Enhanced Viral Intergenic Region–Specific Short Interfering RNA Accumulation and DNA Methylation Correlates with Resistance Against a Geminivirus

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RNA silencing mediated by short-interfering RNA (siRNA) is used by plants as a defense against viruses. In the case of geminiviruses, viral DNA is targeted at the transcriptional level, while virus-derived transcripts are targeted by post-transcriptional silencing. *Mungbean yellow mosaic India virus* (MYMIV), a bipartite geminivirus, causes yellow mosaic disease in soybean (*Glycine max*). A soybean variety resistant to this disease has been identified (line PK416). To understand the molecular mechanism underlying this resistance, distribution of MYMIV-derived siRNAs along the viral genome was compared in resistant and susceptible plants, using samples obtained in the first few days following inoculation. We observed that, in the resistant soybean variety, most of the virus-derived siRNAs were complementary to the intergenic region (IR), while in the susceptible variety (line JS335), a majority of the siRNAs corresponded to coding regions of the viral genome. Most of the IR-specific siRNA molecules produced in the resistant plants were 24 nt in size. Bisulfite sequencing showed that, in the resistant plants, a higher level of methylation occurred in the IR of viral DNA.

Plants can inhibit virus infection using RNA silencing, an evolutionarily conserved set of mechanisms in which small RNAs direct the degradation or inhibit the translation of larger RNA molecules in a sequence-specific fashion (Baulcombe 2004; Carr et al. 2010; Llave 2010). Small RNAs are of two general types, depending on sources. MicroRNAs (miRNA, 21 to 22 nt) arise from non-protein coding parts of the genome, through formation of precursor RNAs followed by processing steps. On the other hand, short interfering RNAs (siRNAs, 21 to 24 nt) are produced by cleavage of double-stranded RNA (dsRNA) (Brodersen and Voinnet 2006; Xie et al. 2004). In the case of viral infection, dsRNAs are generated during virus replication, either by host or virus-derived RNA-dependent RNA polymerases, or by transcription through inverted repeats, or from converging promoters (Blevins et al. 2006; Shivaprasad et al. 2005). The siRNA mediates gene suppression by targeted RNA degradation (posttranscriptional gene silencing [PTGS]). In addition, plants adopt another mode of small RNA-mediated

resistance, referred to as transcriptional gene silencing (TGS), involving methylation of cytosine residues in the viral DNA and viral DNA-associated histones (Akbergenov et al. 2006; Daxinger et al. 2009; Dogar 2006; Mathieu and Bender 2004; Vanitharani et al. 2005).

Geminiviruses are a large and diverse group of plant viruses that infect a broad variety of economically important crops. These viruses have small genomes consisting of either one or two single-stranded circular DNA molecules. Genomes of bipartite geminiviruses consist of two single-stranded covalently closed circular DNA molecules, referred to as DNA A and DNA B. DNA A encodes a total of six proteins, of which four proteins are derived from the complementary-sense strand (AC1, AC2, AC3, and AC4) and two proteins (AV1 and AV2) from the virion strand. Proteins from the complementary strand are necessary for viral DNA replication and transcription, while AV1 is required for encapsidation. DNA B, on the other hand, encodes two proteins (BC1 and BV1), one each from complementary-sense and virion-sense strands. These two proteins are required for intra- and intercellular movement of the viruses. Bidirectional promoters are located in the intergenic regions (IR) between the leftward and the rightward transcription units in both DNA A and DNA B. DNA A and DNA B share a common region (CR) of about 200 nt located within the IR. The CR contains a conserved stem-loop motif required for replication-initiation. The viral genome essentially multiplies by a combination of rolling circle and recombination dependent replication in the nucleus via a double-stranded DNA intermediate. The double-stranded DNA intermediate also serves as a template for bidirectional transcription by the host RNA polymerase (Gutierrez 1999; Hanley-Bowdoin et al. 2000; Jeske 2009; Jeske et al. 2001; Preiss and Jeske 2003; Shivaprasad et al. 2005; Usharani et al. 2006). AC1 (Rep) is the main protein required for the initiation of virus replication (Nash et al. 2011). AC2 and AC4 proteins are known to play a role in the suppression of host-mediated gene silencing (Bisaro 2006). Some of the geminivirus-infected plants show disappearance of symptoms after a few days. Symptom recovery over time has been shown to correlate with the accumulation of siRNAs, viral DNA methylation, and PTGS against the protein-coding genes (Chellappan et al. 2004; Rodriguez-Negrete et al. 2009).

