Viral Genome Methylation as an Epigenetic Defense against Geminiviruses

Priya Raja, Bradley C. Sanville, R. Cody Buchmann, and David M. Bisaro

Department of Molecular Genetics, Plant Biotechnology Center, and Program in Molecular, Cellular and Developmental Biology,
The Ohio State University, Columbus, Ohio 43210

Received 1 April 2008/Accepted 25 June 2008

Geminiviruses encapsidate single-stranded DNA genomes that replicate in plant cell nuclei through double-stranded DNA intermediates that associate with cellular histone proteins to form minichromosomes. Like most plant viruses, geminiviruses are targeted by RNA silencing and encode suppressor proteins such as AL2 and L2 to counter this defense. These related proteins can suppress silencing by multiple mechanisms, one of which involves interacting with and inhibiting adenosine kinase (ADK), a cellular enzyme associated with the methyl cycle that generates S-adenosyl-methionine, an essential methyltransferase cofactor. Thus, we hypothesized that the viral genome is targeted by small-RNA-directed methylation. Here, we show that Arabidopsis plants with mutations in genes encoding cytosine or histone H3 lysine 9 (H3K9) methyltransferases, RNA-directed methylation pathway components, or ADK are hypersensitive to geminivirus infection. We also demonstrate that viral DNA and associated histone H3 are methylated in infected plants and that cytosine methylation levels are significantly reduced in viral DNA isolated from methylation-deficient mutants. Finally, we demonstrate that Beet curly top virus L2 mutant DNA present in tissues that have recovered from infection is hypermethylated and that host recovery requires AGO4, a component of the RNA-directed methylation pathway. We propose that plants use chromatin methylation as a defense against DNA viruses, which geminiviruses counter by inhibiting global methylation. In addition, our results establish that geminiviruses can be useful models for genome methylation in plants and suggest that there are redundant pathways leading to cytosine methylation.
ated at lysine 9 (H3K9), a hallmark of repressed genes, is highly represented in viral chromatin. Finally, we show that host recovery from geminivirus infection, which occurs in plants infected with L2 mutants, correlates with hypermethylation of viral DNA and that recovery requires the RNA-directed methylation pathway component AGO4.

MATERIALS AND METHODS

Arabidopsis mutants. Mutants were obtained from the Arabidopsis Biological Research Center at The Ohio State University or from individuals. The following previously characterized seed stocks were used: wild-type Ler-0 (CS20) Landsberg erecta ecotype; ago 4-1 gl-1 (CS636/A2g277040) AGO4 (63); kyp2 gl-1 (CS636/A1g13960) kryptonite/SuVH4 (26); drm1 drm2 momethylase (33); wild-type Ws-2 (CS22659) Wassilewskija ecotype; met1-7 (27); clear RNA polymerase D 2A (23);

Virus inoculation. Agroinoculation of Nicotiana benthamiana plants with tomato golden mosaic virus, cabbage leaf curl virus (CaLCuV), and beet curly top virus (BCTV) was carried out as described previously (50). Plants were inoculated 30 to 40 days after germination. An overnight culture of Agrobacterium tumefaciens containing tandem repeat copies of the appropriate virus genome was injected into the petiole of leaves using a Hamilton syringe. Three leaves were inoculated per plant, and 30 μl of inoculum was used per leaf. Infection of Arabidopsis plants with CaLCuV and BCTV was carried out mechanically as described for N. benthamiana and sugar beet (24). Arabidopsis plants were inoculated within 5 days of bolting. Bolts were cut where they emerged from the rosette, and inoculum was applied to the freshly cut stem, which was then punctured with an insect pin multiple times. CaLCuV and tomato golden mosaic virus symptoms were observed, and plants were harvested 14 to 21 days postinoculation. Due to an inherently longer latent period, BCTV symptoms were observed, and plants were harvested 21 to 30 days postinoculation. Symptomatic leaf tissue was harvested from N. benthamiana and sugar beet (24). Arabidopsis plants were inoculated within 5 days of bolting. Bolts were cut where they emerged from the rosette, and inoculum was applied to the freshly cut stem, which was then punctured with an insect pin multiple times. CaLCuV and tomato golden mosaic virus symptoms were observed, and plants were harvested 14 to 21 days postinoculation. Due to an inherently longer latent period, BCTV symptoms were observed, and plants were harvested 21 to 30 days postinoculation. Symptomatic leaf tissue was harvested from N. benthamiana and sugar beet (24). Arabidopsis plants were inoculated within 5 days of bolting. Bolts were cut where they emerged from the rosette, and inoculum was applied to the freshly cut stem, which was then punctured with an insect pin multiple times. CaLCuV and tomato golden mosaic virus symptoms were observed, and plants were harvested 14 to 21 days postinoculation. Due to an inherently longer latent period, BCTV symptoms were observed, and plants were harvested 21 to 30 days postinoculation. Symptomatic leaf tissue was harvested from N. benthamiana and sugar beet (24). Arabidopsis plants were inoculated within 5 days of bolting. Bolts were cut where they emerged from the rosette, and inoculum was applied to the freshly cut stem, which was then punctured with an insect pin multiple times. CaLCuV and tomato golden mosaic virus symptoms were observed, and plants were harvested 14 to 21 days postinoculation. Due to an inherently longer latent period, BCTV symptoms were observed, and plants were harvested 21 to 30 days postinoculation. Symptomatic leaf tissue was harvested from N. benthamiana and sugar beet (24).

