95, 461–470.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A., Carrington, J.C., 2003. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Dev. Cell 4, 205–217.

Kjemtrup, S., Sampson, K.S., Peele, C.G., Nguyen, L.V., Conkling, M., Thompson, W.F., Robertson, D., 1998. Gene silencing from plant DNA carried by a geminivirus. Plant J. 14, 91–100.

Krake, L.R., Rezaian, A.M., Dry, I.B., 1998. Expression of tomato leaf curl C4 gene produces virus-like symptoms in transgenic plants. Mol. Plant-Microb. Interact. 11, 413–417.

Lakatos, L., Szittya, G., Silhavy, D., Burgyan, J., 2004. Molecular mechanism of RNA silencing suppression mediated by the p19 protein of tombusviruses. EMBO J. 23, 876–884.

Latham, J.R., Saunders, K., Pinner, M.S., Stanley, J., 1997. Induction of plant cell division by beet curly top virus gene C4. Plant J. 11, 1273–1283.

Lecellier, C.-H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saib, A., Voinnet, O., 2005. A cellular microRNA mediates antiviral defense in human cells. Science 308, 557–560.

Lecoq, K., Belloc, I., Desgranges, C., Daignan-Fornier, B., 2001. Role of adenosine kinase in *Saccharomyces cerevisiae*: identification of the ADO1 gene and study of the mutant phenotypes. Yeast 18, 335–342.

Li, W.X., Ding, S.W., 2001. Viral suppressors of RNA silencing. Curr. Opin. Biotechnol. 12, 150–154.

Li, W.-X., Li, H., Lu, R., Li, F., Dus, M., Atkinson, P., Brydon, E.W.A., Johnson, K.L., Garcia-Sastre, A., Ball, L.A., Palese, P., Ding, S.-W., 2004. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc. Natl. Acad. Sci. U.S.A. 101, 1350–1355.

Li, J., Yang, Z., Yu, B., Liu, J., Chen, X., 2005. Methylation protects miRNAs and siRNAs from a 3′-enf uridylation activity in *Arabidopsis*. Curr. Biol. 15, 1501–1507.

Lichner, Z., Silhavy, D., Burgyan, J., 2003. Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defenses. J. Gen. Virol. 84, 975–980.

Lindbo, J.A., Silva-Rosales, L., Proebsting, W.M., Dougherty, W.G., 1993. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell 5, 1749–1759.

Lippman, Z., Martienssen, R., 2004. The role of RNA interference in heterochromatic silencing. Nature 431, 364–370.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.-J., Hammond, S.M., Joshua-Tor, L., Hannon, G.J., 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science 305, 1437–1441.

Llave, C., 2004. MicroRNAs: more than a role in plant development? Mol. Plant Pathol. 5, 361–366.

Llave, C., Kasschau, K.D., Carrington, J.C., 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proc. Natl. Acad. Sci. U.S.A. 97, 13401–13406.

Llave, C., Xie, Z., Kasschau, K.D., Carrington, J.C., 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. Science 297, 2053–2056.

Lucioli, A., Noris, E., Brunetti, A., Tavazza, R., Ruzza, V., Castillo, A.G., Bejarano, E.R., Accotto, G.P., Tavazza, M., 2003. Tomato *Yellow Leaf Curl Sardinia Virus* Rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. J. Virol. 77, 6785–6798.

Mansoor, S., Briddon, R.W., Zafar, Y., Stanley, J., 2003. Geminivirus disease complexes: an emerging threat. Trends Plant Sci. 8, 128–134.

Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A., Matzke, A.J.M., 2000. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. 19, 5194–5201.

Moffatt, B.A., Stevens, Y.Y., Allen, M.S., Snider, J.D., Periera, L.A., Todorova, M.I., Summers, P.S., Weretilnyk, E.A., Martin-Caffrey, L., Wagner, C., 2002. Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation. Plant Physiol. 128, 812–821.

Molnar, A., Csorba, T., Lakatos, L., Varallyay, E., Lacomme, C., Burgyan, J., 2005. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J. Virol. 79, 7812–7818.

