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Review

RNA silencing directed against geminiviruses: Post-transcriptional and epigenetic components

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ABSTRACT

It is well-established that plants use cytoplasmic, post-transcriptional gene silencing (PTGS) as a defense against RNA viruses and DNA virus transcripts. More recently, it has become clear that small RNA-directed methylation leading to transcriptional gene silencing (TGS) is also used as a defense against DNA virus chromatin. Here we use the DNA-containing geminiviruses as models to discuss what is currently known about both types of antiviral silencing, and viral suppression of PTGS and TGS as a counterdefense.

1. Introduction

Advances in our understanding of small RNA biology and viral pathogenesis over the past decade have led to the current view of RNA silencing as a ubiquitous defense used by plants against RNA viruses and the transcripts produced by DNA viruses. Progress has been accelerated by a useful synergy: studies of cytoplasmic, post-transcriptional silencing (PTGS), RNA-based plant defenses, and counterdefensive viral silencing suppressor proteins have often converged to yield unique insights into pathway mechanisms. A similar convergence is now possible for nuclear processes. Only a few plant virus families, namely the *Geminiviridae*, the *Nanoviridae*, and the *Caulimoviridae*, have DNA genomes and replicate in the nuclear compartment, and none of these families include viruses that directly replicate double-stranded DNA (dsDNA) progeny genomes from an encapsidated dsDNA genome. Both the geminiviruses and the nanoviruses utilize dsDNA intermediates during rolling circle replication but encapsidate single-stranded DNA (ssDNA), and although caulimoviruses package dsDNA, they are pararetroviruses that replicate through an RNA intermediate and use reverse transcriptase to generate genomic DNA. Why true dsDNA viruses are absent in plants is unclear. However, recent studies have revealed that viral chromatin is subject to repressive methylation, which in plants is directed by small RNAs using components of the RNA silencing machinery. Thus both the post-transcriptional and epigenetic arms of RNA silencing are deployed against DNA viruses. Repressive methylation leads to transcriptional gene silencing (TGS) and, not surprisingly, counterdefense includes suppression of methylation and TGS by DNA virus proteins. This review will describe what is currently known about the roles of PTGS and chromatin methylation leading to TGS in defense against the geminiviruses, easily the largest group of plant DNA viruses. Relevant silencing pathways will be briefly discussed, as will recent information about geminivirus suppressor proteins and what is known of their mechanisms of action. For additional detail the reader is referred to more comprehensive reviews of RNA silencing pathways, antiviral silencing and its suppression by viral proteins, and RNA-directed methylation [1–5].

2. The *Geminiviridae*

The geminiviruses comprise a large family of pathogens that infect a wide range of plant species and cause economically significant losses of food and fiber crops (for reviews see [6–11]). The name derives from the unique, geminate (twinned) icosahedral capsid structure that is characteristic of all family members. The individual icosahedra of each pair are incomplete, and the missing faces are joined so that the interior forms a single container that encapsidates one molecule of circular, ssDNA varying in size from 2.5 to 3.0 kilobases (kb), depending on the virus. Genome replication takes place in the nucleus by a rolling circle mechanism that involves dsDNA replicative form (RF) intermediates, although some recombination-mediated replication also occurs [12–15]. The dsDNA RF molecules are templates for replication and transcription, and become associated with cellular histone proteins to form minichromosomes [16,17]. Because geminiviruses encode neither a DNA or an RNA polymerase, they depend almost entirely on host systems to amplify their genomes

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and express their genes. Thus, they are ideal models for cellular replication and transcription and the epigenetic regulation of these processes.

The hundreds of known geminiviruses are placed into four genera, but representatives from only two, which include some of the best-studied viruses of dicotyledonous plants, are considered here [18]. Members of the genus *Begomovirus* have small (~2.5 kb), circular genomes consisting of either one or two similar-sized components. Thus begomovirus genomes range in size from ~2.5 to 5 kb, and have a coding capacity of six to seven genes. In bipartite viruses both genome components, which are separately encapsidated, are required for infectivity. As a consequence, two different geminate particles must arrive in the same cell to initiate a systemic infection. The A genome component contains information necessary for replication and encapsidation, whereas the B component provides functions required for cell-to-cell movement and systemic spread through the vascular tissue of infected plants. In monopartite begomoviruses functions required for virus spread are encoded by viral sense (rightward) genes. A diagram of dsDNA RF intermediates is shown in Fig. 1. In all geminiviruses transcription occurs bidirectionally from an intergenic region (IR) that also contains the origin of replication. Thus the IR constitutes a small (~250 base pairs) but critical master control region comprised of oppositely oriented promoters flanking a replication origin. In bipartite viruses a portion of the IR, referred to as the common region (CR), is nearly identical in the two genome components.

Viral proteins involved in replication and transcription are multifunctional, and some (but almost certainly not all) of their functions have been identified. The AL1 protein (also known as AC1, C1, or replication initiator protein; Rep) is the only viral protein required for replication [19]. This remarkable protein has properties reminiscent of both SV40 T-antigen and the replication initiator proteins of single-stranded bacteriophages such as ϕX174. Like the phage proteins, AL1 carries out the essential cleavage and ligation events that initiate and terminate rolling circle replication [20]. Additionally, it mediates origin recognition by binding within the origin of replication, where it recruits host replication factors including proliferating cell nuclear antigen (PCNA), replication protein A (RPA), and replication factor C (RFC) [21–24]. It also likely serves as a replicative helicase [25,26]. These diverse activities appear to be regulated, at least in part, by the oligomeric state of the protein. In addition, AL1 provides an environment favorable to DNA synthesis by interacting with the plant retinoblastoma-related protein (pRBR) to antagonize E2F-mediated repression of cellular replication genes such as PCNA [27–30], and further conditions cells through interactions with kinases that modulate metabolism [31,32]. The AL3 protein (also known as AC3, C3, or replication enhancer protein; REn) is needed for optimal viral DNA amplification [33]. How AL3 stimulates replication is not entirely clear, however, it is known to interact with AL1 and may assist in recruiting host factors to the origin, and host cell conditioning, through independent interactions with pRBR, PCNA, and possibly other host factors [23,34,35]. Indeed, the importance of AL1/AL3 cell conditioning was underscored by a global analysis of transcription, which confirmed that geminiviruses impact the pRBR/E2F network and promote endoreduplication in infected cells [36]. The AL2 protein (also known as AC2, C2, or transcriptional activator protein; TrAP) is a transcription factor required for the expression of viral late genes [37,38]. It also acts as a pathogenicity factor by inhibiting the cellular stress response mediated by SNF1-related kinase (SnRK1) [39,40]. AL2 also suppresses RNA silencing by multiple mechanisms that appear to involve the activation of cellular genes and the inhibition of adenosine kinase (ADK) [41]. (The role of AL2 in silencing suppression is discussed in detail in later sections.) Thus, the core functions of viral replication and transcription factors are concerned with redirecting host replication and transcription machineries to viral templates, while secondary functions involve reprogramming host cells to provide an environment favorable to the virus and/or countering host defenses. Secondary functions are usually mechanistically unrelated to core activities, but nevertheless are crucial to viral success. A major challenge in geminivirus biology will be to identify and uncover the molecular basis of secondary functions, and to understand how the activities of these multifunctional proteins are regulated.

Among the best-studied bipartite begomoviruses are *Tomato golden mosaic virus* (TGMV), *African cassava mosaic virus* (ACMV), *Abutilon mosaic virus* (AbMV), *Mung bean yellow mosaic virus* (MYMV), and *Cabbage leaf curl virus* (CaLCuV). Well-known monopartite begomoviruses include *Tomato yellow leaf curl virus* (TYLCV), *Tomato leaf curl virus* (TLCV), and their relatives (Fig. 1). All of these viruses infect the model tobacco plant *Nicotiana benthamiana*, but only CaLCuV also causes symptomatic infections in the important genetic model species *Arabidopsis thaliana*. As the early gene products of begomoviruses are highly conserved, for convenience they will be referred to as AL1, AL2, or AL3.

Another virus of interest is *Beet curly top virus* (BCTV), the type member of the genus *Curtovirus*. Like all curtoviruses, BCTV has a monopartite genome and a broad host range that includes both *N. benthamiana* and *Arabidopsis* (Fig. 1). The sequences and functions of the early gene products involved in replication, L1 and L3, are highly conserved with their begomovirus AL1 and AL3 counterparts [42–45]. However, the AL2 and L2 proteins have little direct sequence homology and share some but not all functions [39,40,44,46]. Like the monopartite begomoviruses, some of the rightward curtovirus gene products serve as movement proteins, although they are structurally and probably functionally distinct. Virus movement proteins and mechanisms are not discussed in this review.

3. Viral transcription and its relationship to viral chromatin

Geminivirus genome organization superficially resembles that of the polyomaviruses, with an intergenic region (IR) containing oppositely oriented promoters separated by the origin of replication. The sequences that comprise these functional elements overlap extensively within this small region (Fig. 1). Transcription programs have been best-studied in the bipartite begomoviruses (for review see [7,10]). Viral mRNAs are polyadenylated and initiate downstream of consensus TATA box and initiator sequences typical of RNA polymerase II promoters. Most are unspliced, although transcription patterns are otherwise complex. Overlapping transcripts and transcripts containing multiple open reading frames (ORFs) are common. For example, in the TGMV A component, there are three leftward (complementary sense), 3′ co-terminal transcripts which overlap the 3′ end of the convergent rightward, viral sense transcription unit that encodes the capsid protein (CP) (Fig. 1). The major leftward promoter in the IR, AL-62 (the number refers to the nucleotide coordinate of the transcription start site), sponsors transcription of an mRNA that spans the *AL1*, *AL2*, and *AL3* genes. This transcript also includes *AL4*, which is embedded within *AL1* but in a different reading frame. (AL4 protein is discussed in a later section.) The AL1 and AL3 proteins, and most likely AL4, appear to be expressed from this transcript [47]. Two downstream transcripts driven by individual promoters, AL-1629 and AL-1935, have also been identified in TGMV infected cells. The shorter AL-1629 supports expression of AL2 protein, whereas AL3 is translated from both AL-1629 and AL-1935 [48]. Thus, within individual mRNAs, mechanisms that allow translation of some downstream genes (e.g., leaky scanning) and inhibit the translation of others (e.g., cryptic ORFs) likely operate [49]. Additional study will be needed before these mechanisms are fully elucidated. However, recent evidence suggests that the activities of the downstream promoters are linked to AL-62 promoter activity. As noted earlier, AL1 acts as an origin recognition protein by binding a sequence that lies within the replication origin and immediately upstream of the AL-62 initiation site. As a result, AL1 is autoregulatory

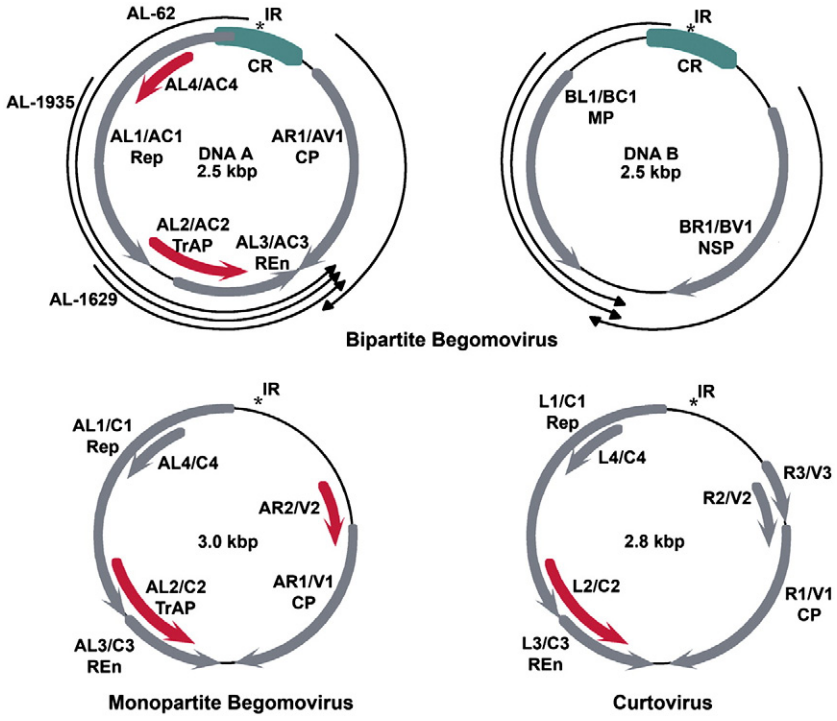

Fig. 1. Geminivirus genome organization. The diagrams depict the dsDNA replicative forms of a typical bipartite begomovirus (e.g., TGMV, CaLCuV), a monopartite begomovirus (e.g., TYLCV), and a curtovirus (e.g., BCTV). Viral genes are indicated by filled arrows. Red arrows indicate that the protein has been directly demonstrated to have silencing suppressor activity in at least one virus. Not all potential suppressors have as yet been tested for activity. There are currently two gene nomenclature systems in use. One refers to genes as leftward (L) or rightward (R) with respect to direction of transcription relative to the conventional map. The other refers to genes as complementary sense (C) or viral sense (V). Some viral proteins have also been named according to core function, including replication initiator protein (Rep), transcriptional activator protein (TrAP), replication enhancer (REn), capsid protein (CP), movement protein (MP), and nuclear shuttle protein (NSP). Major transcripts (indicated by thin arrows) are shown using data from TGMV as a model for bipartite begomoviruses. Note that the bidirectional transcription units overlap at their 3′ ends. In some cases transcript names (AL-61, AL-1935, AL-1629) are provided (see text). The position of a conserved hairpin that marks the site of replication initiation is indicated by an asterisk within the intergenic region (IR). The common region (CR), a sequence of ~230 base pairs that is nearly identical between the A and B genome components of bipartite viruses, is shown as a blue box.

and represses its own transcription [50,51]. Interestingly, activity from the AL-1629 and AL-1935 promoters requires prior AL1-dependent repression of AL-62 [52]. This suggests that bipartite begomovirus transcription occurs in three phases: gene products involved in replication (AL1 and AL3) are expressed early, AL2 expression is delayed-early, and expression of late genes (*CP* and nuclear shuttle protein, *NSP*) from rightward promoters, which require transactivation by AL2, is a late event [52].