DNA isolation and Southern blot analysis. DNA was isolated from homogenized plants samples frozen in liquid nitrogen using DNeasy columns (Qiagen), and 500 to 600 ng of genomic DNA from infected plants was restricted overnight. After digestion, 1 to 1.2% agarose gel electrophoresis was carried out, followed by overnight transfer, using standard protocols, onto Nytran Super Charge membrane. The blot was UV cross-linked (1,700 μJ) and hybridized overnight using a 32P-labeled full-length virus probe at 48°C in Ultralab hybridization buffer (Ambion). Random primer-based 32P labeling was carried out using Strip-EZ DNA (Ambion). Signal intensity was quantitated using a Phosphorimager (Bio-Rad Molecular Imager FX).

Bisulfite sequencing. The bisulfite sequencing method is based on Frommer et al. (16). DNA isolated from infected plant tissue was linearized overnight using appropriate restriction enzymes. Proteinase K digestion was subsequently carried out overnight, followed by bisulfite conversion using CT conversion reagent (EZ-DNA Methylation Gold; Zymo Research). Primers were designed against converted template, and the intergenic region of the viral genome was amplified by PCR. The PCR product was purified using Promega Wizard columns and TA cloned, and individual clones were sequenced at The Ohio State University Plant Microbe Genomics Facility. For conversion control, plasmids containing CaLCuV DNA or BCTV DNA were added to a vast excess of healthy plant DNA extract and treated with bisulfite reagent. The following forward and reverse primers were used to amplify CaLCuV and BCTV intergenic regions (IR) following bisulfite conversion: CaLCuV 2556 CF, GGGGATATGTTAAGAATATGGTTTGG (forward), and CaLCuV 539 CR, TCCCCACATATAAACAACCA (reverse); BCTV 2640 CF, GGGGATATGTTAAGAATATGGTTTGG (forward), and BCTV 147 CR, TCCCCCTCTTTATTAAACAATCAC (reverse). The chromatin immunoprecipitation (ChIP) protocol was based on the method described by Johnson et al. (29). Symptomatic Arabidopsis inflorescence or N. benthamiana leaf tissue (0.3 g) was cross-linked with formaldehyde for 20 min and then quenched with glycine for 10 min under vacuum. Tissue was then ground in liquid nitrogen and sonicated in lysis buffer under shearing conditions that resulted in fragments about 500 bp long (range, 250 to 1,000 bp). Protein A agarose beads and salmon sperm DNA were used for preclearing. Immunoprecipitation was carried out overnight at 4°C using commercially available antibodies (from Abcam or Upstate Biotechnologies) targeted to specific histone modifications. Cross-links were reversed at 65°C for 16 h, and then DNA was extracted using phenol-chloroform, followed by clean-up with Promega mini prep columns. Purified DNA (2 to 4 μl) was used as a PCR template with primers specific for viral DNA and a control sequence. Primers used for CaLCuV amplified exactly the same region used in the bisulfite PCR but were designed to original virus template and not bisulfite-converted template. The following forward and reverse primers were used to amplify Tnt, Ta3, and actin controls: TntF, CAGTTGTCCTCTAAAAGATGTTGCC (forward), and TntR, GAAATCTCATCTTTGCCTGC (reverse); Ta3F, GATTCTTACTGTAAAGAACATGGCATTGAGAGA (forward), and Ta3R, TCCAAATTTCTGAGGGTCTTGAAACC (reverse); actinF, CGTTTCGCTTTCCTTAGT and actinR, AGGCGAACGCGATCTAGAGACTCACCTTGG (reverse).

RESULTS

Methylation-deficient Arabidopsis mutants are hypersusceptible to geminivirus infection. We hypothesized that plant hosts use methylation as a defense against geminiviruses. A prediction of this hypothesis is that methylation-deficient mutant plants should display enhanced susceptibility to geminivirus infection. We tested this using Arabidopsis-infecting geminiviruses of two distinct genera. CaLCuV (genus Begomovirus) is a bipartite virus that produces an AL2 protein, and BCTV (genus Curtovirus) is a monopartite virus that encodes the related L2 protein. Arabidopsis mutants were selected to represent an array of defects in RNA-directed methylation pathway components, the methyl cycle, and downstream effectors (Fig. 1) (21, 54). The last group included the DNA methyltransferase mutants dml1 dml2, cmt3, and met1. The enzymes encoded by these genes carry out de novo and/or maintenance methylation of cytosine to 5-methyl cytosine in a variety of sequence contexts, including CG, CNG (where N is A, T, or C), or CHH (where H is A, T, or C). MET1 primarily maintains CG methylation, whereas the DRM1/2 and CMT3 methyltransferases are important for methylation at non-CG sites (3, 11). CMT3 additionally maintains CNG methylation in cooperation with H3K9 and H3K27 methyltransferases, indicating linkage between these epigenetic marks (26, 34, 35). Consequently, we also tested a histone H3K9 methyltransferase mutant (kyp2) for geminivirus sensitivity. RNA-directed methylation pathway mutants included plants lacking a functional chromatin remodeling enzyme (dml1) (27), the AGO4 protein (ago4) (65, 64), and a subunit common to RNA polymerases IVa and IVb (mrp2da) (23, 39). Selected methyl cycle activities included ADK and methionine methyltransferase. Arabidopsis has two ADK genes, and the loss of all ADK function is lethal (36).
Geminivirus