Muangsan, N., Beclin, C., Vaucheret, H., Robertson, D., 2004. Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. Plant J. 38, 1004–1014.

Noris, E., Jupin, I., Accotto, G.P., Gronenborn, B., 1996. DNA-binding activity of the C2 protein of tomato yellow leaf curl virus. Virology 217, 607–612.

Noris, E., Lucioli, A., Tavazza, R., Caciagli, P., Accotto, G.P., Tavazza, M., 2004. *Tomato yellow leaf curl Sardinia virus* can overcome transgene-mediated RNA silencing of two essential genes. J. Gen. Virol. 85, 1745–1749.

Palaqui, J.-C., Elmayan, T., Pollien, J.-M., Vaucheret, H., 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. 16, 4738–4745.

Peele, C., Jordan, C.V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., Robertson, D., 2001. Silencing of a meristematic gene using geminivirus-derived vectors. Plant J. 27, 357–366.

Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C., Tuschl, T., 2004. Identification of virus-encoded microRNAs. Science 304, 734–736.

Pilartz, M., Jeske, H., 2003. Mapping of Abutilon mosaic geminivirus minichromosomes. J. Virol. 77, 10808–10818.

Pooggin, M., Hohn, T., 2003. RNAi targeting of DNA virus in plants. Nat. Biotechnol. 21, 131–132.

Pruss, G., Shi, X.M., Carrington, J.C., Vance, V.B., 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell 9, 859–868.

Qi, Y., Zhong, X., Itaya, A., Ding, B., 2004. Dissecting RNA silencing in protoplasts uncovers novel effects of viral suppressors on the silencing pathway at the cellular level. Nucleic Acids Res. 32, e179.

Qi, Y., Denli, A.M., Hannon, G.J., 2005. Biochemical specialization within *Arabidopsis* RNA silencing pathways. Mol. Cell 19, 421–428.

Rocha, P.S.C.F., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., Moffatt, B.A., Wagner, C., Vaucheret, H., Furner, I., 2005. An *Arabidopsis* homology-dependent gene silencing1 gene codes for an *S*-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. Plant Cell 17, 404–417.

Roth, B.M., Pruss, G.J., Vance, V.B., 2004. Plant viral suppressors of RNA silencing. Virus Res. 102, 97–108.

Ruiz, M.T., Voinnet, O., Baulcombe, D.C., 1998. Initiation and maintenance of virus-induced gene silencing. Plant Cell 10, 937–946.

Saeed, M., Behjatnia, S.A.A., Mansoor, S., Zafar, Y., Hasnain, S., Rezaian, A.M., 2005. A single complementary-sense transcript of a geminivirus DNA β satellite is a determinant of pathogenicity. Mol. Plant-Microb. Interact. 18, 7–14.

Saunders, K., Bedford, I.D., Briddon, R.W., Markham, P.G., Wong, S.M., Stanley, J., 2000. A unique virus complex causes Ageratum yellow vein disease. Proc. Natl. Acad. Sci. U.S.A. 97, 6890–6895.

Saunders, K., Norman, A., Gucciardo, S., Stanley, J., 2004. The DNA β satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity determinant (βC1). Virology 324, 37–47.

Seemanpillai, M., Dry, I., Randles, J., Rezaian, A., 2003. Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. Mol. Plant-Microb. Interact. 16, 429–438.

Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M., Burgyan, J., 2002. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. 21, 3070–3080.

Stanley, J., Latham, J., Pinner, M.S., Bedford, I., Markham, P.G., 1992. Mutational analysis of the monopartite geminivirus beet curly top virus. Virology 191, 396–405.

Sunter, G., Bisaro, D.M., 1992. Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. Plant Cell 4, 1321–1331.

Sunter, G., Bisaro, D.M., 1997. Regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): evidence for activation and derepression mechanisms. Virology 232, 269–280.

Sunter, G., Bisaro, D.M., 2003. Identification of a minimal sequence required for activation of the tomato golden mosaic virus coat protein promoter in protoplasts. Virology 305, 452–462.

Sunter, G., Stenger, D.C., Bisaro, D.M., 1994. Heterologous complementation by geminivirus AL2 and AL3 genes. Virology 203, 203–210.