Mungbean yellow mosaic India virus (MYMIV), a bipartite geminivirus, causes yellow mosaic disease in a number of legume plants, such as soybean (*Glycine max*), blackgram (*Vigna mungo*), mungbean (*Vigna radiata*), and cowpea (*Vigna unguiculata*). Plants infected by MYMIV do not show symptom recovery (Boulton 2003; Varma and Malathi, 2003). We have

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earlier reported that a soybean variety resistant to MYMIV showed a rapid degradation of viral RNAs in comparison with a susceptible variety (Yadav et al. 2009). In this report, we compared distribution of the virus-derived siRNAs along the viral genome in the resistant and susceptible soybean varieties. We further demonstrate that, in the resistant variety, there is a higher level of IR-specific 24-nt siRNA accumulation and DNA methylation.

RESULTS

Distribution pattern of MYMIV-derived siRNA.

We reported earlier that agroinoculation of the two-leaf stage soybean seedlings with a single uncut recombinant binary plasmid containing tandem repeats of both DNA A and DNA B of MYMIV (pCAMBIA-A2B2) at the stem-node region resulted in appearance of yellow mosaic symptoms in all the infected plants of a susceptible variety (JS335) within 10 to 12 days. A

resistant variety (PK416), however, did not develop any yellow mosaic symptoms, even after 21 days postinfection (Yadav et al. 2009). For all the experiments described in this report, we collected hundreds of inoculated tissue samples at different timepoints after inoculation and bulked them into two parts for replicate experiments. Accumulation of the viral transcripts at different timepoints after inoculation was established by reverse transcription followed by polymerase chain reaction (RT-PCR). Parallel reactions lacking reverse transcriptase were performed to ensure that the origin of the PCR product was from RNA only. The early transcripts, AC1 (Rep) and AC2 (TrAP) appeared within 2 days postinoculation (dpi) in both the susceptible (JS335) and resistant (PK416) varieties. However, in the resistant variety, these transcripts disappeared after 6 dpi. The late transcripts (AV1, BC1, and BV1) appeared from the sixth day onwards in JS335, while in PK416, only the BV1 (NSP) transcript could be detected only at 6 dpi (Fig. 1A). To assess the ability of the host plants to produce the virus-spe-

Fig. 1. Accumulation of *Mungbean yellow mosaic India virus* (MYMIV) transcripts and MYMIV DNA A-specific siRNA in the soybean varieties JS335 and PK416. **A**, Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to detect the accumulation of viral gene-derived transcripts. Total RNA was extracted from the inoculated tissues of the plants agroinoculated with pCAMBIA-A2B2 at 0, 2, 4, 6, 8, and 10 days postinoculation (dpi). Total RNA (100 ng each) was used for RT, followed by PCR amplification. PCR (25 cycles) was performed to view the product in each case. RNA extracted from 15 dpi from the inoculated tissues of JS335 agroinoculated with empty vector (pCAMBIA 1305.1) was used as the mock. RNA extracted from the pCAMBIA-A2B2-inoculated JS335 leaves showing yellow mosaic symptoms after 15 dpi was used as positive control sample (+ control). Reactions with reverse transcriptase (+RT) or with water replacing reverse transcriptase (–RT) were performed for each sample to ensure that the products were derived from RNA only. Amplification of actin transcript was kept as internal control. **B**, Northern hybridization shows accumulation of the virus-derived siRNA in the soybean varieties. Low molecular weight RNA (20 µg) isolated from inoculated stem nodes of JS335 and PK416 agroinoculated with pCAMBIA-A2B2 or vector only (mock) at 6 dpi was separated on a 15% polyacrylamide and 7 M urea gel. RNAs transferred on a membrane were hybridized with radiolabeled full-length DNA A molecule as a probe. Size of the standard oligonucleotides is mentioned. Ethidium bromide–stained gel is shown for equivalent loading.

cific siRNA synthesis, we hybridized the low molecular weight (LMW) RNAs isolated from the inoculated plants at 6 dpi with the DNA A molecule as a probe. Figure 1B shows that both varieties produced viral DNA-specific 21 to 24-nt small RNAs. Higher accumulation of the siRNAs in the susceptible variety (JS335) was most probably due to multiplication and movement of the viral DNA in the surrounding cells.