ssDNA

ds replicative form

Geminivirus minichromosome

Cellular heterochromatin

PolIVa

ssRNA

dsRNA

dCMB

siRNA duplex

AGO4

RNA directed DNA methylation

DDM1

DRD1

ddm1

CMT3

met1

H3K9 methylation

Histone methylation DNA methylation maintenance

FIG. 1. The methylation pathway in plants. A putative pathway for RNA-directed DNA methylation in Arabidopsis is illustrated. Genomic and viral genome targets may be transcribed by an RNA polymerase IVa complex (Pol IVa; containing NRPD1A and NRPD2). Resulting single-stranded RNA (ssRNA) is converted to dsRNA by complexes containing RDR2. The 24-nt siRNAs processed from dsRNA by DCL3 are loaded into complexes containing AGO4, which subsequently associates with Pol IVb (containing NRPD1B and NRPD2). The AGO4-associated siRNAs target the complex to homologous DNA sequences, where cytosine methyltransferases (e.g., DRM1/2) are recruited. Methylation also involves the SWI-SNF chromatin remodeling activities DRD1 and DDM1. Cytosine methyltransferases CMT3 and MET1 are primarily involved in methylation maintenance at CNG and CG sites, respectively. CNG methylation by CMT3 is also linked to H3K9 methylation carried out by KYP2.

However, it was possible to separately test adk1 and adk2 mutants, which are expected to have reduced levels of SAM and reduced global methylation. By contrast, mmt mutants show slightly elevated levels of SAM (30). We were unable to test mutants deficient for the methyl cycle enzyme S-adenosyl homocysteine hydrolase (hog1) (45) and the siRNA/miRNA methyltransferase HEN1 (hen1) (40) due to severe morphological defects. Otherwise, the selected mutants did not exhibit phenotypes that complicated observation of symptom development. However, the timing of flowering is known to be altered in Arabidopsis methylation mutants, hastening it in some but delaying it in others, depending on the ecotype (18). To control for differences in developmental timing, wild-type and mutant plants were infected after they had both undergone the vegetative to floral transition. Because of this, rosette tissue was very much reduced by the time symptoms appeared, making floral tissue a better choice than leaves to assess disease development. In all cases, mutant responses to infection were compared with wild-type plants of the same ecotype. Importantly, to observe a range of symptom enhancement, plants were inoculated under conditions that normally produce relatively mild symptoms (24, 50).

As summarized in Table 1, we found that methylation-deficient plants were highly susceptible to geminivirus infection and typically developed severe symptoms compared to infected wild-type plants. Major differences were manifest in the marked stunting and enhanced deformation of flowers and siliques. Each mutant was tested with both viruses, and in all cases, both viruses yielded similar results. CaLCuV typically causes curling of the inflorescence and of siliques near the inflorescence. BCTV infection additionally results in shortened internodes and outgrowths on the inflorescence stem with increased anthocyanin pigmentation. These symptoms were far more pronounced in most of the mutants, and extensive stunting and deformation of inflorescence structures were observed (Fig. 2).

Most susceptible were the methyltransferase mutants drm1, cmt3, and kyp2; the methylation pathway mutants ddm1, ago4, and nrd2a (polymerase IV); and the methyl cycle mutants adk1 and adk2 (Fig. 2 and Table 1). Symptom enhancement was less extreme in met1 mutant plants, suggesting that maintenance of CG methylation is less important than non-CG and H3K9 methylation in limiting infection. However, the plants used in these experiments were heterozygous for the

| TABLE 1. Methylation-deficient mutants are hypersusceptible to geminivirus infection |

<table>
<thead>
<tr>
<th>Gene function and mutation(s)</th>
<th>Relative severity of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyltransferase components</td>
<td></td>
</tr>
<tr>
<td>nrd2a (Pol IV)</td>
<td>++</td>
</tr>
<tr>
<td>ago4</td>
<td>++</td>
</tr>
<tr>
<td>ddm1</td>
<td>++</td>
</tr>
<tr>
<td>Cytosine methyltransferases</td>
<td></td>
</tr>
<tr>
<td>drm1</td>
<td>+++</td>
</tr>
<tr>
<td>drm2</td>
<td>+++</td>
</tr>
<tr>
<td>cmt3</td>
<td>+++</td>
</tr>
<tr>
<td>met1</td>
<td>+</td>
</tr>
<tr>
<td>H3K9 methyltransferase, kyp2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Methyl cycle enzymes</td>
<td></td>
</tr>
<tr>
<td>adk1</td>
<td>+++</td>
</tr>
<tr>
<td>adk2</td>
<td>+++</td>
</tr>
<tr>
<td>mmt</td>
<td>+</td>
</tr>
<tr>
<td>DCL and RDR enzymes</td>
<td></td>
</tr>
<tr>
<td>dcl2</td>
<td>+</td>
</tr>
<tr>
<td>dcl3</td>
<td>+</td>
</tr>
<tr>
<td>dcl4</td>
<td>+</td>
</tr>
<tr>
<td>dcl2 dcl3 dcl4</td>
<td>+</td>
</tr>
<tr>
<td>rdr2</td>
<td>+</td>
</tr>
<tr>
<td>rdr6</td>
<td>+</td>
</tr>
</tbody>
</table>