The structure of geminivirus chromatin has not been extensively studied, but what is known is consistent with the viral transcription program, and further suggests that viral nucleosome organization is similar to that of host chromatin. A circular DNA molecule 2.6 kb in size could accommodate 10–15 nucleosomes, depending on the length of linker DNA. Assuming an average of 200 bp associated with each, one would expect the viral genome to be complexed with a maximum of 13 nucleosomes. An early electron microscope study showed geminivirus nucleoprotein complexes with a "beads-on-a-string" appearance typical of open chromatin, with an average of 12 beads and a maximum of 13 per ring [53]. Later studies employing nuclease digestion and topoisomer analysis confirmed this general structure [16,17]. In addition, nuclease hypersensitive sites were detected at regions corresponding to the IR and at least one of the downstream leftward promoters, consistent with the notion that these regions can exist in a more open and active conformation. Closer inspection of nuclease digestion patterns also provided evidence for populations of genomes with alternative nucleosome phasing, suggesting that nucleosome positions might not be fixed [17]. The overall picture that emerges from these studies is one of dynamic viral chromosomes, possibly varying between a repressed state (13 nucleosomes) and active states with one or more relatively open sites corresponding to promoter regions (11–12 nucleosomes). It will be interesting to confirm this, and to determine whether and how viral proteins might influence chromatin structure. It also remains to be seen whether histone variants that could stabilize nucleosome arrangements characteristic of active or repressed states are incorporated into viral chromatin. What little is presently known concerning the modification of histone tails is described in a later section.

4. Major RNA silencing pathways in plants

Plants have multiple antiviral defenses, but perhaps the most effective and ubiquitous are those based on RNA silencing. Key components of silencing pathways include Dicer-like (DCL) ribonucleases and associated dsRNA binding (DRB) proteins, RNA-dependent RNA polymerases (RDR), and Argonaute (AGO) proteins. *Arabidopsis* encodes four DCL, five DRB, six RDR, and ten AGO proteins which functionally partner in specific ways to create distinct but partially overlapping pathways commonly triggered by dsRNA. For a comprehensive picture of the silencing pathways outlined here, the reader is referred to several excellent reviews [54–58].

RNA silencing is mediated by 18–25 nucleotide (nt) RNAs of two main types; the small interfering RNAs (siRNA) and the microRNAs (miRNA). Both are processed (diced) from larger dsRNA precursors by DCL proteins with the assistance of DRB proteins. In *Arabidopsis*, DCL1, DCL2, DCL3, and DCL4 generate RNAs that are mostly 21 nt, 22 nt, 24 nt, and 21 nt in length, respectively. The small RNA duplexes they produce have two nucleotide 3′ overhangs, and the terminal nucleotide is 2′-O-methylated by HUA ENHANCER 1 (HEN1). HEN1-mediated methylation of siRNAs and miRNAs stabilizes their ends by blocking oligouridylation and subsequent degradation [59,60]. The guide strand of an siRNA or miRNA is incorporated into an RNA induced silencing complex (RISC) and targets this effector to

complementary RNA. RISC complexes may function in the nucleus or the cytoplasm, and always include an AGO protein. Possible outcomes of small RNA-directed RISC activity include cleavage (slicing) of target RNAs and/or translational repression, depending on the degree of homology between the small RNA and target RNA. Generally perfect complementarity leads to target cleavage whereas imperfect base pairing to mRNA causes translational repression by unknown mechanisms. In addition, specialized nuclear RISC directs methylation of cytosine residues and/or histone methylation, leading to TGS and assembly of heterochromatin.

Most endogenous siRNAs produced in *Arabidopsis* are directed against transposons and inverted repeat sequences, and thus are part of heterochromatic pathways that will be discussed later [61]. However, some loci produce small RNAs that direct PTGS (e.g., trans-acting siRNAs and natural antisense RNAs), and it is pathways similar to these that have been adapted for defense against viral RNA (see following sections). The miRNAs, which play important roles in regulating developmental processes and certain stress responses, comprise another endogenous PTGS pathway that is potentially antiviral or could be manipulated by viruses. These small RNAs arise from non-coding RNA polymerase II transcripts (primary miRNAs) that contain stem–loop structures resulting from imperfect intramolecular base pairing. These are processed into pre-miRNA and finally into mature, 21 nt miRNA by DCL1 in collaboration with HYL1 (DRB1) [57]. One strand of the miRNA programs an AGO1 (or AGO10)-containing RISC. In plants, most miRNAs are perfectly complementary to their target mRNA and cleavage is the usual outcome, although translational repression also occurs [62].

There is growing evidence that the miRNA and related silencing pathways have antiviral roles in mammalian cells, and that mammalian viruses produce proteins that are able to suppress these responses [63–68]. In addition, some viruses (e.g., herpesviruses) encode miRNAs of their own that regulate viral or host gene expression [69–74], and some may alter the expression of cellular miRNAs to aid their replication [75]. It has been suggested that endogenous miRNAs may be antiviral in plants [76], and transgenic expression of artificial miRNAs has been shown to confer virus resistance [77]. To date, however, a natural plant antiviral miRNA has not been reported, and there is no direct evidence that changes in cellular miRNA profiles caused by plant viruses promote virus replication. Although some plant virus silencing suppressors globally interfere with miRNA metabolism, this is generally considered a side-effect caused by the interaction of viral suppressors with components shared by the miRNA and antiviral siRNA pathways [78–81]. Plant virus-encoded miRNAs also have not been reported, although there is evidence that a highly structured region of the *Cauliflower mosaic virus* genome (CaMV, a pararetrovirus) spawns siRNAs that could function like miRNAs and target host transcripts. A direct effect of these small RNA species on virus replication has yet to be demonstrated [82].

5. Silencing pathways that target RNA viruses

RNA silencing was first recognized as an antiviral defense in plants [83–85]. Its importance is supported by the observation that virus infection is invariably accompanied by the appearance of siRNAs derived from the viral genome, and even more so by the fact that all successful plant viruses examined to date encode at least one silencing suppressor. Because the vast majority of plant viruses have genomes composed of RNA, it is logical to begin with a brief discussion of cytoplasmic, antiviral PTGS pathways (for review see [2,5,55,86]). A diagram illustrating cytoplasmic and nuclear antiviral silencing pathways, and sites of action of selected viral suppressor proteins, is presented in Fig. 2.

Analysis of siRNAs in plants infected with RNA viruses has revealed that substrates for DCL activities include inverted repeat structures in viral transcripts as well as double-stranded replicative intermediates [87,88]. Thus the dsRNA that serves as the source of primary viral siRNAs is produced by viral RDRs. A defining feature of PTGS is the appearance of 21–22 nt siRNAs, and although functional redundancy obscured the issue for some time, recent evidence indicates that DCL4, in many cases, is the primary antiviral Dicer [89–93]. The 21 nt DCL4-related siRNAs are typically more abundant than the 22 nt siRNAs generated by DCL2. However, DCL2 redundantly participates in antiviral silencing when DCL4 is inactivated by mutation or by a viral silencing suppressor. Significantly enhanced host susceptibility, with dramatic reductions in virus-derived siRNA, are usually observed only in *dcl2*/*dcl4* double mutants [91–93]. DRB4, which associates with DCL4 [94,95], also contributes to antiviral silencing [93]. DCL1, which generates miRNAs, and DCL3, which produces siRNAs associated with methylation, do not appear to be directly involved in defense against RNA viruses but may have regulatory roles [92,93].

Amplification of viral siRNA reinforces the antiviral silencing response in plants. Source material for secondary virus-specific siRNAs is generated primarily by the action of RDR6, and redundantly by RDR1, which can synthesize dsRNA using primed or unprimed ssRNA templates [92,93,96]. These cellular RDRs are activated by "aberrant" RNAs, including those that are not capped or lack polyadenylation, a characteristic of many viral RNAs. Sliced viral transcripts may also share these features. Synthesis of dsRNA additionally involves multiple host factors, including SUPPRESSOR OF GENE SILENCING 3 (SGS3) [97]. The resulting dsRNA is subsequently processed by DCL4 (and redundantly by DCL2) to generate secondary siRNAs [96,98]. Both primary and secondary siRNAs are ultimately loaded into RISC complexes, and several lines of evidence indicate that AGO1 is the major antiviral slicer [93,99–101]. However, AGO7 can redundantly serve, particularly in the absence of AGO1 function, although AGO1- and AGO7-containing RISC complexes prefer slightly different substrates [93].

A remarkable feature of RNA silencing is the ability of a silencing signal (likely small RNA) to spread cell-to-cell and systemically in the host plant, a process that can immunize tissues in advance of an invading virus [102–104]. Although mechanistic details are still emerging, this non-cell-autonomous pathway is conditioned by DCL4 (and redundantly by DCL2), in concert with other cellular factors. In particular, long-range systemic spread depends on siRNA amplification mediated by RDR6, possibly both at the site where the signal is created and where it is received. Because *rdr6* mutant plants show enhanced susceptibility to multiple RNA viruses and are often unable to exclude viruses from the apical meristem, siRNA amplification and systemic spread are clearly key components of the antiviral response [105–107]. In addition, several viral silencing suppressors, including p19 encoded by *Cymbidium ringspot virus* (CymRSV) and the 2b protein of *Cucumber mosaic virus* (CMV), can prevent the systemic spread of silencing, providing further evidence for its importance in antiviral defense.

6. PTGS pathways that target DNA virus transcripts

Transcripts produced by viruses that package DNA genomes, including the geminiviruses and pararetroviruses such as CaMV, are also targeted by cytoplasmic PTGS (Fig. 2). Transcript secondary structure and convergent transcripts provide dsRNA substrates for DCL activity [82,108,109]. Aberrant transcripts are likely also produced, both as a result of over-expression and by RISC-mediated transcript cleavage. However, unlike RNA viruses, DNA virus transcripts are generated by RNA polymerase II, and so the initial substrates for both primary and secondary siRNA production are produced by a cellular enzyme. Also unlike RNA viruses, all four Dicers appear to work in concert to generate the antiviral response, and 24 nt viral siRNA produced by DCL3 is the most abundant species in infected tissues [82,89]. As DCL3 is a methylation pathway component, this

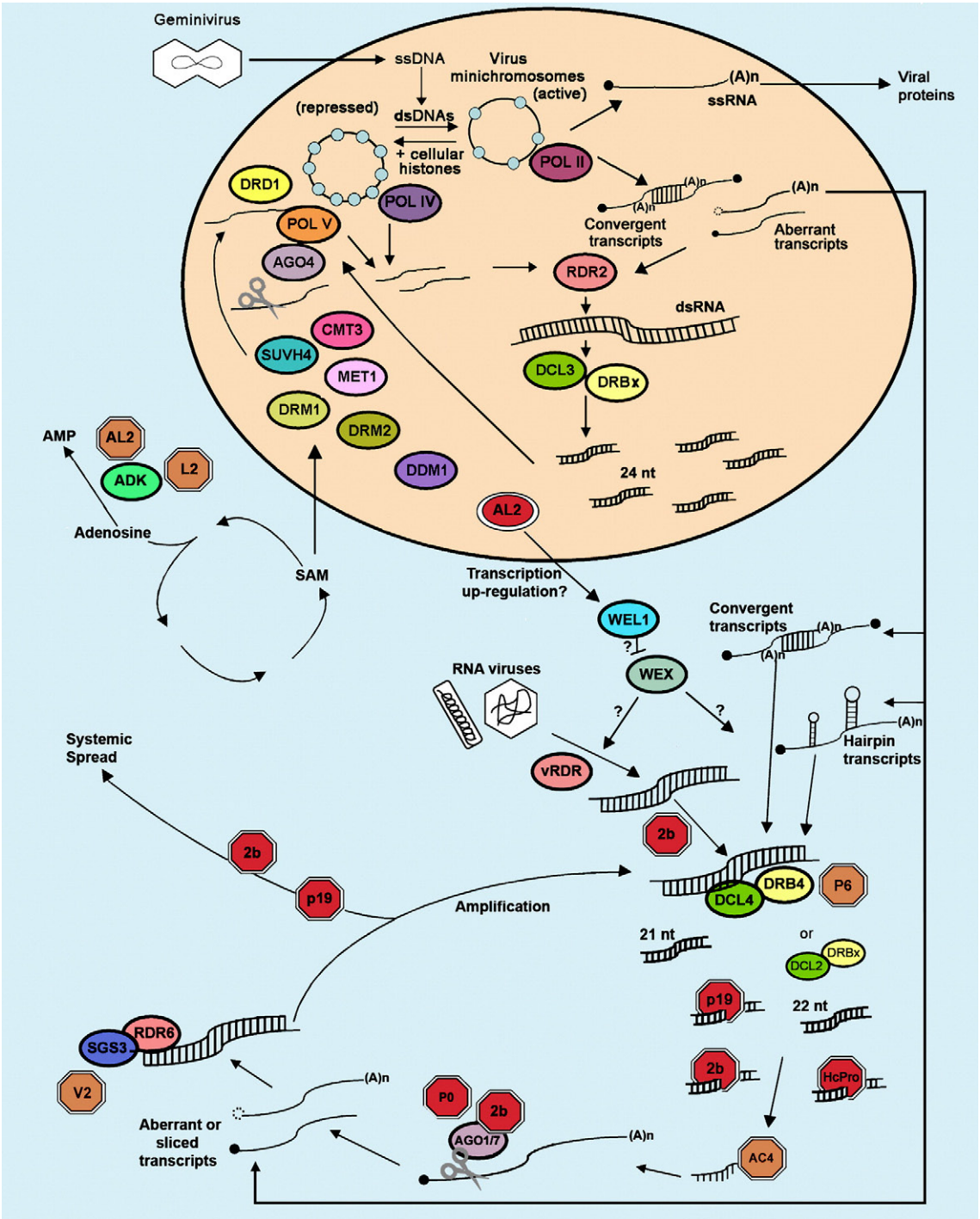

Fig. 2. Antiviral silencing pathways in *Arabidopsis*. Cytoplasmic events (blue background) illustrated include the generation of primary viral siRNAs by DCL4 in collaboration with DRB4. DCL2 can redundantly function if DCL4 is inactivated. The sources of primary dsRNA include the products of viral RDRs (vRDR) for RNA viruses or RNA polymerase II (POL II) for geminiviruses and other DNA viruses. The siRNAs enter RISC complexes that employ AGO1 as the primary slicer activity (indicated by scissors), with redundant activity provided by AGO7. RDR6-mediated secondary siRNA production, in conjunction with SGS3 and other proteins, is also shown. Transcripts normally possess a cap (black circle) and a polyA tail (A_n). Aberrant or sliced transcripts which lack these features may directly serve as substrates for dsRNA synthesis by RDR6 (or redundantly by RDR1, not shown). Cell-to-cell and systemic spread of silencing is presumably mediated by small RNAs and enhanced by amplification. Nuclear events (brown circle) depicted include POL II mediated transcription of viral mRNAs from active geminivirus minichromosomes (nucleosomes illustrated as blue circles) and non-coding POL IV transcription from methylated, repressed minichromosomes. Transcripts produced by either enzyme are subject to DCL3-mediated processing either directly (if they possess sufficient double-stranded character) or following conversion to dsRNA by RDR2. The 24 nt siRNA products of DCL3 guide docking of AGO4-containing RISC onto nascent transcripts generated by POL V. Subsequent methylation of viral DNA and associated histones involves recruiting cytosine (DRM1, DRM2, CMT3, MET1) and histone (e.g. SUVH4) methyltransferases, and requires chromatin remodelers, including DRM1 and DDM1. POL V transcripts may also be sliced by AGO4 (scissors) to act as additional RDR2 substrates. Suppressor proteins encoded by RNA viruses (red stop signs) or DNA viruses (orange stop signs) interfere with silencing pathways either by binding small RNAs or interacting with pathway components, as indicated. Note that the geminivirus AL2 and L2 proteins suppress silencing in a transcription-independent manner by inhibiting the methyl cycle that generates S-adenosyl methionine (SAM). Methyl cycle inhibition is accomplished by inactivation of adenosine kinase (ADK) (see Fig. 4). A transcription-dependent mechanism unique to AL2 that presumably involves transcriptional up-regulation of a cellular gene (WEL1) is also illustrated.

implicates methylation in DNA virus defense, as will be elaborated later.