Sunter, G., Sunter, J., Bisaro, D.M., 2001. Plants expressing tomato golden mosaic virus AL2 or beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. Virology 285, 59–70.

Szittya, G., Molnar, A., Silhavy, D., Hornyik, C., Burgyan, J., 2002. Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. Plant Cell 14, 359–372.

Tanaka, H., Masuta, C., 1997. Morphological changes and hypomethylation of DNA in transgenic tobacco expressing antisense RNA of the *S*-adnosyl-L-homocysteine hydrolase gene. Plant Mol. Biol. 35, 981–986.

Tao, X., Zhou, X., 2004. A modified viral satellite DNA that suppresses gene expression in plants. Plant J. 38, 850–860.

Trinks, D., Rajeswaran, R., Shivaprasad, P.V., Akbergenov, R., Oakeley, E.J., Veluthambi, K., Hohn, T., Pooggin, M., 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. J. Virol. 79, 2517–2527.

Vaistij, F.E., Jones, L., Baulcombe, D.C., 2002. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell 14, 857–867.

Vanitharani, R., Chellappan, P., Fauquet, C., 2003. Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. Proc. Natl. Acad. Sci. U.S.A. 100, 9632–9636.

Vanitharani, R., Chellappan, P., Pita, J.S., Fauquet, C., 2004. Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. J. Virol. 78, 9487–9498.

Vanitharani, R., Chellappan, P., Fauquet, C.M., 2005. Geminiviruses and RNA silencing. Trends Plant Sci. 10, 144–151.

van Wezel, R., Liu, H., Tien, P., Stanley, J., Hong, Y., 2001. Gene C2 of the monopartite geminivirus tomato yellow leaf curl virus-China encodes a pathogenicity determinant that is localized in the nucleus. Mol. Plant-Microb. Interact. 14, 1125–1128.

van Wezel, R., Liu, H., Tien, P., Stanley, J., Hong, Y., 2002. Mutation of three cysteine residues in tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene silencing-suppression. Mol. Plant-Microb. Interact. 15, 203–208.

van Wezel, R., Liu, H., Wu, Z., Stanley, J., Hong, Y., 2003. Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttrancriptional gene silencing. J. Virol. 77, 696–700.

Vargason, J.M., Szittya, G., Burgyan, J., Tanaka Hall, T.M., 2003. Size selective recognition of siRNA by an RNA silencing suppressor. Cell 115, 799–811.

Voinnet, O., 2005. Induction and suppression of RNA silencing: insights from viral infections. Nat. Rev., Genet. 6, 206–221.

Voinnet, O., Baulcombe, D.C., 1997. Systemic signalling in gene silencing. Nature 389, 553.

Voinnet, O., Pinto, Y.M., Baulcombe, D.C., 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. U.S.A. 96, 14147–14152.

Wang, H., Hao, L., Shung, C.-Y., Sunter, G., Bisaro, D.M., 2003. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. Plant Cell 15, 3020–3032.

Wang, H., Buckley, K.J., Yang, X., Buchmann, R.C., Bisaro, D.M., 2005. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. J. Virol. 79, 7410–7418.

Weretilnyk, E.A., Alexander, K.J., Drebenstedt, M., Snider, J.D., Summers, P.S., Moffatt, B.A., 2001. Maintaining methylation activities during salt stress. The involvement of adenosine kinase. Plant Physiol. 125, 856–865.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., Carrington, J.C., 2004. Genetic and functional diversification of small RNA pathways in plants. PLOS Biol. 2, 0642–0652.

Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R.W., Steward, R., Chen, X., 2005. Methylation as a crucial step in plant microRNA biogenesis. Science 307, 932–935.

Zhou, X.P., Xie, Y., Tao, X.R., Zhang, Z.K., Li, Z.H., Fauquet, C.M., 2003. Characterization of DNAβ associated with begomoviruses in China and evidence for co-evolution with their cognate DNA A. J. Gen. Virol. 84, 237–247.

Zilberman, D., Cao, X., Jacobsen, S.E., 2003. *Argonaute4* control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299, 716–771.