*Severity of disease symptoms in mutant Arabidopsis plants was based on the degree of stunting and floral deformation relative to wild-type plants of the appropriate ecotype (Col-0, Ler-0, or Ws-2) according to the following rating scale: +, typical, wild-type symptoms; ++, moderately enhanced; ++++, severe; ++++, very severe with extensive stunting. Pol IV, polymerase IV.
met1-7 allele, so the symptom phenotype could have been moderated by remaining MET1 activity. Slight enhancement of the methyl cycle conditioned by the mmt mutation had no apparent effect on the outcome of geminivirus infections. Importantly, no disease enhancement was noted when *Turnip crinkle virus*, an RNA virus, was used to infect *drm1 drm2* mutants (data not shown), indicating that the enhanced symptoms observed following geminivirus infection of mutant plants were not a general effect of reduced stress tolerance. Although Southern blot analysis of infected plant DNA obtained from floral tissues did not reveal increases in viral DNA levels beyond that observed in normal plant-to-plant variation (data not shown), the nature of the enhanced symptoms suggests that CaLCuV and BCTV are more meristem-invasive in methylation mutants than in wild-type plants. Further work is needed to confirm this.

These experiments provide strong genetic evidence that RNA-directed methylation of cytosine residues and H3K9

FIG. 2. Methylation-deficient mutant plants are hypersusceptible to geminivirus infection. Photographs are illustrative of geminivirus disease symptoms on wild-type and selected mutant plants. Plants were mock inoculated or inoculated with virus within 5 days of bolting, and symptoms were observed after 10 to 14 days for CaLCuV and 14 to 21 days for BCTV. Methylation-deficient mutants showed greater stunting and increased inflorescence deformation in response to geminivirus infection than wild-type (WT) plants of the same ecotype. (A) *Arabidopsis* Ler-0 and *cmt3* mutant plants inoculated with BCTV. (B) Col-0 and *adk1* mutant plants inoculated with CaLCuV. (C) Typical inflorescence structures of BCTV-infected *kyp2*, *cmt3*, and *ago4* mutants in the Ler-0 background. (D) Inflorescence structures of CaLCuV-infected *adk2*, *nrpd2a*, and *met1* mutants in the Col-0 background. (E) Inflorescence structures of BCTV-infected *ddm1*, *rdr2*, *rdr6*, *dcl2*, *dcl3*, *dcl4*, and *dcl2 dcl3 dcl4* mutants in the Col-0 background. (F) Inflorescence structures of BCTV-infected *drm1 drm2* and *mmt* mutants in the Ws-2 background.
plays an important role in defense against geminiviruses. Further, the sensitivity of adk mutants is in accord with AL2- and L2-mediated reduction of ADK activity during geminivirus infection (57, 58).

Plants deficient in DCL and RDR enzyme activities show moderately increased susceptibility to geminiviruses. In an attempt to identify the DCL and RDR enzymes most relevant to geminivirus defense, we examined additional mutant plants for susceptibility to CaLCuV and BCTV. These included individual and multiple Dicer mutants (dc12, dc13, dc14, and dc12 dc13 dc14) and two RDR polymerase mutants (rdr2 and rdr6). DCL3 generates the larger 24-nucleotide (nt) siRNAs associated with DNA methylation in plants (60). RDR2 is also involved in methylation whereas RDR6 has been linked to PTGS and defense against meristem invasion by certain RNA viruses (48, 60). We were unable to study the miRNA-associated Dicer (dc11) because morphological defects associated with this mutation complicated observation of viral disease symptoms.

As indicated in Table 1, little or no disease enhancement was seen in rdr2 and rdr6 mutants, suggesting either that de novo dsRNA synthesis does not play a significant role in geminivirus defense or that there is considerable functional redundancy between the several RDR proteins in Arabidopsis. Functional redundancy among the four Arabidopsis DCL enzymes in the biogenesis of endogenous siRNAs has been previously reported and was also apparent here in the response of dcl mutants to geminiviruses (Table 1) (17, 22, 60). The moderately enhanced susceptibility of dcl3 plants to BCTV and CaLCuV is consistent with the involvement of this enzyme in the methylation pathway but at the same time suggests that other DCL activities are able to partially fulfill its role. The sensitivity of dcl2 mutants is in keeping with the observation that geminivirus transcripts are targeted by PTGS and that this activity produces most of the PTGS-associated 22-nt viral siRNAs during geminivirus infection (1). The dcl4 mutants showed no obvious symptom enhancement, and this enzyme may play a little role in geminivirus defense when other DCL activities are present, at least in floral tissues. Interestingly, dcl2 dcl3 dcl4 triple mutants displayed only moderate symptom enhancement, suggesting a role for dcl1 when other Dicers are disabled. This is consistent with recent reports suggesting that all four DCL enzymes are involved in the biogenesis of virus-specific siRNAs against DNA viruses and that, in the absence of the others, DCL1 can generate small RNAs in response to CaLCuV (6, 37).