One way to separate PTGS pathways from methylation and TGS is to examine the requirements for virus induced gene silencing (VIGS) from geminivirus vectors. VIGS is a process in which over-expression of a coding region fragment from a replicating virus vector causes PTGS-mediated silencing of the cognate endogenous gene [110]. Studies using a CaLCuV-based geminivirus vector have clearly shown that VIGS does not require genes in the methylation pathway, but silencing initiation and spread are compromised in *Arabidopsis dcl4*, *rdr6*, and *sgs3* mutant plants [89,111]. Silencing directed against CaMV transcripts is also mediated mostly by DCL4 and redundantly by DCL2 [82]. These observations suggest that silencing pathways deployed against RNA viruses and DNA virus transcripts are similar. Evidence to support this hypothesis comes from studies of viral silencing suppressors. The CaMV P6 suppressor has been reported to interact with DRB4, which is known to collaborate with DCL4 [112], and SGS3 is a target of the TYLCV V2 suppressor [113].

A role for DCL1 is no doubt made possible by the presence of DNA virus transcripts in the nucleus. Nevertheless, it is remarkable that *Arabidopsis dcl2*/*dcl3*/*dcl4* triple mutants, which have only DCL1 activity, accumulate 21 nt viral siRNAs and display only moderately enhanced susceptibility to CaMV and the geminiviruses CaLCuV and BCTV [82,89,114]. At this time it is not known which antiviral pathways DCL1 alone can support. However, in the case of CaMV, it was suggested that DCL1 might also facilitate siRNA biogenesis by other DCL proteins, possibly by cleaving the leader of the CaMV 35S transcript, a region of extensive secondary structure. DCL1-mediated excision of the leader, an event that is perhaps similar to primary- or pre-miRNA maturation, might stimulate further processing by other Dicers [82]. In any event, it seems that DCL1 alone is able to condition a partial, small RNA-based defense against DNA viruses. While the significance of this in wild-type plants is unclear, these observations highlight DCL1 as a versatile enzyme capable of producing small RNAs that can mediate multiple processes.

7. Methylation pathways and TGS in plants

The covalent modification of DNA and associated histones is a fundamental epigenetic mechanism that regulates gene expression during normal development, plays a major role in preventing the rearrangement of repeated sequences and in silencing transposons, and distinguishes heterochromatic and euchromatic regions of the genome. How specific sequences are targeted for repressive methylation is not completely understood, but in some organisms, including the fission yeast *Schizosaccharomyces pombe* and plants, siRNAs guide methylation to homologous sequences in a process termed RNA-directed DNA methylation (RdDM) [1,4,115,116].

Genome-wide profiling studies have shown that cytosine methylation is highly associated with heterochromatic sequences, including centromeric repeats, transposons, and retroelements [117–119]. The scale of the effort to silence these regions is reflected by the fact that methylation-associated 24 nt siRNAs, most of which map to centromeric and pericentromeric regions, easily represent the most abundant size class in wild-type *Arabidopsis* [61]. Methylation, especially in promoter regions, can silence transcription, and it is somewhat counterintuitive that TGS can be triggered and maintained by transcription of inverted or tandemly repeated sequences. TGS can also be initiated experimentally by ectopic expression of RNA corresponding to promoters [120,121]. Key players in the RdDM pathway include DCL3 and an as yet unidentified DRB protein(s), as well as RDR2 and AGO4 [54,122–128]. In addition, the plant-specific RNA polymerases IV and V (formerly POL IVa and POL IVb, respectively) are also central to methylation [127,129–131]. POL IV and POL V are not essential for viability in *Arabidopsis*, but appear to be specialized forms of RNA POL II that have evolved to function in siRNA-mediated methylation [132]. Because they likely have the ability to transcribe methylated DNA, their activities may provide an answer to the paradox that transcription is required for TGS [133].

A model for siRNA biogenesis and methylation posits that POL IV transcribes ssRNA from transposons and other methylated and repressed sequences, likely including geminivirus genomes (see below) (Fig. 2). These non-coding transcripts are recruited to sites of ribonucleoprotein complex assembly in the nucleolus (Cajal bodies) where they are converted to dsRNA by RDR2. The dsRNA is processed by DCL3 and incorporated into an AGO4-containing RISC complex which leaves the nucleolus and, programmed by the siRNA, directs methylation of complementary sequences, presumably by recruiting cytosine and histone methyltransferases [134,135]. Downstream components include the SWI2/SNF2 chromatin remodeling enzyme DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (DRD1) [136,137], and DOMAINS REARRANGED METHYLTRANSFERASE 1 and 2 (DRM1, DRM2), cytosine methyltransferases similar to mammalian Dnmt3 that are specialized for *de novo* methylation in all sequence contexts [138]. Methylation of histone H3 at lysine 9 (H3K9) also occurs in both plants and *S. pombe* [115,139–144].

An important issue is how siRNAs guide RISC to specific sequences. Genomic DNA might unwind to allow base pairing with siRNA, or transcription might produce a nascent, non-coding scaffold RNA to which siRNAs could pair. Either mechanism provides a sequence-specific interaction for docking AGO4-containing RISC, where it could serve as a platform to recruit chromatin modifying enzymes. Recent evidence from *S. pombe* and plants favors the RNA scaffold model, with RNA POL V likely producing the scaffold transcript in *Arabidopsis* [145–148]. In addition to recruiting methyltransferases, AGO4 also has slicer activity, and sliced scaffold transcripts could serve as a template for RDR-mediated dsRNA production that could amplify siRNAs to reinforce and maintain methylation [146,149].

In plants, cytosine methylation occurs in all sequence contexts: CG, CNG, and CHH (where H is A, T or C). CG methylation is symmetric and can be maintained by METHYLTYRANSFERASE1 (MET1) after the initial trigger is removed, in a manner that depends on the SWI2/SNF2-related protein DECREASE IN DNA METHYLATION1 (DDM1) [121,150]. MET1, a homolog of mammalian Dnmt1, prefers hemi-methylated substrates such as those present immediately following DNA replication [151]. In contrast, methylation occurring at CNG requires protein signals to target CHROMOMETHYLASE3 (CMT3) [152,153]. These signals include histone H3K9 dimethylation (H3K9me2) by SUVH4 (SU(VAR)3-9 HOMOLOG4; also known as KRYPTONITE2) [154–157]. CMT3 can bind methylated histone peptides *in vitro*, and SUVH4 in turn can bind CNG-methylated oligonucleotides, suggesting a positive feedback loop involving these two methyltransferases [157,158]. H3K9 methylation also depends on DDM1 [125,139,140]. Not surprisingly, H3K9me2 is associated with CNG methylation throughout the genome, and particularly at centromeric and pericentromeric regions [159]. CHH methylation is mostly carried out by DRM2 (*DRM1* is not highly expressed in *Arabidopsis*) and requires continual methylation signals provided by the *de novo* methylation pathway described above [138]. Thus, methylation pathway components are important for the establishment of cytosine and H3K9 methylation in all sequence contexts, and for maintenance at non-CG sites. Given the critical role of methylation in establishing genome organization and maintaining genome integrity, it is not surprising that considerable redundancy is built into the system. For example, DRM2 and CMT3 can function redundantly to initiate methylation and maintain non-CG methylation [138]. Similarly, SUVH4 and other activities, including SUVH5 and SUVH6, can methylate H3K9 [160,161]. AGO6 also functions in a partially redundant manner with AGO4 [162]. Further, CG methylation, which is more robustly maintained, appears to coordinate non-CG methylation and other epigenetic events, allowing them to be stably inherited [163].

8. RNA-directed methylation as a defense against geminiviruses

Given that plants use methylation to suppress the activity of endogenous invasive DNAs such as transposons, it is reasonable that they would also use methylation to repress viral minichromosomes. Early support for this came from studies which demonstrated that *in vitro* methylation of geminivirus DNA greatly reduced replication in tobacco protoplasts, mostly by inhibiting transcription [164,165]. Other indirect evidence followed. Transcriptional silencing of transgenes driven by the CaMV 35S promoter was observed in CaMV infected plants [166]. Similarly, it was found that transgenes driven by geminivirus promoters could be hypermethylated and transcriptionally silenced in plants inoculated with cognate, but not heterologous, geminiviruses [167,168]. These observations suggested that sequence-specific signals capable of eliciting TGS are generated during infection, and that these signals can target homologous promoters in cellular chromatin. It was also reported that geminivirus-infected plants can recover from infection following transient expression of a construct designed to express dsRNA corresponding to the viral IR, the master control region that contains divergent early and late gene promoters. Thus TGS signals could also act on promoters in replicating virus chromatin [169]. Similar experiments further suggested that silencing was associated with cytosine and H3K9 methylation of the viral genome [168,170].

A recent study has provided direct, genetic evidence for chromatin methylation as an epigenetic defense against geminiviruses [114]. As illustrated by representative experiments shown in Fig. 3, this study showed that methylation-deficient *Arabidopsis* mutants exhibit hypersensitivity to the begomovirus CaLCuV and the curtovirus BCTV. All of the methylation pathway mutants examined, including *nrpd2a* (deficient for both POL IV and POL V), *ago4*, and *ddm1*, proved hypersusceptible to CaLCuV and BCTV (Fig. 3A). Similar enhanced susceptibility was observed in mutant plants lacking the non-CG methyltransferases *drm1*/*drm2* and *cmt3*, and the H3K9 methyltransferase *kyp2*/*suvh4*. Hypersusceptibility was also evident in adenosine kinase mutants (*adk1* and *adk2*). ADK increases the efficiency of the methyl cycle that generates the essential methyltransferase co-factor S-adenosyl methionine (SAM), and is a target of the CaLCuV AL2 and BCTV L2 silencing suppressors (discussed further below). Experiments with *met1* mutants (CG maintenance methyltransferase) proved difficult because a complete loss of MET1 function results in severe growth defects. However, *met1* heterozygotes exhibit normal growth and showed moderately enhanced susceptibility to the geminiviruses. Importantly, bisulfite sequencing revealed that increased disease severity was in all cases accompanied by reduced cytosine methylation in the CaLCuV and BCTV intergenic regions. Compared to wild-type plants, total methylation was reduced approximately 10 to 25%, depending on the mutant, with most reductions occurring at non-CG sites. That larger reductions were not observed is testimony to the functional redundancy of silencing pathway components. Nevertheless, these findings suggest that non-CG methylation is an important aspect of host defense against geminiviruses, since even relatively modest reductions are sufficient to cause a severe infection phenotype [114].

Analysis of histone methylation has so far been limited to wild-type plants. As shown in Fig. 3B, the available evidence indicates that both abundant H3K9me2 and H3 acetylated at K9 and/or K14 are associated with the CaLCuV IR. Although it is possible that viral genomes contain mixed histone modifications, the presence of both active (acetylated H3) and repressive (H3K9) marks associated with the viral control region suggests that populations of active and repressed genomes co-exist in infected plants [114]. This would be consistent with the detection of viral chromosomes with different nucleosome densities [17].

While the sensitivity of methylation-deficient mutants to geminiviruses is quite striking, the most compelling argument for methylation as an antiviral defense comes from studies that have associated the methylation pathway with host recovery from infection. Recovery is a phenomenon where new tissues or shoots initiated after the establishment of a symptomatic infection exhibit symptom remission and contain very little virus. Recovery has been observed in several geminivirus–host combinations. For example, wild-type *N. benthamiana* and *Arabidopsis* plants can recover from infection with BCTV $L2^{-}$ mutant virus, which lacks a pathogenicity factor that opposes methylation (discussed further below) [45,46]. Remarkably, however, *Arabidopsis ago4* plants cannot recover from infection with the mutant virus (Fig. 3C), conclusively linking recovery and L2 function with the methylation pathway. Further, the IR of BCTV $L2^{-}$ genomes obtained from recovered tissues of wild-type plants was found to be hypermethylated, with nearly 80% of cytosines in all sequence contexts affected (Fig. 3D) [114].

Increased methylation of viral DNA, as judged by methylation-sensitive restriction analysis, has also been observed following recovery of watermelon plants from infection with the begomovirus *Cucurbit leaf crumple virus*. In addition, symptom remission was enhanced in zucchini plants (which normally exhibit weak recovery) by transient expression of dsRNA corresponding to the viral IR [171]. Another study found that recovery of pepper plants from *Pepper golden mosaic virus* infection was likewise accompanied by increased viral DNA methylation. Interestingly, bisulfite sequencing revealed that cytosine methylation was denser in the IR than in the CP coding region. Consistent with this observation, the bulk of the 24 nt viral siRNAs in infected plants mapped to the IR, while most of the 21 and 22 nt PTGS-associated viral siRNAs originated from coding regions [172].

Together, the studies summarized in this section establish a strong correlation between increased viral genome methylation and inhibition of virus replication. In addition, genetic analysis has provided conclusive evidence that the methylation pathway is an antiviral defense that limits the severity of a primary infection, and at later times is required for recovery. Because recovered tissues flower normally, methylation-based defenses may improve the long-term reproductive success of the host. The genetic evidence further indicates that pathways mediating viral chromatin methylation are similar to those employed at inverted repeats and transposons in host chromatin. This, coupled with the observation that geminiviruses respond aggressively to even small reductions in cytosine methylation, suggests that these viruses can serve as sensitive models for the analysis of fundamental epigenetic phenomena [114].