Geminivirus genomes are methylated in vivo. If methylation is a host defense, it should be possible to directly observe methylation of viral DNA in vivo. To assess methylation at high resolution, we carried out bisulfite sequencing of the CaLCuV genome, examining the IR that contains divergent viral promoters flanking the origin of replication. The experimental protocol involved treatment of DNA obtained from infected plants with bisulfite reagent to convert unmethylated cytosines to uracil, followed by PCR to amplify the viral strand of the IR. PCR products were cloned, and the sequences of 12 to 18 clones were compiled for each treatment.

Because any cytosine can potentially be methylated in plants, PCR primer design is critical. Primers that assume different amounts of cytosine conversion can differentially amplify relatively hypo- or hypermethylated templates. For CaLCuV we selected a primer set biased for templates with a relatively low density of methylation, which allowed us to examine the propensity for methylation at individual sites. The data generated showed evidence of methylation at some but not all cytosines in the IR (Fig. 3A) (see the supplemental material for primary sequence data). Methylation patterns appeared somewhat stochastic in that a larger sampling tended to yield more sites, but distinct patterns emerged. First, we observed that viral DNA methylation in wild-type Arabidopsis plants of different ecotypes (Ler-0, Ws-2, and Col-0) was variable in terms of the overall number of sites methylated (33 to 44% of the 79 cytosine residues surveyed) and that much of this difference could be attributed to lower levels of CG methylation in the Ws-2 ecotype (Fig. 3B). In addition, a larger proportion of non-CG relative to CG sites was methylated in all ecotypes. Similar methylation patterns were observed in CaLCuV IR DNA obtained from N. benthamiana plants although overall methylation was somewhat greater (~50%).

Interesting patterns could also be discerned at the level of individual cytosine residues (Fig. 3A). Methylation was detected at all but 12 of the 79 cytosines surveyed, and 10 were methylated in all three ecotypes. A region centered on the conserved hairpin showed a relatively high density of methylation in all ecotypes. In addition, a CNG site immediately adjacent to the directly repeated AL1 (replication initiator protein) binding sites was frequently methylated. A smaller region of high-density methylation adjacent to the coat protein start codon was also apparent. Importantly, several of the highly methylated cytosines were unmethylated or hypomethylated in DNA from the majority of the mutant plants tested (adk2, ago4, cmt3, drm1 drm2, and kyp2) (Fig. 3A).

Cytosine methylation levels in the IR of CaLCuV isolated from infected mutant plants are summarized in Fig. 3B. Reduced methylation in drm1 drm2 and cmt3 mutants confirms that these cytosine methyltransferases target viral DNA and is consistent with their known activities and partially overlapping functions (9). CHH methylation lacks a known maintenance mechanism and should be most sensitive to the drm1 drm2 mutations. Also as expected, viral DNA from cmt3 mutants showed greater reduction at CNG sites than CHH sites. CaLCuV DNA from kyp2 mutants displayed an equivalent reduction in CNG methylation, reinforcing the linkage between H3K9 methylation and CMT3-mediated maintenance of CNG methylation (26, 34, 35). The methylation pathway mutant ago4 showed significant reductions in both types of non-CG methylation, consistent with previous reports (63). The largest reduction in methylation at non-CG sites was observed in adk2 mutants, which underscores the role of ADK in maintaining the methyl cycle and its value as a target for the AL2 and L2 proteins. By contrast, most of the mutations did not lead to significant reductions in CG methylation, suggesting that the CG maintenance pathway is relatively robust.

Analysis of cytosine methylation in the BCTV genome. To further examine the correlation between cytosine methylation and enhanced susceptibility, studies were extended to include bisulfite sequencing of the BCTV IR from N. benthamiana, wild-type Arabidopsis Ler-0, and methylation-deficient mutants in this ecotype background (ago4, kyp2, and cmt3). In this case PCR primers used with bisulfite-treated DNA amplified a mixture of relatively densely and lightly methylated templates.
FIG. 3. The IRs of CaLCuV and BCTV are methylated in infected plants, and methylation levels are reduced in methylation-deficient mutants. Viral DNA isolated from floral tissues of infected wild-type plants or infected mutant plants was treated with bisulfite, and the IR was amplified by PCR. Amplified fragments were cloned, and 12 to 18 (CaLCuV) or 7 to 10 (BCTV) independent clones representing the encapsidated, viral strand were sequenced for each treatment. (A) The sequence of the CaLCuV IR, summarizing methylation detected in viral DNA isolated from wild-type plants of the ecotypes Ler-0, Ws-2, and Col-0, is shown. Methylated cytosines are indicated in red. Cytosines that were unmethylated in all three ecotypes are indicated in green. Cytosines methylated in 5 to 10% of the clones are additionally noted by a dot, and more frequently methylated cytosines (>10 to 20% of the clones) are marked by an asterisk. Sites that show reduced methylation (zero to one clone) in the mutants are underlined. The locations of the conserved geminivirus hairpin and the putative, imperfectly repeated AL1 binding sites (AGGGGAG/AGGAGAG) are shown. The locations of start codons for the divergent AL1 and coat protein genes are also indicated. (B) The histograms represent the percentages of cytosine sites methylated in different sequence contexts in CaLCuV IR DNA isolated from N. benthamiana (N. benth.) and Arabidopsis wild-type (Ler-0, Ws-2, and Col-0) plants and the indicated mutant plants (adk2, ago4, cmt3, drm1/2, drm2, and kyp2). Mutants are compared to the appropriate wild-type background. (C) Histograms represent the percentages of total cytosine residues methylated in different sequence contexts in BCTV IR DNA isolated from N. benthamiana and Arabidopsis wild-type Ler-0 plants, and ago4, kyp2, and cmt3 mutants.
More specifically, cytosine residues in individual clones tended to be either mostly unmethylated or mostly methylated, suggesting that populations of active and repressed dsDNA replicative forms coexist in infected plants. As a consequence, all but two of the cytosines (total examined, 44) were methylated in at least one of the 7 to 10 clones examined for all treatments. Thus, data obtained for BCTV did not permit analysis of methylation at individual sites, but it was possible to discern changes in methylation density.

As illustrated in Fig. 3C, the overall methylation density in the BCTV IR was similar in N. benthamiana and wild-type Arabidopsis Ler-0 plants, and in each species 50 to 55% of the total cytosines represented in the 10 sample clones were methylated (see the supplemental material for primary sequence data). However, the amount of CG methylation was relatively greater, and CNG methylation was lower in N. benthamiana than Arabidopsis plants. Similar to wild-type plants, virtually all cytosine residues in BCTV IR DNA obtained from ago4, kyp2, and cmt3 mutant plants was methylated in at least 1 of the 10 clones examined for each treatment. But the proportion of relatively undermethylated clones was greater, and viral DNA from mutant plants thus displayed significant reductions in methylation density (20 to 25%) in all sequence contexts (Fig. 3C).

Taken together, analysis of the CaLCuV and BCTV intergenic regions clearly indicated that hypersusceptibility of methylation-deficient Arabidopsis mutants to geminivirus infection was correlated with reduced cytosine methylation. Depending on the experimental conditions, either a reduced number of sites (Fig. 3B) or reduced methylation density (Fig. 3C) was observed.

ChIP reveals both active and repressive marks on geminivirus DNA. Geminivirus dsDNA replicative forms associate with histones and exist as minichromosomes. We also observed that kyp2 mutants show enhanced susceptibility to geminivirus infection. Hence, we used ChIP to investigate modification of histone H3 associated with the CaLCuV IR in wild-type N. benthamiana and Arabidopsis plants. Viral DNA was sonicated to an average size of 500 bp (range, 250 to 1 kbp) and, following cross-linking and immunoprecipitation with antibodies specific for different histone H3 modifications, a 405-bp fragment spanning the 305-bp IR was amplified to determine whether viral DNA was associated with the modified histones.

H3K4 is associated with active genes, and H3K9 is associated with gene silencing (28). Further, there is cross talk between histone and DNA methylation, as CMT3 interacts with histone H3 only when it is methylated at H3K9 and H3K27 (34). Using ChIP, we detected all three types of dimethylated histone H3 associated with CaLCuV DNA, and similar results were obtained with extracts from N. benthamiana and Arabidopsis (Fig. 4). Detection of H3K9 (and H3K27) is consistent with the enhanced susceptibility of kyp2 mutants and with the idea that these histone modifications are a component of defense against geminiviruses. In particular, H3K9 methylation appeared to be especially abundant in both host plants. On the other hand, H3K4 methylation and H3 acetylation are indicative of viral gene expression, which is to be expected during an infection. The presence of both active and repressive histone H3 marks within the viral control region again suggests that populations of active and repressed genomes are present in infected plants.

Recovery of Arabidopsis plants from infection with BCTV L2− mutant virus requires AGO4 and is associated with greatly increased cytosine methylation. Begomovirus AL2− mutants are not systemically infectious. By contrast, infections of N. benthamiana or Arabidopsis with BCTV or BCTV L2− null mutants proceed similarly in primary infected tissue (25). However, after removal of the primary infected shoots, new growth (secondary infected tissue) is symptomatic in BCTV-infected plants but exhibits recovery (no symptoms) in BCTV L2− mutant-infected plants. This recovery phenomenon is highly reproducible. In Arabidopsis, recovery rarely occurs in plants infected with wild-type BCTV but nearly always occurs in wild-type plants infected with the BCTV L2− mutant virus. Viral DNA is present in recovered tissue but in much reduced amounts.