9. Suppression of PTGS by geminivirus proteins

Perhaps the strongest evidence that RNA silencing is a potent antiviral defense is the fact that all plant viruses examined to date encode at least one protein that has silencing suppressor activity. Viral suppressors are structurally diverse and can affect different and sometimes multiple points in silencing pathways. Their purpose is to promote viral invasiveness by enhancing virus replication in infected cells, and/or by inhibiting local or long-distance spread of antiviral silencing. They may act by interfering with small RNA production, by binding and sequestering small RNA, or by directly or indirectly inhibiting the activity of silencing-related proteins (for review see [3,64,173]). To use some RNA virus suppressors as examples, the helper component-protease (HC-Pro) found in *Potato virus Y* and *Tobacco etch virus* (TEV), the p19 protein of *Cymbidium ringspot virus* (CymRSV), and the 2b protein of *Cucumber mosaic virus* (CMV) bind and sequester siRNAs (and miRNAs), inhibiting their incorporation into RISC and methylation by HEN1 [174–176]. In contrast, P0 of poleroviruses (e.g. *Beet western yellows virus*) is an F-box containing protein that targets and mediates degradation of AGO1 [177,178]. Remarkably, some proteins act by multiple mechanisms. The small RNA-binding CMV 2b protein can also interact with AGO1 and

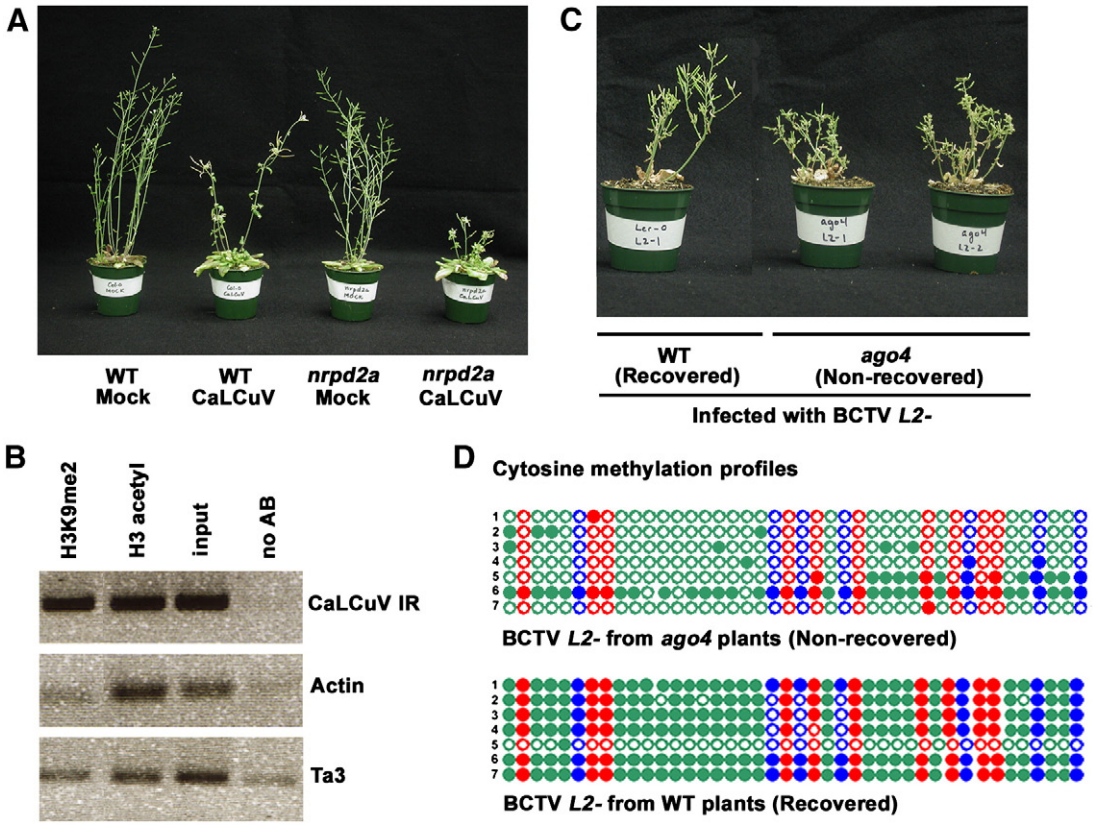

Fig. 3. Genetic analysis shows that viral chromatin methylation acts as a defense against geminiviruses. (A) An *Arabidopsis* POL IV/V mutant (*nrpd2a*) infected with CaLCuV displays enhanced symptoms, including increased stunting and severe floral deformation, compared with a wild-type (WT) infected plant. Control mock-inoculated plants are also shown. Similar results were obtained with CaLCuV and BCTV on all methylation mutants tested (see text). Enhanced susceptibility was accompanied by reduced cytosine methylation in the viral IR (not shown). (B) Chromatin immunoprecipitation (ChIP) using antibodies specific for dimethyl H3K9 (H3K9me2) or H3 acetylated at K9 or K14 (H3 acetyl) indicates that both marks are associated with the CaLCuV IR. Actin and transposon Ta3 were used as control sequences, and no AB indicates controls without antibody. (C) Wild-type plants infected with BCTV *L2*$^{-}$ mutant virus can recover (left), an ability that is lost in *ago4* mutants (right) that continue to show severe disease. (D) Cytosine methylation profiles confirm that recovery is characterized by increased methylation. Methylation of the BCTV IR was assessed by bisulfite sequencing. The dots represent all cytosines in the IR and are color coded according to sequence context (red: CG, blue: CNG, green: CHH). Filled circles indicate methylation, and each line represents the sequence of an individual clone. Viral DNA is sporadically methylated in non-recovered tissue from *ago4* plants, with most genomes lightly methylated and presumably active. The opposite is true in recovered tissue from wild-type plants, where nearly all cytosines are methylated and most genomes are repressed.

interfere with slicer activity [100], and HC-Pro interacts with a cellular protein (rgs-CaM) that appears to negatively regulate silencing [179].

Comparatively little is known about the activities of DNA virus silencing suppressors, but what is understood so far indicates that they are quite unique. The Baulcombe laboratory first showed that geminivirus AL2/AC2 protein could reverse established PTGS [180]. This ~15 kDa begomovirus protein was initially characterized as a transcription factor that stimulates the expression of virus late genes by both activation and derepression mechanisms [38,181–183]. AL2 has a basic N-terminal region that contains a nuclear localization signal (NLS), a central zinc finger-like domain (CCHC motif), and a C-terminal acidic-type transcription activation domain [184–186]. However, AL2 is a non-canonical transcription factor in that it only weakly binds ssDNA and dsDNA in a sequence non-specific manner, and is likely recruited to responsive promoters by interactions with cellular proteins [183,184,187]. AL2 does not bind siRNA or miRNA [188,189]. Rather, several studies have demonstrated that the ability of AL2 to reverse PTGS and inhibit its systemic spread requires an intact NLS, the CCHC motif, and the transcription activation domain, suggesting that silencing suppression in these contexts requires AL2 to stimulate transcription. More specifically, it is presumed that AL2 activates the transcription of cellular genes that negatively regulate silencing pathways [190–192]. This mode of silencing suppression has been termed transcription-dependent, although interactions with silencing pathway components through the activation domain cannot formally be ruled out [41].

That AL2 can, in principle, alter the host transcriptome comes from studies showing that geminivirus infection can activate the expression of reporter transgenes driven by the cognate viral CP promoter in an AL2-dependent fashion [181,193]. Transcriptional profiling following transient expression of AL2 in *Arabidopsis* protoplasts identified several genes whose expression was increased. One of these was *Werner exonuclease-like 1* (*WEL1*), a homologue of *Werner syndrome-like exonuclease* (*WEX*) [192]. Although its role is unclear, WEX is required for PTGS (but not TGS) directed against transgenes [194], and it has been proposed that WEL1 over-expression might compete for factors needed for WEX function [192]. Direct evidence to support this hypothesis, and for up-regulation of *WEL1* transcription during virus infection, is needed. Nevertheless, the transcription-dependent suppression mechanism in general is experimentally well-supported.

Evidence for transcription-independent silencing suppression by AL2 and the related L2/C2 protein from the curtovirus BCTV has also been obtained [189]. AL2 and L2 share a similar genome position and overlap, in different reading frames, the *AL1*/*L1* and *AL3*/*L3* genes that are highly conserved between begomoviruses and curtoviruses (Fig. 1). However, L2 shares little direct homology with its begomovirus counterparts, except for the central CCHC motif. In addition, unlike AL2, L2 lacks an obvious activation domain and is not a transcription factor [43–45]. Despite these differences, AL2 and L2 share pathogenicity functions, and both interact with and inactivate SNF1-related kinase (SnRK1) and adenosine kinase (ADK) [39,40,46,195]. The SnRK1

interactions inhibit the cellular stress response, which appears to be a component of plant basal defenses [40]. SnRK1 will not be discussed further here. However, the interaction with ADK, a nucleoside kinase that phosphorylates adenosine to 5′-AMP, offers several advantages, and of these silencing suppression is most germane to this review.

AL2 and L2 inactivate ADK *in vitro* and when co-expressed with ADK in *E. coli* and yeast, and ADK activity is reduced in an AL2/L2-dependent manner in geminivirus-infected plant tissues [46]. Bimolecular fluorescence complementation studies in *N. benthamiana* cells have also demonstrated that dimeric AL2 moves to the nucleus to activate transcription, whereas AL2:ADK and L2:ADK complexes accumulate mainly in the cytoplasm [196]. A link between ADK activity and silencing comes from the observation that ADK is needed to maintain the methyl cycle responsible for generating S-adenosyl-methionine (SAM), a methyl donor and essential methyltransferase co-factor [197–199] (Fig. 4). It is likely for this reason that ADK-deficient mutant plants display silencing defects [199]. A connection between methylation and PTGS was established by early observations that post-transcriptional silencing is frequently associated with methylation of the coding regions of targeted genes, and particularly reporter transgenes [200–203]. Such body methylation can interfere with gene expression [204], possibly by promoting the production of aberrant transcripts that could serve as substrates for dsRNA synthesis and thereby amplify siRNA production.

Evidence that AL2 and L2 can suppress PTGS in a transcription-independent manner by ADK inhibition was obtained from studies of transient local silencing in *N. benthamiana* leaves. These experiments showed that silencing directed against GFP could be suppressed by wild-type AL2 and L2 proteins, and also by a missense mutant (AL2 C33A) impaired for transcription activation or a truncated mutant lacking the activation domain ($AL2_{1-114}$) [189,196]. Further, local suppression could be phenocopied by ADK knock-down and by an adenosine analogue (A-134974) that inhibits ADK activity [189]. Knock-down of S-adenosyl-homocysteine hydrolase (SAHH) also results in suppression of PTGS directed against GFP (R.C. Buchmann and D.M. Bisaro, unpublished). Thus both intrinsic (SAHH) and associated (ADK) methyl cycle enzymes are important for PTGS, and AL2 and L2 inhibit the methyl cycle and PTGS in a transcription-independent manner by inactivating ADK. To summarize, begomovirus AL2 proteins appear to suppress silencing by activating the expression of cellular genes and by inhibiting ADK, whereas curtovirus L2 is limited to the latter mechanism [41].

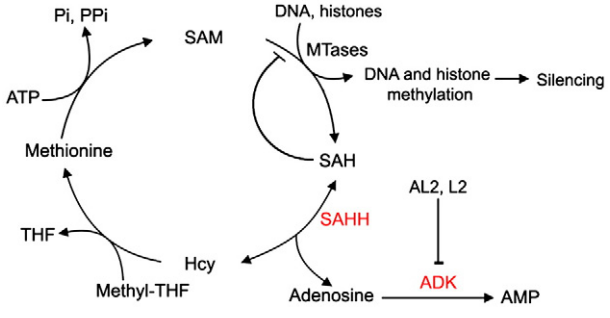

Fig. 4. The methyl cycle and its inhibition by AL2 and L2. S-adenosyl methionine (SAM) is the methyl donor for most transmethylation reactions. The product, S-adenosyl homocysteine (SAH), effectively competes with SAM for methyltransferases (MTases). SAH is converted to homocysteine (Hcy) and adenosine by S-adenosyl homocysteine hydrolase (SAHH). Phosphorylation of adenosine by adenosine kinase (ADK) is critical because the SAHH-catalyzed reaction is reversible and the equilibrium lies in the direction of SAH synthesis. By removing adenosine, ADK both promotes flux through the cycle and minimizes competitive inhibition of methyltransferase reactions by SAH. Thus, ADK inactivation by geminivirus AL2 and L2 proteins globally interferes with methylation. Both SAHH and ADK (shown in red) are required for methylation-dependent silencing (see text). Here only DNA and histones are listed as methyl-transferase substrates, although any type of transmethylation reaction may require SAM as a co-factor. THF: tetrahydrofolate.

The AL4/AC4 protein encoded by begomoviruses has also been demonstrated to have suppressor activity. The *AL4* gene is embedded within *AL1*, but in a different reading frame (Fig. 1). However, despite the highly conserved nature of AL1, AL4 is among the least conserved geminivirus proteins. The PTGS suppression activities of both AL4 and AL2 from four different cassava-infecting begomoviruses were initially examined in transient assays using *N. benthamiana* leaves [205]. Two of the AL4 proteins, from viruses that elicit recovery-type symptoms (severe disease followed by host recovery), had suppressor activity in this assay. The remaining two from non-recovery-type viruses (which elicit disease from which host plants do not recover) had little or no activity. Conversely, the AL2 proteins of non-recovery viruses were effective suppressors, while AL2 proteins of recovery-type viruses were not. This study was instructive for several reasons. First, it demonstrated that geminiviruses can encode more than one suppressor (in this case AL2 and AL4). Second, it showed that similar proteins from different viruses do not necessarily have equivalent suppressor activities. Third, it suggested that the AL2 and AL4 proteins primarily affect different silencing pathways, providing a mechanistic basis for observed severe disease synergy sometimes observed in the field. Severe disease can result when mixed infections, or recombination, combine active AL2 and AL4 suppressors. Finally, the different disease phenotypes induced by this panel of viruses suggested that while the counterdefense effects of AL4 are somewhat transient, an effective AL2 suppressor is associated with the absence of host recovery [205,206]. This is consistent with genetic data linking AL2/L2 function with recovery [45,114].

Insight into the AL4 suppression mechanism has been gained from the finding that the silencing-active protein associates with single-stranded, but not double-stranded, siRNAs and miRNAs *in vitro* and *in vivo* [188]. Thus AL4 is the only known protein that is apparently able to suppress PTGS, and coincidentally the miRNA pathway, by binding small RNAs in single-stranded form. This suggests that AL4 acts downstream of small RNA biogenesis and unwinding, and implies that RISC loading and unwinding are not necessarily coupled in plants. Presumably single-stranded small RNAs are accessible at some point between these events. Alternatively, small ssRNA may be recycled following release from AGO complexes. In any case, before more can be said about the mechanism it will be important to carefully define the affinity of AL4 for RNAs of varying structures and sizes and to determine whether the protein is able to interfere with RISC loading or disrupt previously formed RISC complexes.

The AR2/V2 protein (hereafter V2) that is unique to monopartite begomoviruses such as TYLCV has also been shown to suppress PTGS in transient assays [207]. Thus TYLCV and its relatives could potentially express three suppressor proteins (AL2, AL4, and V2). However, the AL2 version of the protein encoded by the virus used in this study (TYLCV-Israel) lacks activity, further demonstrating that different begomoviruses may rely on different suppressor functions. AL4 has not yet been tested. More recently, it was elegantly demonstrated that suppression of PTGS by V2 depends on its ability to interact with SISG3, the tomato homologue of *Arabidopsis* SGS3 [113]. Thus, V2 could interfere with siRNA amplification.