To determine if methylation plays a role in recovery, wild-type Arabidopsis (ecotype Ler-0) and plants lacking the methylation pathway component AGO4 were inoculated with wild-type BCTV and two different BCTV L2− null mutants (L2-1 and L2-2) (25). Primary infected tissue of wild-type and mutant plants showed symptoms of infection, and as before the symptoms on ago4 mutant plants were considerably more severe (Fig. 2C). After removal of primary infected tissue, secondary
shoots of wild-type and ago4 plants infected with wild-type BCTV again showed severe symptoms (10 to 16 plants per treatment) (data not shown). As expected, wild-type plants infected with BCTV L2-1 or BCTV L2-2 mutant virus showed recovery, with little or no evidence of symptoms. Remarkably, however, ago4 mutant plants infected with these mutant viruses did not recover, and severe symptoms appeared in nearly all secondary shoots in all of the 16 plants inoculated with each mutant virus (Fig. 5A). The symptomatic secondary tissues in ago4 plants contained much higher levels of BCTV L2-1 and BCTV L2-2 DNA than asymptomatic, recovered secondary tissue from wild-type plants (Fig. 5B). We concluded that AGO4 is required for recovery, linking L2 function and recovery with the methylation pathway.

The BCTV IR in viral DNA isolated from secondary infected tissues was examined for cytosine methylation by bisulfite sequencing (see the supplemental material for primary sequence data). We found that BCTV L2-1 and BCTV L2-2 genomes obtained from recovered, secondary tissue of wild-type plants were hypermethylated. In the seven clones examined for each mutant virus, nearly all were highly methylated (about 80% of all cytosine residues examined) (Fig. 5C). Increased methylation was readily apparent in both non-CG and CG contexts.

**DISCUSSION**

In this report, several lines of evidence are presented to support the hypothesis that plant hosts use small RNA-directed methylation as a defense against geminiviruses. We demonstrate that *Arabidopsis* plants harboring inactivating mutations in cytokine methyltransferases (*drm1 drm2, cmt3, and met1*), histone H3K9 methyltransferase (*kyp2*), methylation pathway components (*ago4, ddm1, and nrpd2A*), or methyl cycle enzymes (*adk1 and adk2*) are hypersusceptible to infection with the geminiviruses CaLCuV and BCTV. We show that cytosine residues in viral genomes are methylated in infected plants and that methylation is significantly reduced in methylation-deficient mutant plants that display enhanced susceptibility. We present evidence for populations of viral minichromosomes carrying marks associated with repressed chromatin (H3K9) or active chromatin (acetylated H3) in infected plants. Finally, we demonstrate that BCTV L2+ mutant virus genomes are hypermethylated in tissue that has recovered from infection and that a methylation pathway component (AGO4) is required for *Arabidopsis* plants to undergo recovery. L2 is a silencing suppressor that interacts with and inhibits ADK (58). Thus, the absence of a viral pathogenicity factor that can oppose methylation is rendered redundant in plants containing a mutation that compromises methylation. In total, these observations indicate that plants employ an epigenetic mechanism, chromatin methylation, as a defense against DNA viruses.

Bisulfite sequencing of the CaLCuV IR, which was biased for relatively undermethylated clones, was revealing in several respects. First, it showed a greater proportion of non-CG methylation than CG methylation, as was previously observed within the C4 gene of *Tomato leaf curl virus* DNA from *N. benthamiana* plants (4). Further, cytosine residues in the vicinity of the conserved hairpin and AL1 binding sites were the most frequently methylated. The hairpin and AL1 binding sites lie within the divergent early and late gene promoters and in addition are integral components of the replication origin core (20). Thus, these sequences are potentially high-value targets for defensive purposes. Increased methylation in this critical viral control region and reduced density in more susceptible mutants are consistent with a role for methylation in defense and with earlier findings that methylation negatively impacts viral transcription and replication (8, 15). However, how these sites are targeted remains unclear. The hairpin has a duplex stem consisting of only 11 bp, and the core AL1 binding site direct repeats are approximately 7 bp; thus, transcripts across this region would not be expected to possess a great deal of repetitive character or secondary structure.
It is also interesting that most of the mutants tested did not reduce CG methylation in the CaLCuV IR, yet all of them showed extreme sensitivity to geminiviruses. This suggests that in the context of a virus infection, methylation at non-CG sites is relatively more important for defense than maintenance methylation at CG sites. In addition, the relative consistency of CG methylation suggests that its maintenance is robust and less sensitive to perturbations in de novo methylation. Another striking observation is that while the methylation mutants displayed extreme sensitivity to geminiviruses, overall methylation levels in the CaLCuV IR were reduced only about 10% to 20% (Fig. 3B). Thus, geminiviruses are able to exploit relatively small changes in cytosine methylation. The relatively high levels of residual methylation also prompt us to conclude that there are redundant pathways leading to cytosine methylation, as suggested by others (22, 63). Finally, in mutant plants, methylation was usually reduced in a manner consistent with known activities of the inactivated genes. For example, cmt3 and kyp2 mutants showed the largest reductions in CNG methylation, while CHH methylation was most affected in drm1 drm2 mutant plants (Fig. 3B). Thus, methylation of geminivirus chromatin appears to mirror host chromatin methylation in many respects, and it is likely that geminiviruses will prove to be useful models for the study of RNA-directed methylation pathways.