Perhaps the most intriguing example of silencing suppression in the *Geminiviridae* is observed with certain viruses that associate with a satellite DNA known as DNA β. While most monopartite begomoviruses produce symptomatic infections, some do not. In these cases, disease complexes consisting of the virus and DNA β are sometimes found (for review see [208]). DNA β is about half the size (~1.3 kb) of the helper virus on which it depends for replication, encapsidation, and systemic spread. In return, the sole satellite-encoded protein, βC1, is the pathogenicity determinant responsible for increased virus titers and symptoms induced by the disease complex [209,210]. Remarkably, βC1 pathogenicity may be partly explained by its interaction

with ASYMMETRIC LEAVES 1 (AS1), a protein capable of inhibiting jasmonic acid-mediated defense responses that could interfere with the whitefly vectors that transmit the virus and satellite. AS1 also regulates leaf polarity and so βC1:AS1 interaction is at least partly responsible for symptom development [211]. However, viral invasiveness is no doubt promoted by the ability of βC1 to suppress PTGS, which is apparently unrelated to the AS1 interaction [212]. The βC1 protein binds ssDNA and dsDNA in a sequence non-specific fashion and localizes to the nucleus, and its suppression activity requires the NLS. Beyond this little is known about the mechanism of suppression. However, it can be speculated that dependence on βC1 correlates with attenuated suppressor functions in the helper viruses.

10. Suppression of TGS by AL2 and L2

Given that plants employ viral chromatin methylation as a defense against geminiviruses, that the methylation pathway is required for recovery, and that AL2 and L2 could inhibit methylation by interfering with ADK activity, it was reasonable to ask whether these proteins suppress TGS. A recent study used two different experimental strategies to address this question [213]. The first employed a transgenic *N. benthamiana* line (16-TGS) containing a transcriptionally silenced 35S promoter-GFP transgene, in which the geminivirus proteins were expressed using the RNA virus *Potato virus X* (PVX) as a vector. Expression of wild-type AL2, transcriptionally compromised AL2 mutant proteins (e.g., $AL2_{1-114}$), or L2 in the 16-TGS plants restored GFP expression, indicating that the geminivirus proteins can reverse TGS in a transcription-independent manner (Fig. 5A). Using a VIGS vector based on the RNA virus *Tobacco rattle virus*, knock-down of SAHH (not shown) and ADK expression in 16-TGS plants also resulted in TGS reversal, further implicating the transcription-independent mechanism involving methyl cycle inhibition (Fig. 5A). SAHH was previously identified in a genetic screen designed to detect genes required for methylation-dependent silencing [214]. TGS reversal in a more biologically relevant context was also demonstrated. Inoculation of 16-TGS plants with wild-type begomoviruses (including CaLCuV and TGMV) and the curtovirus BCTV reversed TGS in a manner consistent with the tissue tropism of these viruses. GFP expression was observed in both vascular tissue and mesophyll cells in symptomatic areas of begomovirus-infected plants, but was mainly confined to the vascular tissue following infection with the more tightly phloem-limited BCTV (Fig. 5A). Importantly, infection of 16-TGS plants with a BCTV $L2^{-}$ mutant did not result in silencing suppression, confirming its dependence on L2 (and by extension AL2) function.

In a second approach, *Arabidopsis* lines containing transgenes driven by a dexamethasone (dex)-inducible promoter were established. Included were AL2, $AL2_{1-114}$, and L2 expressing transgenes, as well as a dsADK transgene capable of producing dsRNA to knock-down ADK expression. Following dex-induction, the effects of the viral proteins on the expression of *Arabidopsis* loci known to be transcriptionally silenced by methylation were examined by reverse transcription-PCR (RT-PCR) [213]. The silenced loci examined included a putative *F-box* family gene, the retrotransposons *AtSN1* (SINE element) and *Athila* (LTR element), and a DNA transposon (*CACTA*-like). It was found that all three viral proteins, and dsADK RNA expression, caused ectopic expression of *F-box* and the retrotransposons, indicating that TGS reversal occurred via the transcription-independent mechanism. However, only AL2 was able to overcome TGS of the *CACTA*-like transposon, suggesting that this required the transcription-dependent mechanism (Fig. 5B). The reason for this difference is not presently known, however, *CACTA*-like silencing requires CG methylation while the other loci are expressed in genetic backgrounds that reduce non-CG methylation [117,128]. Because CG methylation is more robustly maintained than non-CG methylation, it may be less sensitive to methyl cycle inhibition.

Subsequent analysis of methylation at the *AtSN1* locus using bisulfite sequencing revealed that transgenic expression of AL2 reduced methylation by about 15%. The reductions were observed primarily at non-CG sites, although the number of CG sites examined was insufficient for definitive conclusions regarding sequence context to be drawn (Fig. 5C). However, methylation reduction by ADK inhibition is not expected to be locus-specific, and a quantitative, analysis capable of assessing CNG methylation status within MspI restriction sites genome-wide showed that methylation was globally reduced (1.5 to 2.5-fold) in transgenic plants expressing AL2 or L2 (Fig. 5D). Taken together, these studies provide additional evidence that methylation of viral chromatin is an important defense by showing that as a countermeasure, geminivirus proteins can globally reduce methylation and reverse TGS by nonspecifically inhibiting cellular transmethylation reactions. No doubt other geminivirus proteins and proteins encoded by other DNA viruses will also prove capable of suppressing TGS by this or other mechanisms. In addition, given the considerable overlap between silencing pathways and the variety of silencing suppression mechanisms employed by viruses, it is likely that some encoded by RNA viruses will be capable of inhibiting methylation and TGS, even if only as an unintended consequence. In this context, it is interesting to speculate that some RNA viruses might profit by inhibiting SAM-dependent, HEN1-mediated 2′-O-methylation of siRNAs, thereby destabilizing and reducing pools of functional viral siRNAs in infected cells. There is some evidence for reduced methylation and enhanced degradation of small RNAs in *Tobacco mosaic virus* (TMV) infected plants, although the same study did not detect a similar destabilization in geminivirus-infected plants [89]. This is one of many issues that warrants further study.

11. Summary

It now seems clear that geminiviruses, and most likely other DNA viruses that infect plants, confront both PTGS and TGS-based host defenses, which may partly explain the preponderance of RNA viruses in the plant virus world. To be successful then, a plant DNA virus must be able to suppress both types of defenses. This could be accomplished by suppressor proteins with activity against both pathways, or by multiple proteins more or less specialized to act on different pathways. For example, the begomovirus AL2 and L2 proteins are relatively weak PTGS suppressors in most assays, yet both AL2 and L2 oppose methylation and strongly suppress TGS. Because methylation-deficient mutant plants display significantly enhanced disease symptoms as well as a loss of recovery, it could be argued that a primary role for AL2 and L2 is to inhibit the methylation-based defenses that lead to TGS, with PTGS suppression being somewhat less important. On the other hand, the effects of the AL4 protein appear to be more transient in nature, and perhaps the role of this protein is primarily to oppose PTGS. However, it should be cautioned that this is a simplistic explanation, as the existence of successful begomoviruses with relatively stronger and weaker AL2 and AL4 suppressors suggests some functional overlap. No doubt this is a complex issue, and the relative importance of PTGS and TGS to any particular virus could vary depending on the host plant and aspects of agro-ecosystems that are currently not understood. In any event, now that we know viral proteins can suppress TGS, it will be interesting to identify additional TGS suppressors and determine their mechanisms of action. If experience with PTGS suppressors is any guide, we can anticipate a greater appreciation of host methylation pathways and their roles in antiviral defense.

Genetic analysis indicates that the methylation pathway used in defense against geminiviruses is similar to that used by the host to suppress genomic repeat sequences and transposons usually found in heterochromatin. This, and the extreme sensitivity of methylation-deficient mutants to geminiviruses, indicates that they will be useful

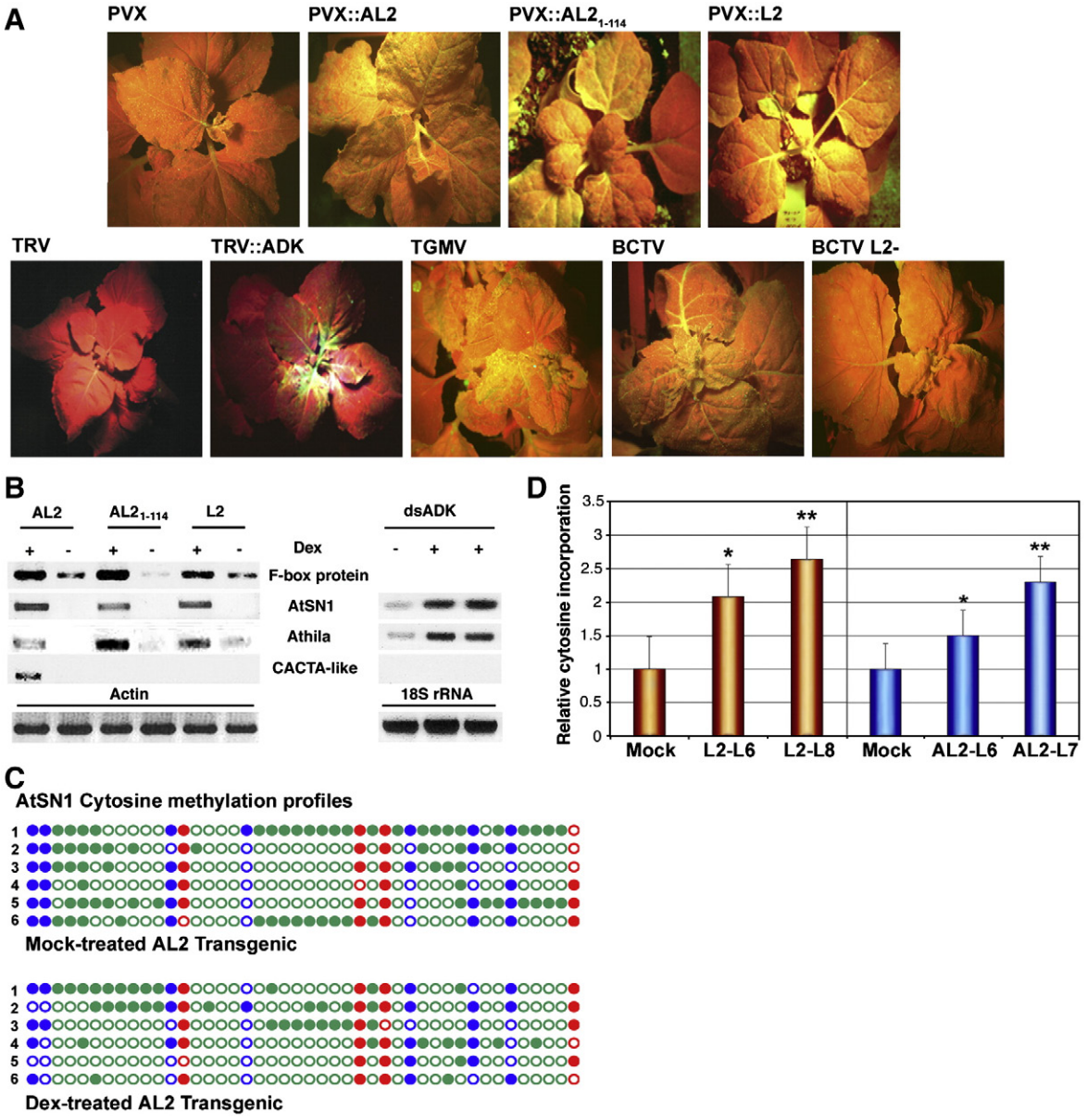

Fig. 5. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing (TGS). (A) *N. benthamiana* plants containing a transcriptionally silenced, 35S-GFP transgene (line 16-TGS) were inoculated with a *Potato virus X* (PVX) vector, or PVX expressing the geminivirus proteins AL2, transcription-defective AL2 mutants (e.g., $AL2_{1-114}$), or L2 (see text). All geminivirus proteins could restore GFP expression, as indicated by yellow-green fluorescence under UV illumination, and subsequent Northern blot analysis with a GFP-specific probe (not shown). Silenced plants appear red under UV light due to chlorophyll autofluorescence. Experiments using a *Tobacco rattle virus* (TRV) VIGS vector showed that ADK knock-down also reversed TGS, as did infections with wild-type geminiviruses TGMV and BCTV, but not a BCTV *L2*$^{-}$ mutant. (B) *Arabidopsis* plants containing dexamethasone (dex) inducible transgenes expressing AL2, $AL2_{1-114}$, L2, or dsRNA corresponding to ADK (dsADK) were treated with dex or water, and expression levels of endogenous genes known to be methylated and transcriptionally silenced were assessed by RT-PCR. All transgenes caused ectopic expression of all tested loci except *CACTA-like*, which was expressed only in the presence of AL2. Actin or 18S rRNA served as controls. (C) Bisulfite sequencing revealed that transgenic expression of AL2 caused a reduction in cytosine methylation at the *AtSN1* locus. Methylation profiles are as described in Fig. 3D. (D) Total genomic DNA was isolated from water or dex-treated *Arabidopsis* plants containing dex-inducible AL2 or L2 transgenes, digested to completion with MspI, and incubated with labeled dCTP and Taq polymerase to allow single-nucleotide extension. MspI (C$^{\downarrow}$CGG) does not cleave if the external cytosine is methylated. Reduced methylation results in greater cleavage and hence greater incorporation of labeled nucleotide. Incorporation was increased from 1.5 to 2.5-fold by the geminivirus proteins. Experiments were performed with three individual plants from each of two transgenic lines. Asterisks indicate significant differences between dex- and mock-treated samples at the 95% (*) or 99% (**) confidence level, as determined by Student's *t* test.

models for this type of repressive methylation. It is interesting to note that elements from DNA viruses (e.g., geminivirus promoters and particularly the CaMV 35S promoter) are often used to drive the constitutive expression of transgenes, and clearly are not targeted for repressive methylation in the context of host chromatin. It will be especially important to determine the specific features or properties of replicating viral genomes that cause these same sequences to be identified as methylation targets in viral chromatin. Conversely, because geminivirus proteins can actively suppress methylation, it is worth considering whether host genome instability due to transposon reactivation is an aspect of pathogenesis. Answering these and other challenges that have been raised by the discovery of methylation-based antiviral defenses and counter-defenses will take time and require the efforts of many investigators.

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References

[1] S.W.-L. Chan, I.R. Henderson, S.E. Jacobsen, Gardening the genome: DNA methylation in *Arabidopsis thaliana*, Nat. Rev. 6 (2005) 351–360.
[2] S.-W. Ding, O. Voinnet, Antiviral immunity directed by small RNAs, Cell 130 (2007) 413–426.
[3] J.A. Diaz-Pendon, S.-W. Ding, Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis, Annu. Rev. Phytopathol. 46 (2008) 303–326.
[4] I.R. Henderson, S.E. Jacobsen, Epigenetic inheritance in plants, Nature 447 (2007) 418–424.
[5] V. Ruiz-Ferrer, O. Voinnet, Roles of plant small RNAs in biotic stress responses, Annu. Rev. Plant Biol. 60 (2009) 485–510.
[6] C. Gutierrez, Geminiviruses and the plant cell cycle, Plant Mol. Biol. 43 (2000) 763–772.
[7] L. Hanley-Bowdoin, S.B. Settlage, B.M. Orozco, S. Nagar, D. Robertson, Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation, Crit. Rev. Biochem. Mol. Biol. 35 (2000) 105–140.
[8] L. Hanley-Bowdoin, S. Settlage, D. Robertson, Reprogramming plant gene expression: a prerequisite to geminivirus replication, Mol. Plant Pathol. 5 (2004) 149–156.
[9] M.R. Rojas, C. Hagen, W.J. Lucas, R.L. Gilbertson, Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses, Annu. Rev. Phytopathol. 43 (2005) 361–394.
[10] H. Jeske, Geminiviruses, Curr. Top. Immunol. Microbiol. 331 (2009) 185–226.
[11] M.S. Nawaz-ul-Rehman, C.M. Fauquet, Evolution of geminiviruses and their satellites, FEBS Lett. 583 (2009) 1825–1832.
[12] K. Saunders, A. Lucy, J. Stanley, DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication, Nucleic Acids Res. 19 (1991) 2325–2330.
[13] D.C. Stenger, G.N. Revington, M.C. Stevenson, D.M. Bisaro, Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling circle replication of a plant viral DNA, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8029–8033.
[14] H. Jeske, M. Lutgemeier, W. Preiss, DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus, EMBO J. 20 (2001) 6158–6167.
[15] W. Preiss, H. Jeske, Multitasking in replication is common among geminiviruses, J. Virol. 2003 (2003) 2972–2980.
[16] M. Pilartz, H. Jeske, Abutilon mosaic virus double-stranded DNA is packed into minichromosomes, Virology 189 (1992) 800–802.
[17] M. Pilartz, H. Jeske, Mapping of Abutilon mosaic geminivirus minichromosomes, J. Virol. 77 (2003) 10808–10818.
[18] C.M. Fauquet, D.M. Bisaro, R.W. Briddon, J.K. Brown, B.D. Harrison, E.P. Rybicki, D.C. Stenger, J. Stanley, Revision of taxonomic criteria for species demarcation in the family Geminiviridae, and an updated list of begomovirus species, Arch. Virol. 148 (2003) 405–421.
[19] J.S. Elmer, L. Brand, G. Sunter, W.E. Gardiner, D.M. Bisaro, S.G. Rogers, Genetic analysis of tomato golden mosaic virus II. The product of the AL1 coding sequence is required for replication, Nucleic Acids Res. 16 (1988) 7043–7060.
[20] J. Laufs, W. Traut, F. Heyraud, V. Matzeit, S.G. Rogers, J. Schell, B. Gronenborn, *In vitro* cleavage and ligation at the viral origin of replication by the replication protein of tomato yellow leaf curl virus, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 3879–3883.
[21] E.P.B. Fontes, P.A. Eagle, P.S. Sipe, V.A. Luckow, L. Hanley-Bowdoin, Interaction between a geminivirus replication protein and origin DNA is essential for viral replication, J. Biol. Chem. 269 (1994) 8459–8465.
[22] A. Luque, A.P. Sanz-Burgos, E. Ramirez-Parra, M.M. Castellano, C. Gutierrez, Interaction of geminivirus Rep protein with replication factor C and its potential role during geminivirus replication, Virology 302 (2002) 83–94.
[23] A.G. Castillo, D. Collinet, S. Deret, A. Kashoggi, E.R. Bejarano, Dual interaction of plant PCNA with geminivirus replication accessory protein (REn) and viral replication protein (Rep), Virology 312 (2003) 381–394.
[24] D.K. Singh, M.N. Islam, N.R. Choudhury, S. Karjee, S.K. Mukherjee, The 32 kDa subunit of replication protein A (RPA) participates in the DNA replication of *Mung bean yellow mosaic India virus* (MYMIV) by interacting with the viral Rep protein, Nucleic Acids Res. 2007 (2006) 755–770.
[25] N.R. Choudhury, P.S. Malik, D.K. Singh, M.N. Islam, K. Kaliappan, S.K. Mukherjee, The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase, Nucleic Acids Res. 34 (2006) 6362–6377.
[26] D. Clerot, F. Bernardi, DNA helicase activity is associated with the replication initiator protein Rep of Tomato yellow leaf curl geminivirus, J. Virol. 80 (2006) 11322–11330.
[27] S. Nagar, T.J. Pedersen, K.M. Carrick, L. Hanley-Bowdoin, D. Robertson, A geminivirus induces expression of host DNA synthesis protein in terminally differentiated plant cells, Plant Cell 7 (1995) 705–719.
[28] R.A. Ach, T. Durfee, A.B. Miller, P. Taranto, L. Hanley-Bowdoin, P.C. Zambriski, W. Gruissem, An alternatively-spliced, multigene family in maize encodes retinoblastoma-related proteins which can interact with a plant D-type cyclin and a geminivirus replication protein, Mol. Cell. Biol. 17 (1997) 5077–5086.
[29] L.J. Kong, B.M. Orozco, J.L. Roe, S. Nagar, S. Ou, H.S. Feiler, T. Durfee, A.B. Miller, W. Gruissem, D. Robertson, L. Hanley-Bowdoin, A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants, EMBO J. 19 (2000) 3485–3494.
[30] E.M. Egelkrout, D. Robertson, L. Hanley-Bowdoin, Proliferating cell nuclear antigen transcription is repressed through an E2F consensus element and activated by geminivirus infection in mature leaves, Plant Cell 13 (2001) 1437–1452.
[31] W. Shen, L. Hanley-Bowdoin, Geminivirus infection up-regulates the expression of two *Arabidopsis* protein kinases related to yeast SNF1- and mammalian AMPK activating kinases, Plant Physiol. 142 (2006) 1642–1655.
[32] W. Shen, M.I. Reyes, L. Hanley-Bowdoin, *Arabidopsis* protein kinases GRIK1 and GRIK2 specifically activate SnRK1 by phosphorylating its activation loop, Plant Physiol. 150 (2009) 996–1005.
[33] G. Sunter, M.D. Hartitz, S.G. Hormuzdi, C.L. Brough, D.M. Bisaro, Genetic analysis of tomato golden mosaic virus. ORF AL2 is required for coat protein accumulation while ORF AL3 is necessary for efficient DNA replication, Virology 179 (1990) 69–77.
[34] S.B. Settlage, A.B. Miller, W. Gruissem, L. Hanley-Bowdoin, Dual interaction of a geminivirus replication accessory factor with a viral replication protein and a plant cell cycle regulator, Virology 279 (2001) 570–576.
[35] S.B. Settlage, R.G. See, L. Hanley-Bowdoin, Geminivirus C3 protein: replication enhancement and protein interactions, J. Virol. 79 (2005) 9885–9895.
[36] J.T. Ascencio-Ibanez, R. Sozzani, T.-J. Lee, T.-M. Chu, R.D. Wolfinger, R. Cella, L. Hanley-Bowdoin, Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection, Plant Physiol. 148 (2008) 436–454.
[37] G. Sunter, D.M. Bisaro, Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression, Virology 180 (1991) 416–419.
[38] G. Sunter, D.M. Bisaro, Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription, Plant Cell 4 (1992) 1321–1331.
[39] G. Sunter, J. Sunter, D.M. Bisaro, 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 (2001) 59–70.
[40] L. Hao, H. Wang, G. Sunter, D.M. Bisaro, Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase, Plant Cell 15 (2003) 1034–1048.
[41] D.M. Bisaro, Silencing suppression by geminivirus proteins, Virology 344 (2006) 158–168.
[42] I.-R. Choi, D.C. Stenger, Strain-specific determinants of beet curly top geminivirus DNA replication, Virology 206 (1995) 904–912.
[43] J. Stanley, J. Latham, M.S. Pinner, I. Bedford, P.G. Markham, Mutational analysis of the monopartite geminivirus beet curly top virus, Virology 191 (1992) 396–405.
[44] G. Sunter, D.C. Stenger, D.M. Bisaro, Heterologous complementation by geminivirus AL2 and AL3 genes, Virology 203 (1994) 203–210.
[45] S.G. Hormuzdi, D.M. Bisaro, Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis, Virology 206 (1995) 1044–1054.
[46] H. Wang, L. Hao, C.-Y. Shung, G. Sunter, D.M. Bisaro, Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins, Plant Cell 15 (2003) 3020–3032.
[47] L. Hanley-Bowdoin, J.S. Elmer, S.G. Rogers, Functional expression of the leftward open reading frames of the A component of tomato golden mosaic virus in transgenic plants, Plant Cell 1 (1989) 1057–1067.
[48] C.-Y. Shung, J. Sunter, S.S. Sirasanagandla, G. Sunter, Distinct viral sequence elements are necessary for expression of *Tomato golden mosaic virus* complementary sense transcripts that direct AL2 and AL3 gene expression, Mol. Plant-Microb. Interact. 19 (2006) 1394–1405.
[49] C.-Y. Shung, G. Sunter, Regulation of *Tomato golden mosaic virus AL2* and *AL3* gene expression by a conserved upstream open reading frame, Virology 383 (2009) 310–318.
[50] G. Sunter, M.D. Hartitz, D.M. Bisaro, Tomato golden mosaic virus leftward gene expression: autoregulation of geminivirus replication protein, Virology 195 (1993) 275–280.
[51] P.A. Eagle, B.M. Orozco, L. Hanley-Bowdoin, A DNA sequence required for geminivirus replication also mediates transcriptional regulation, Plant Cell 6 (1994) 1157–1170.
[52] C.-Y. Shung, G. Sunter, AL1-dependent repression of transcription enhances expression of *Tomato golden mosaic virus* AL2 and AL3, Virology 364 (2007) 112–122.
[53] A.M. Abouzid, T. Frischmuth, H. Jeske, A putative replicative form of the abutilon mosaic virus (gemini group) in a chromatin-like structure, Mol. Gen. Genet. 212 (1988) 252–258.
[54] Z. Xie, L.K. Johansen, A.M. Gustafson, K.D. Kasschau, A.D. Lellis, D. Zilberman, S.E. Jacobsen, J.C. Carrington, Genetic and functional diversification of small RNA pathways in plants, PLOS Biol. 2 (2004) e104, doi:10.1371/journal.pbio.0020104.
[55] O. Voinnet, Induction and suppression of RNA silencing: insights from viral infections, Nat. Rev., Genet. 6 (2005) 206–220.
[56] P. Broderson, O. Voinnet, The diversity of RNA silencing pathways in plants, Trends Genet. 22 (2006) 268–280.
[57] M.W. Jones-Rhoades, D. Bartel, B. Bartel, MicroRNAs and their regulatory roles in plants, Annu. Rev. Plant Biol. 57 (2006) 19–53.
[58] H. Vaucheret, Post-transcriptional small RNA pathways in plants: mechanisms and regulations, Genes Dev. 20 (2006) 759–771.
[59] J. Li, Z. Yang, B. Yu, J. Liu, X. Chen, Methylation protects miRNAs and siRNAs from a 3′-end uridylation activity in *Arabidopsis*, Curr. Biol. 15 (2005) 1501–1507.
[60] B. Yu, Z. Yang, J. Li, S. Minakhina, M. Yang, R.W. Padgett, R. Steward, X. Chen, Methylation as a crucial step in plant microRNA biogenesis, Science 307 (2005) 932–935.