Bisulfite sequencing of the BCTV IR, which was biased toward more heavily methylated clones, was also revealing as it showed that viral genomes can be either mostly unmethylated or mostly methylated. This is in accord with the results of ChiP experiments, which showed that both active (acetylated H3) and repressive (H3K9) marks are associated with viral minichromosomes. Together, these results suggest that populations of active and repressed genomes are present in infected plants. Further, a larger proportion of relatively unmethylated clones was observed in DNA from mutant plants (ago4, kyp2, and cmt3), and overall cytosine methylation was reduced ~20 to 25%, confirming the correlation between enhanced susceptibility and reduced methylation. Conversely, most BCTV L2− mutant clones obtained from asymptomatic, recovered tissue were nearly completely methylated (~80% of cytosines methylated), underscoring the role of methylation in inhibiting geminivirus replication.

It bears repeating that differences in CaLCuV and BCTV IR methylation reported here likely stem from the PCR primers chosen for amplification of bisulfite-treated DNA, with the former primer set being biased for relatively unmethylated templates. Indeed, experiments with a different CaLCuV IR primer set produced a mixture of mostly undermethylated and mostly methylated clones, similar to what was observed with BCTV (see supplemental material). A truer picture of cytosine methylation awaits analysis using unbiased methods. However, given the differences observed between Arabidopsis ecotypes and N. benthamiana and the dramatic hypermethylation of viral DNA in recovered tissue, it is clear that viral cytosine methylation is dynamic. Factors that may influence methylation levels include the particular virus-host combination, tissue type, developmental age, environmental conditions, and the status of host defenses.

If plants employ both PTGS and methylation against geminiviruses, is it possible to evaluate the relative contributions of these pathways to antiviral defense? Earlier studies have shown that specific components of PTGS pathways, namely DCL4, RDR6, and SGS3, are required for silencing endogenous genes (virus-induced gene silencing) using geminivirus vectors (6, 38). In particular, these enzymes are required for geminivirus virus-induced gene silencing in newly developed leaves, suggesting that systemic spread of the PTGS signal elicited by geminiviruses involves a pathway that utilizes these enzymes. In addition, slight symptom enhancement was observed in dcl4, rdr6, and sgs3 mutant plants although viral DNA levels, at best, were only marginally enhanced. In contrast, we observed some enhancement of floral symptoms in dcl2 and dcl3 but not dcl4 mutants, and there were no obvious differences between rdr2 and rdr6 mutants and wild-type plants (Table 1). Given the functional redundancy that exists among the DCL and RDR proteins and our incomplete knowledge of antiviral silencing systems, we find it difficult to make definitive statements about the relative importance of specific pathways at this time. However, one could speculate that, in Arabidopsis, both the methylation pathway (likely involving DCL3 and RDR2) and a pathway involving DCL4/RDR6/SGS3 are important for limiting the spread of geminiviruses, with perhaps different roles in different tissues. The latter pathway may be more important for limiting geminivirus spread to shoot apical meristems, while the methylation pathway may play a greater role in preventing spread to secondary tissues (and conditioning recovery) and floral meristems. Further study is needed to answer these questions.

In summary, we conclude that geminivirus genomes are targeted for de novo methylation by both cytosine and histone methyltransferases and that geminivirus genomes will be useful models for examining RNA-directed methylation pathways. The antiviral nature of this methylation is clear from the hypersusceptibility of the methylation-deficient Arabidopsis mutants examined in this study and from the reductions in viral DNA methylation observed in the mutants. In addition, the reduced accumulation and hypermethylation of BCTV L2-1 and L2-2 mutant genomes in recovered tissue from wild-type plants and the absence of recovery in ago4 mutant plants provide compelling evidence that host defense pathways leading to recovery require AGO4-mediated methylation, which can be opposed by the BCTV L2 protein. Taken together, our genetic and biochemical data strongly support the hypothesis that plants use cytosine and histone methylation as an epigenetic defense against invading DNA virus genomes, which most likely results in transcriptional gene silencing or direct inhibition of replication. This methylation-based defense occurs in addition to PTGS, which results in degradation of viral mRNA.As. It follows that the geminivirus AL2 and L2 proteins, which are known to suppress PTGS and can inhibit methylation by inactivating ADK, may also be capable of interfering with transcriptional gene silencing, and preliminary evidence indicates that this is the case.

ACKNOWLEDGMENTS

We thank Kenn Buckley, Jim Carrington, Biao Ding, Erich Grotewold, John Lindbo, Laura Rush, Debbie Parris, and Garry Sunter for thoughtful discussions. We also thank the Arabidopsis Biological Resource Center, Jim Carrington, Steve Jacobsen, and Jack Morris for the gifts of mutant seeds. We are grateful to Kengo Morohashi for advice on ChiP experiments and Matt Balasco for technical assistance.