[61] K.D. Kasschau, N. Fahlgren, E.J. Chapman, C.M. Sullivan, J.S. Cumbie, S.A. Givan, J.C. Carrington, Genome-wide profiling and analysis of *Arabidopsis* siRNAs, PLoS Biol. 5 (2007) e57, doi:10.1371/journal.pbio.0050057.
[62] P. Broderson, L. Sakvarelidze-Achard, M. Bruun-Rasmussen, P. Dunoyer, Y.Y. Yamamoto, L. Sieburth, O. Voinnet, Widespread translational inhibition by plant miRNAs and siRNAs, Science 320 (2008) 1185–1190.
[63] C.-H. Lecellier, P. Dunoyer, K. Arar, L.-C.J.S.Please check the author names given if captured correctly. Eyquem, C. Himber, A. Saib, O. Voinnet, A cellular microRNA mediates antiviral defense in human cells, Science 308 (2005) 557–560.
[64] F. Li, S.-W. Ding, Virus counterdefense: diverse strategies for evading the RNA-silencing immunity, Annu. Rev. Microbiol. 60 (2006) 503–531.
[65] R. Triboulet, B. Mari, Y.-L. Lin, C. Chable-Bessia, Y. Bennasser, K. Lebrigand, B. Cardinaud, T. Maurin, P. Barbry, V. Baillat, J. Reynes, P. Corbeau, K.-T. Jeang, M. Benkirane, Suppression of microRNA-silencing pathway by HIV-1 during virus replication, Science 315 (2007) 1579–1582.
[66] W. de Vries, B. Berkhout, RNAi suppressors encoded by pathogenic human viruses, Int. J. Biochem. Cell Biol. 40 (2008) 2007–2012.
[67] S. Qian, X. Zhong, L. Yu, B. Ding, P. de Haan, K. Boris-Lawrie, HIV-1 Tat RNA silencing suppressor activity is conserved across kingdoms and counteracts translational repression of HIV-1, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 605–610.
[68] Z. Wu, Y. Zhu, D.M. Bisaro, D.S. Parris, Herpes simplex virus type 1 suppresses RNA-induced gene silencing in mammalian cells, J. Virol. 83 (2009) 6652–6663.
[69] S. Pfeffer, M. Zavolan, F.A. Grasser, M. Chien, J.J. Russo, J. Ju, B. John, A.J. Enright, D. Marks, C. Sander, T. Tuschl, Identification of virus-encoded microRNAs, Science 304 (2004) 734–736.
[70] F. Grey, A. Antoniewicz, E. Allen, J. Saugstad, A. McShea, J.C. Carrington, J. Nelson, Identification and characterization of human cytomegalovirus-encoded micro-RNAs, J. Virol. 79 (2005) 12095–12099.
[71] N. Stern-Ginossar, N. Elefant, A. Zimmerman, D.G. Wolf, N. Saleh, M. Biton, E. Horwitz, Z. Prokocimer, M. Prichard, G. Hahn, D. Goldman-Wohl, C. Greenfield, S. Yagel, H. Hengel, Y. Altuvia, H. Margalit, O. Mandelboim, Host immune system gene targeting by a viral miRNA, Science 317 (2007) 376–381.
[72] R.W. Morgan, A. Anderson, E. Bernberg, S. Kamboj, E. Huang, G. Lagasse, G. Isaacs, M. Parcells, B.C. Meyers, P.J. Green, J. Burnside, Sequence conservation and differential expression of Marek's disease virus microRNAs, J. Virol. 82 (2008) 12213–12220.
[73] E. Murphy, J. Vanicek, H. Robins, T. Shenk, A.J. Levine, Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 5453–5458.
[74] J.L. Umbach, M.F. Kramer, I. Jurak, H.W. Karnowski, D.M. Coen, B.R. Cullen, MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs, Nature 454 (2008) 780–783.
[75] F.-Z. Wang, F. Weber, C. Croce, C.-G. Liu, X. Liao, P.E. Pellett, Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication, J. Virol. 82 (2008) 9065–9074.
[76] C. Llave, MicroRNAs: more than a role in plant development? Mol. Plant Pathol. 5 (2004) 361–366.
[77] Q.-W. Niu, S.-S. Lin, J.L. Reyes, K.-C. Chen, H.-W. Wu, S.-D. Yeh, N.-H. Chua, Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance, Nat. Biotechnol. 24 (2006) 1420–1428.
[78] K.D. Kasschau, Z. Xie, E. Allen, C. Llave, E.J. Chapman, K.A. Krizan, J.C. Carrington, P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function, Dev. Cell 4 (2003) 205–217.
[79] E.J. Chapman, A.I. Prokhnevsky, K. Gopinath, V.V. Dolja, J.C. Carrington, Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step, Genes Dev. 18 (2004) 1179–1186.
[80] P. Dunoyer, C.-H. Lecellier, E.A. Parizotto, C. Himber, O. Voinnet, Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing, Plant Cell 16 (2004) 1235–1250.
[81] L. Martinez-Priego, L. Donaire, C. Llave, Silencing suppressor activity of *Tobacco rattle virus*-encoded 16-kDa protein and interference with endogenous small RNA-guided regulatory pathways, Virology 376 (2008) 346–356.
[82] G. Moissard, O. Voinnet, RNA silencing of host transcripts by cauliflower mosaic virus requires coordinated action of the four *Arabidopsis* Dicer-like proteins, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 19593–19598.
[83] J.A. Lindbo, L. Silva-Rosales, W.M. Proebsting, W.G. Dougherty, Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance, Plant Cell 5 (1993) 1749–1759.
[84] F. Ratcliff, B.D. Harrison, D.C. Baulcombe, A similarity between viral defense and gene silencing in plants, Science 276 (1997) 1558–1560.
[85] M.T. Ruiz, O. Voinnet, D.C. Baulcombe, Initiation and maintenance of virus-induced gene silencing, Plant Cell 10 (1998) 937–946.
[86] S. Mlotshwa, G.J. Pruss, V. Vance, Small RNAs in viral infection and host defense, Trends Plant Sci. 13 (2008) 375–382.
[87] A. Molnar, T. Csorba, L. Lakatos, E. Varallyay, C. Lacomme, J. Burgyan, Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs, J. Virol. 79 (2005) 7812–7818.
[88] T. Ho, D. Pallett, R. Rusholme, T. Dalmay, H. Wang, A simplified method for cloning short interfering RNAs from *Brassica juncea* infected with *Turnip mosaic potyvirus* and *Turnip crinkle carmovirus*, J. Virol. Methods 136 (2006) 217–223.
[89] T. Blevins, R. Rajeswaran, P.V. Shivaprasad, D. Beknazariants, A. Si-Ammour, H.-S. Park, F. Vazquez, D. Robertson, F. Meins, T. Hohn, M.M. Pooggin, Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing, Nucleic Acids Res. 34 (2006) 6233–6246.
[90] N. Bouche, D. Lauressergues, V. Gasciolli, H. Vaucheret, An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs, EMBO J. 25 (2006) 3347–3356.
[91] A. Deleris, J. Gallego-Bartolome, J. Bao, K. Kasschau, J.C. Carrington, O. Voinnet, Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense, Science 313 (2006) 68–71.
[92] J.A. Diaz-Pendon, F. Li, W.-X. Li, S.-W. Ding, Suppression of antiviral silencing by Cucumber Mosaic Virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs, Plant Cell 19 (2007) 2053–2063.
[93] F. Qu, X. Ye, T.J. Morris, *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14732–14737.
[94] X. Adenot, T. Elmayan, D. Lauressergues, S. Boutet, N. Bouche, V. Gasciolli, H. Vaucheret, DRB4-dependent *TAS3 trans*-acting siRNAs control leaf morphology through AGO7, Curr. Biol. 16 (2006) 927–932.
[95] Y. Nakazawa, A. Hiraguri, H. Moriyama, T. Fukuhara, The dsRNA-binding protein DRB4 interacts with the Dicer-like protein DCL4 in vivo and functions in the trans-acting siRNA pathway, Plant Mol. Biol. 63 (2007) 777–785.
[96] O. Voinnet, Use, tolerance and avoidance of amplified RNA silencing by plants, Trends Plant Sci. 13 (2008) 317–328.
[97] P. Mourrain, C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J.-B. Morel, D. Jouette, A.M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T.A. Vo, H. Vaucheret, *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance, Cell 101 (2000) 533–542.
[98] G. Moissard, E.A. Parizotto, C. Himber, O. Voinnet, Transitivity in *Arabidopsis* can be primed, requires the redundant action of the antiviral Dicer-like 4 and Dicer-like 2, and is compromised by viral suppressor proteins, RNA 13 (2007) 1268–1278.
[99] N. Baumberger, D.C. Baulcombe, *Arabidopsis* ARGONAUTE1 is an RNA slicer that selectively recruits microRNAs and short interfering RNAs, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 11928–11933.
[100] X. Zhang, Y.-R. Yuan, Y. Pei, S.-S. Lin, T. Tuschl, D.J. Patel, N.-H. Chua, *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense, Genes Dev. 20 (2006) 3255–3268.
[101] V. Pantoleo, G. Szittya, J. Burgyan, Molecular basis of viral RNA targeting by viral small interfering RNA-programmed RISC, J. Virol. 81 (2007) 3797–3806.
[102] J.-C. Palaqui, T. Elmayan, J.-M. Pollien, H. Vaucheret, Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions, EMBO J. 16 (1997) 4738–4745.
[103] C. Himber, P. Dunoyer, G. Moissard, C. Ritzenthaler, O. Voinnet, Transitivity-dependent and -independent cell-to-cell movement of RNA silencing, EMBO J. 22 (2003) 4523–4533.
[104] O. Voinnet, Non-cell autonomous RNA silencing, FEBS Lett. 579 (2005) 5858–5871.
[105] F. Qu, X. Ye, G. Hou, S. Sato, T.E. Clemente, T.J. Morris, RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*, J. Virol. 79 (2005) 15209–15217.
[106] F. Schwach, F.E. Vaistij, L. Jones, D.C. Baulcombe, An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal, Plant Physiol. 138 (2005) 1842–1852.
[107] F.E. Vaistij, L. Jones, Compromised virus-induced gene silencing in RDR6-deficient plants, Plant Physiol. 149 (2009) 1399–1407.
[108] P. Chellappan, R. Vanitharani, C.M. Fauquet, Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences, J. Virol. 78 (2004) 7465–7477.
[109] R. Akbergenov, A. Si-Ammour, T. Blevins, I. Amin, C. Kutter, H. Vanderschuren, P. Zhang, W. Gruissem, F. Meins Jr., T. Hohn, M.M. Pooggin, Molecular characterization of geminivirus-derived small RNAs in different plant species, Nucleic Acids Res. 34 (2006) 462–471.
[110] D.C. Baulcombe, Fast forward genetics based on virus-induced gene silencing, Curr. Opin. Plant Biol. 2 (1999) 109–113.
[111] N. Muangsan, C. Beclin, H. Vaucheret, D. Robertson, 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 (2004) 1004–1014.
[112] G. Haas, J. Azevado, G. Moissard, A. Geldreich, C. Himber, M. Bureau, T. Fukuhara, M. Keller, O. Voinnet, Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4, EMBO J. 27 (2008) 2102–2112.
[113] E. Glick, A. Zrachya, Y. Levy, A. Mett, D. Gidoni, E. Belausov, V. Citovsky, Y. Gafni, Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 157–161.
[114] P. Raja, B.C. Sanville, R.C. Buchmann, D.M. Bisaro, Viral genome methylation as an epigenetic defense against geminiviruses, J. Virol. 82 (2008) 8997–9007.
[115] R.A. Martienssen, M. Zaratiegui, D.B. Goto, RNA interference and heterochromatin in the fission yeast *Schizosaccharomyces pombe*, Trends Genet. 21 (2005) 450–456.
[116] S.W.-L. Chan, Inputs and outputs for chromatin-targeted RNAi, Trends Plant Sci. 13 (2008) 383–389.
[117] X. Zhang, J. Yazaki, A. Sundaresan, S. Cokus, S.W.-L. Chan, H. Chen, I.R. Henderson, P. Shinn, M. Pellegrini, S.E. Jacobsen, J.R. Ecker, Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*, Cell 126 (2006) 1189–1201.
[118] S.J. Cokus, S. Feng, X. Zhang, Z. Chen, B. Merriman, C.D. Haudenschild, S. Pradhan, S.F. Nelson, M. Pellegrini, S.E. Jacobsen, Shotgun bisuphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning, Nature 452 (2008) 215–219.

[119] R. Lister, R.C. O'Malley, J. Tonti-Filippini, B.D. Gregory, C.C. Berry, A.H. Millar, J.R. Ecker, Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*, Cell 133 (2008) 523–536.
[120] M.F. Mette, W. Aufsatz, J. van der Winden, M.A. Matzke, A.J.M. Matzke, Transcriptional silencing and promoter methylation triggered by double-stranded RNA, EMBO J. 19 (2000) 5194–5201.
[121] L. Jones, F. Ratcliff, D.C. Baulcombe, RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance, Curr. Biol. 11 (2001) 747–757.
[122] A. Hamilton, O. Voinnet, L. Chappell, D.C. Baulcombe, Two classes of short interfering RNA in RNA silencing, EMBO J. 21 (2002) 4671–4679.
[123] D. Zilberman, X. Cao, S.E. Jacobsen, *Argonaute4* control of locus-specific siRNA accumulation and DNA and histone methylation, Science 299 (2003) 716–719.
[124] S.W.-L. Chan, D. Zilberman, Z. Xie, L.K. Johansen, J.C. Carrington, S.E. Jacobsen, RNA silencing genes control de novo DNA methylation, Science 303 (2004) 1336.
[125] Z. Lippman, A.-V. Gendrel, M. Black, M.W. Vaughn, N. Dedhia, W.R. McCrombie, K. Lavine, V. Mittal, B. May, K.D. Kasschau, J.C. Carrington, R.W. Doerge, V. Colot, R. Martienssen, Role of transposable elements in heterochromatin and epigenetic control, Nature 430 (2004) 471–476.
[126] D. Zilberman, X. Cao, L.K. Johansen, Z. Xie, J.C. Carrington, S.E. Jacobsen, Role of *Arabidopsis ARGONAUTE4* in RNA-directed DNA methylation triggered by inverted repeats, Curr. Biol. 14 (2004) 1214–1220.
[127] Y. Qi, A.M. Denli, G.J. Hannon, Biochemical specialization within *Arabidopsis* RNA silencing pathways, Mol. Cell 19 (2005) 421–428.
[128] R.K. Tran, D. Zilberman, C. de Bustos, R.F. Ditt, J.G. Henikoff, A.M. Lindroth, J. Delrow, T. Boyle, S. Kwong, T.D. Bryson, S.E. Jacobsen, S. Henikoff, Chromatin and siRNA pathways cooperate to maintain DNA methylation of small transposable elements in *Arabidopsis*, Genome Biol. 6 (2005) R90.01–R90.11.
[129] A.J. Herr, M.B. Jensen, T. Dalmay, D.C. Baulcombe, RNA polymerase IV directs silencing of endogenous DNA, Science 308 (2005) 118–120.
[130] T. Kanno, B. Huettel, M.F. Mette, W. Aufsatz, E. Jaligot, L. Daxinger, D.P. Kreil, M. Matzke, A.J. Matzke, Atypical RNA polymerase subunits required for RNA-directed DNA methylation, Nat. Genet. 37 (2005) 761–765.
[131] Y. Onodera, J.R. Haag, T. Ream, P.C. Nunes, O. Pontes, C.S. Pikaard, Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation, Cell 120 (2005) 613–622.
[132] T.S. Ream, J.R. Haag, A.T. Wierzbicki, C.D. Nicora, A.D. Norbeck, J.-K. Zhu, G. Hagen, T.J. Guilfoyle, L. Pasa-Tolic, C.S. Pikaard, Subunit compositions of the RNA-silencing enzymes Pol IV and Pol V reveal their origins as specialized forms of RNA polymerase II, Mol. Cell 33 (2009) 192–203.
[133] C.S. Pikaard, J.R. Haag, T. Ream, A.T. Wierzbicki, Roles of RNA polymerase IV in gene silencing, Trends Plant Sci. 13 (2008) 390–397.
[134] C.F. Li, O. Pontes, M. El-Shami, I.R. Henderson, Y.V. Bernatavichute, S.W.-L. Chan, T. Lagrange, C.S. Pikaard, S.E. Jacobsen, An ARGONAUTE4-containing nuclear processing center colocalized with Cajal bodies in *Arabidopsis thaliana*, Cell 126 (2006) 93–106.
[135] O. Pontes, C.F. Li, P.C. Nunes, J. Haag, T. Ream, A. Vitins, S.E. Jacobsen, C.S. Pikaard, The *Arabidopsis* chromatin-modifying nuclear siRNA pathway involves a nucleolar RNA processing center, Cell 126 (2006) 79–92.
[136] T. Kanno, M.F. Mette, D.P. Kreil, W. Aufsatz, M. Matzke, A.J.M. Matzke, Involvement of a putative SNF2 chromatin remodeling protein DRD1 in RNA-directed DNA methylation, Curr. Biol. 14 (2004) 801–805.
[137] S.W.-L. Chan, I.R. Henderson, X. Zhang, G. Shah, J.S.-C. Chien, S.E. Jacobsen, RNAi, DRD1, and histone methylation actively target developmentally important non-CG DNA methylation in *Arabidopsis*, PLoS Genet. 2 (2006) e83, doi:10.1371/journal.pgen.0020083.
[138] X. Cao, W. Aufsatz, D. Zilberman, M.F. Mette, M.S. Huang, M. Matzke, S.E. Jacobsen, Role of *DRM* and *CMT3* methyltransferases in RNA-directed DNA methylation, Curr. Biol. 13 (2003) 2212–2217.
[139] A.-V. Gendrel, Z. Lippman, C. Yordan, V. Colot, R.A. Martienssen, Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*, Science 297 (2002) 1871–1873.
[140] L. Johnson, X. Cao, S.E. Jacobsen, Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation, Curr. Biol. 12 (2002) 1360.
[141] T.A. Volpe, C. Kidner, I.M. Hall, G. Teng, S.I.S. Grewal, R.A. Martienssen, Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi, Science 297 (2002) 1833–1837.
[142] T. Volpe, V. Schramke, G.L. Hamilton, S.A. White, G. Teng, R.A. Marteinssen, R.A. Allshire, RNA interference is required for normal centromere function in fission yeast, Chromosome Res. 11 (2003) 137–146.
[143] A. Verdel, S. Jia, S. Gerber, T. Sugiyama, S. Gygi, S.I. Grewal, D. Moazed, RNAi-mediated targeting of heterochromatin by the RITS complex, Science 303 (2004) 672–676.
[144] S.I.S. Grewal, S.C.R. Elgin, Transcription and RNA interference in the formation of heterochromatin, Nature 447 (2007) 399–406.
[145] M. Buhler, A. Verdel, D. Moazed, Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing, Cell 125 (2006) 873–886.
[146] D.V. Irvine, M. Zaratiegui, N.H. Tolia, D.B. Goto, D.H. Chitwood, M.W. Vaughn, L. Joshua-Tor, R.A. Martienssen, Argonaute slicing is required for heterochromatic silencing and spreading, Science 313 (2006) 1134–1137.
[147] A.T. Wierzbicki, J.R. Haag, C.S. Pikaard, Noncoding transcription by RNA polymerase IVb/PolV mediates transcriptional silencing of overlapping and adjacent genes, Cell 135 (2008) 635–648.
[148] A.T. Wierzbicki, T. Ream, J.R. Haag, C.S. Pikaard, RNA polymerase V transcription guides ARGONAUTE4 to chromatin, Nat. Genet. 41 (2009) 630–634.
[149] Y. Qi, X. He, X.-J. Wang, O. Kohany, J. Jurka, G.J. Hannon, Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation, Nature 443 (2006) 1008–1012.
[150] J.A. Jeddeloh, T.L. Stokes, E.J. Richards, Maintenance of genomic methylation requires a SWI2/SNF2-like protein, Nat. Genet. 22 (1999) 94–97.
[151] X. Cao, S.E. Jacobsen, Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 16491–16498.
[152] L. Bartee, F. Malagnac, J. Bender, *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene, Genes Dev. 15 (2001) 1753–1758.
[153] A.M. Lindroth, X. Cao, J.P. Jackson, D. Zilberman, C.M. McCallum, S. Henikoff, S.E. Jacobsen, Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation, Science 292 (2001) 2077–2080.
[154] J.P. Jackson, A.M. Lindroth, X. Cao, S.E. Jacobsen, Control of CpNpG methylation by the KRYPTONITE histone H3 methyltransferase, Nature 416 (2002) 556–560.
[155] F. Malagnac, L. Bartee, J. Bender, An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation, EMBO J. 21 (2002) 6842–6852.
[156] J.P. Jackson, L. Johnson, Z. Jasencakova, X. Zhang, L. PerezBurgos, P.B. Singh, X. Cheng, I. Schubert, T. Jenuwein, S.E. Jacobsen, Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*, Chromosoma 112 (2004) 308–315.
[157] A.M. Lindroth, D. Shultis, Z. Jasencakova, J. Fuchs, L. Johnson, D. Schubert, D. Patnaik, S. Prakhan, J. Goodrich, I. Schubert, T. Jenuwein, S. Khorasanizadeh, S.E. Jacobsen, Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3, EMBO J. 23 (2004) 4286–4296.
[158] L.M. Johnson, M. Bostick, X. Zhang, E. Kraft, I.R. Henderson, J. Callis, S.E. Jacobsen, The SRA methyl-cytosine-binding domain links DNA and histone methylation, Curr. Biol. 17 (2007) 379–384.
[159] Y.V. Bernatavichute, X. Zhang, S. Cokus, M. Pelligrini, S.E. Jacobsen, Genome-wide association of histone H3 lysine nine methylation with CHG DNA methylation in *Arabidopsis thaliana*, PLoS ONE 3 (2008) e3156, doi:10.1371/journal.pone.0003156.
[160] M.L. Ebbs, L. Bartee, J. Bender, H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of SUVH6 and SUVH4 methyltransferases, Mol. Cell. Biol. 25 (2005) 10507–10515.
[161] M.L. Ebbs, J. Bender, Locus-specific control of DNA methylation by the *Arabidopsis* SUVH5 histone methyltransferase, Plant Cell 18 (2006) 1166–1176.
[162] X. Zheng, J. Zhu, A. Kapoor, J.-K. Zhu, Role of *Arabidopsis* AGO6 in siRNA accumulation, DNA methylation, and transcriptional gene silencing, EMBO J. 26 (2007) 1691–1701.
[163] O. Mathieu, J. Reinders, M. Caikovski, C. Smathajitt, J. Paszkowski, Transgenerational stability of the *Arabidopsis* epigenome is coordinated by CG methylation, Cell 130 (2007) 851–862.
[164] C.L. Brough, W.E. Gardiner, N. Inamdar, X.Y. Zhang, M. Ehrlich, D.M. Bisaro, DNA methylation inhibits propagation of tomato golden mosaic virus DNA in transfected protoplasts, Plant Mol. Biol. 18 (1992) 703–712.
[165] G. Ermak, U. Paszkowski, M. Wohlmuth, O.M. Scheid, J. Paszkowski, Cytosine methylation inhibits replication of African cassava mosaic virus by two distinct mechanisms, Nucleic Acids Res. 21 (1993) 3445–3450.
[166] N.S. Al-Kaff, S.N. Covey, M.M. Kreike, A.M. Page, R. Pinder, P.J. Dale, Transcriptional and posttranscriptional plant gene silencing in response to a pathogen, Science 279 (1998) 2113–2115.
[167] M. Seemanpillai, I. Dry, J. Randles, A. Rezaian, Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection, Mol. Plant-Microb. Interact. 16 (2003) 429–438.
[168] X.-Y. Bian, M.S. Rasheed, M.J. Seemanpillai, M.A. Rezaian, Analysis of silencing escape of *Tomato leaf curl virus*: an evaluation of the role of DNA methylation, Mol. Plant-Microb. Interact. 19 (2006) 614–624.
[169] M. Pooggin, T. Hohn, RNAi targeting of DNA virus in plants, Nat. Biotechnol. 21 (2003) 131–132.
[170] A.M. Dogar, RNAi dependent epigenetic marks on a geminivirus promoter, Virol. J. 3 (2006) doi:10.11 186/1743-1422X-1743-1745.
[171] C. Hagen, M.R. Rojas, T. Kon, R.L. Gilbertson, Recovery from *Cucurbit leaf crumple virus* (family *Geminiviridae*, genus *Begomovirus*) infection is an adaptive antiviral response associated with changes in small viral RNAs, Phytopathol. 98 (2008) 1029–1037.
[172] E.A. Rodriguez-Negrete, J. Carrillo-Trip, R.F. Rivera-Bustamante, RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery, J. Virol. 83 (2009) 1332–1340.
[173] B.M. Roth, G.J. Pruss, V.B. Vance, Plant viral suppressors of RNA silencing, Virus Res. 102 (2004) 97–108.
[174] J.M. Vargason, G. Szittya, J. Burgyan, T.M. Tanaka Hall, Size selective recognition of siRNA by an RNA silencing suppressor, Cell 115 (2003) 799–811.
[175] L. Lakatos, T. Csorba, V. Pantaleo, E. Chapman, J.C. Carrington, Y.-P. Liu, V. Dolja, L.F. Calvino, J. Lopez-Moya, J. Burgyan, Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors, EMBO J. 25 (2006) 2768–2780.
[176] K. Goto, T. Kobori, Y. Kosaka, T. Natsuaki, C. Masuta, Characterization of the silencing suppressor 2b of Cucumber mosaic virus based on examination of its small RNA-binding abilities, Plant Cell Physiol. 48 (2007) 1050–1060.
[177] N. Baumberger, C.-H. Tsai, M. Lie, E. Havecker, D.C. Baulcombe, The polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation, Curr. Biol. 17 (2007) 1609–1614.
[178] D. Bortolamiol, M. Pazhouhandeh, K. Marrocco, P. Genschik, V. Ziegler-Graff, The polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing, Curr. Biol. 17 (2007) 1615–1621.

[179] R. Anandalakshmi, G.J. Pruss, X. Ge, R. Marathe, A.C. Mallory, T.H. Smith, V.B. Vance, A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants, Science 290 (2000) 142–144.
[180] O. Voinnet, Y.M. Pinto, D.C. Baulcombe, Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 14147–14152.
[181] G. Sunter, D.M. Bisaro, Regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): evidence for activation and derepression mechanisms, Virology 232 (1997) 269–280.
[182] G. Sunter, D.M. Bisaro, Identification of a minimal sequence required for activation of the tomato golden mosaic virus coat protein promoter in protoplasts, Virology 305 (2003) 452–462.
[183] G. Lacatus, G. Sunter, Functional analysis of bipartite begomovirus coat protein sequences, Virology 376 (2008) 79–89.
[184] M.D. Hartitz, G. Sunter, D.M. Bisaro, The geminivirus transactivator (TrAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain, Virology 263 (1999) 1–14.
[185] R. van Wezel, H. Liu, P. Tien, J. Stanley, Y. Hong, 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 (2001) 1125–1128.
[186] R. van Wezel, H. Liu, Z. Wu, J. Stanley, Y. Hong, Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttrancriptional gene silencing, J. Virol. 77 (2003) 696–700.
[187] G. Lacatus, G. Sunter, The *Arabidopsis* PEAPOD2 transcription factor interacts with geminivirus AL2 protein and the coat protein promoter, Virology 392 (2009) 196–202.
[188] P. Chellappan, R. Vanitharani, C.M. Fauquet, MicroRNA-binding viral protein interferes with *Arabidopsis* development, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 10381–10386.
[189] H. Wang, K.J. Buckley, X. Yang, R.C. Buchmann, D.M. Bisaro, Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins, J. Virol. 79 (2005) 7410–7418.
[190] R. van Wezel, H. Liu, P. Tien, J. Stanley, Y. Hong, 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 (2002) 203–208.
[191] X. Dong, R. van Wezel, J. Stanley, Y. Hong, Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing, J. Virol. 77 (2003) 7026–7033.
[192] D. Trinks, R. Rajeswaran, P.V. Shivaprasad, R. Akbergenov, E.J. Oakeley, K. Veluthambi, T. Hohn, M. Pooggin, Supression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes, J. Virol. 79 (2005) 2517–2527.
[193] Y. Hong, K. Saunders, J. Stanley, Transactivation of dianthin transgene expression by African cassava mosaic virus AC2, Virology 228 (1997) 383–387.
[194] E. Glazov, K. Phillips, G.J. Budziszewski, F. Meins, J. Levin, A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in *Arabidopsis*, Plant J. 35 (2003) 342–349.
[195] S. Baliji, J. Sunter, G. Sunter, Transcriptional analysis of complementary sense genes in *Spinach curly top virus* and functional role of C2 in pathogenesis, Mol. Plant-Microb. Interact. 20 (2007) 194–206.
[196] X. Yang, S. Baliji, R.C. Buchmann, H. Wang, J.A. Lindbo, G. Sunter, D.M. Bisaro, Functional modulation of the geminivirus AL2 transcription factor and silencing suppressor by self-interaction, J. Virol. 81 (2007) 11972–11981.
[197] K. Lecoq, I. Belloc, C. Desgranges, B. Daignan-Fornier, Role of adenosine kinase in *Saccharomyces cerevisiae*: identification of the *ADO1* gene and study of the mutant phenotypes, Yeast 18 (2001) 335–342.
[198] E.A. Weretilnyk, K.J. Alexander, M. Drebenstedt, J.D. Snider, P.S. Summers, B.A. Moffatt, Maintaining methylation activities during salt stress. The involvement of adenosine kinase, Plant Physiol. 125 (2001) 856–865.
[199] B.A. Moffatt, Y.Y. Stevens, M.S. Allen, J.D. Snider, L.A. Periera, M.I. Todorova, P.S. Summers, E.A. Weretilnyk, L. Martin-Caffrey, C. Wagner, Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation, Plant Physiol. 128 (2002) 812–821.
[200] I. Ingelbrecht, H. Van Houdt, M. Van Montagu, A. Depicker, Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 10502–10506.
[201] H. Smith, S.L. Swaney, T.D. Parks, E.A. Wernsman, W.G. Dougherty, Trangenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs, Plant Cell 6 (1994) 1441–1453.
[202] J.J. English, E. Mueller, D.C. Baulcombe, Suppression of virus accumulation in plants exhibiting silencing of nuclear genes, Plant Cell 8 (1996) 179–188.
[203] A.L. Jones, C.L. Thomas, A.J. Maule, *De novo* methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus, EMBO J. 17 (1998) 6385–6393.
[204] T. Hohn, S. Corsten, S. Rieke, M. Muller, H. Rothnie, Methylation of coding region alone inhibits gene expression in plant protoplasts, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 8334–8339.
[205] R. Vanitharani, P. Chellappan, J.S. Pita, C. Fauquet, Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing, J. Virol. 78 (2004) 9487–9498.
[206] R. Vanitharani, P. Chellappan, C.M. Fauquet, Geminiviruses and RNA silencing, Trends Plant Sci. 10 (2005) 144–151.
[207] A. Zrachya, E. Glick, Y. Levy, T. Arazi, V. Citovsky, Y. Gafni, Suppressor of RNA silencing encoded by *Tomato yellow leaf curl virus*-Israel, Virology 358 (2007) 159–165.
[208] R.W. Briddon, J. Stanley, Subviral agents associated with plant single-stranded DNA viruses, Virology 344 (2006) 198–210.
[209] X. Cui, X. Tao, Y. Yie, C.M. Fauquet, X. Zhou, A DNAβ associated with Tomato yellow leaf curl China virus is required for symptom induction, J. Virol. 78 (2004) 13966–13974.
[210] K. Saunders, A. Norman, S. Gucciardo, J. Stanley, The DNA β satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity determinant (βC1), Virology 324 (2004) 37–47.
[211] J.-Y. Yang, M. Iwasaki, C. Machida, Y. Machida, X. Zhou, N.-H. Chua, βC1, the pathogenicity factor of TYLCCNV, interacts with AS1 to alter leaf development and suppress selective jasmonic acid responses, Genes Dev. 22 (2008) 2564–2577.
[212] X. Cui, G. Li, D. Wang, D. Hu, X. Zhou, A begomovirus DNAβ-encoded protein binds DNA, functions as a suppressor of RNA silencing, and targets the cell nucleus, J. Virol. 79 (2005) 10764–10775.
[213] R.C. Buchmann, S. Asad, J.N. Wolf, G. Mohannath, D.M. Bisaro, Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation, J. Virol. 83 (2009) 5005–5013.
[214] P.S.C.F. Rocha, M. Sheikh, R. Melchiorre, M. Fagard, S. Boutet, R. Loach, B.A. Moffatt, C. Wagner, H. Vaucheret, I. Furner, 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 (2005) 404–417.