To investigate the distribution of virus-derived siRNAs along the MYMIV genomic components, 10 PCR fragments, each of 275 bp, were generated covering the entire DNA A. The PCR fragments were immobilized on a membrane and were hybridized with radiolabeled small RNAs isolated from inoculated tissues of the susceptible (JS335) or resistant (PK416) variety at 6 dpi. Equal amounts of radiolabeled

Fig. 2. Distribution pattern of the virus-derived siRNAs along the DNA A and DNA B components of *Mungbean yellow mosaic India virus* (MYMIV) in JS335 and PK416 varieties. Reverse Northern hybridization was performed to explore siRNA distribution. A total of 20 polymerase chain reaction products covering the entire DNA A and DNA B molecules of MYMIV were transferred to nylon membrane and were hybridized with 5' end-labeled siRNA isolated from pCambia-A2B2 and mock-inoculated stem nodes of JS335 and PK416 at 6 days postinoculation (dpi). Hybridization signals were quantified using the Quantity One software (Bio-Rad, Hercules, CA, U.S.A.) and were plotted in a bar diagram. The relative percent intensities of the signals were estimated and the highest intensity value was scored as 100%. **A**, Autoradiograms show the viral DNA A-specific siRNA accumulation in JS335 (i) and in PK416 (ii). Relative percent accumulations of DNA A-specific siRNA in JS335 (iii) and in PK416 (iv) are shown in a bar diagram. **B**, Autoradiograms show viral DNA B-specific siRNA accumulation in JS335 (i) and in PK416 (ii). Relative percent accumulations of DNA B-specific siRNA in JS335 (iii) and in PK416 (iv) are shown in a bar diagram. As control, membranes were deprobed and hybridized with siRNAs isolated from the mock-inoculated JS335 and PK416. Ethidium bromide-stained DNA gels are shown for equivalent loading.

small RNAs from the two varieties were used for hybridization. It is evident from Figure 2A that the majority of the siRNAs generated in JS335 were complementary to the genomic component covering the overlapping region of pre-coat protein (AV2) and coat protein (AV1). Considerable amounts of siRNAs were also targeted against the overlapping regions of AC2 (TrAP) and AC3 (REn) and AC1 (Rep) and AC4. The region of TrAP that induced more siRNA accumulation encodes a domain that is rich in cysteines and histidines and might form zinc finger to mediate DNA binding. This region also overlaps with Rep-binding domain of REn (Hanley-Bowdoin et al. 2000). Similar accumulation of siRNAs against a region of Rep that encodes a domain for AC3 (REn) interaction and oligomerization was also detected. In contrast, most of the siRNAs were targeted against the IR in PK416. Much lower amounts of siRNAs were also produced against the early transcripts like AC2/AC3 (TrAP/REn) and AC1 (Rep). The same experiment was carried out to determine siRNA distribution along the DNA B molecule. Ten PCR fragments, each of 267 bp covering the entire DNA B molecule, was hybridized with radiolabeled small RNA. In JS335, it was observed that majority of the siRNAs was targeted against BV1 (nuclear shuttle protein). A much lower amount of siRNA against the IR and other regions was observed in JS335. Surprisingly, most of the DNA B-specific siRNAs in PK416 were targeted against the CR in IR (Fig. 2B). The presence of NSP/BV1-specific siRNAs corroborated the accumulation of BV1 transcript in the resistant variety.

The major difference between the two varieties in this regard is that most of the siRNA produced in the resistant variety were targeted against the IR. Therefore, fine mapping of the IR was performed to locate the precise region that induced siRNA formation in both varieties. DNA A and DNA B contain IR of 275 and 972 nt in length, respectively, which share a CR about 200 nt long. Five PCR fragments, each of 55 bp, were generated covering the entire IR of DNA A and were hybridized with the radiolabeled small RNAs purified from infected tissues at 6 dpi. Differential signal intensities against the different IR-derived fragments were observed. Interestingly, the highest hybridization signal was obtained against a fragment representing the loop and one strand of the stem in the CR. Surprisingly, in the resistant variety, only this region of the IR registered a strong hybridization signal, without any detectable signals from the surrounding regions (Fig. 3A).

The IR of DNA B is 972 nt in length. In the previous experiment (Fig. 2B), it was shown that, of three PCR fragments covering most of the IR, only one fragment (position 2,551 to 146) was hybridized with siRNA with a strong signal. Therefore, only that region was studied further. Five PCR fragments (three of 53 bp and two of 54 bp) were generated to cover this 267-nt region and were hybridized with the radiolabeled small RNA. As in the case of IR of DNA A, the susceptible variety exhibited differential signal intensities against all five PCR products. Interestingly, in contrast to that of DNA A, the lowest signal was registered for the stem-loop region (Fig. 3A) in the susceptible variety. On the other hand, only this region showed a strong hybridization signal in the resistant one (Fig. 3B).

Size and relative concentration of siRNA.

Plants produce three distinct classes of siRNAs (21, 22, and 24 nt) against geminiviruses (Akbergenov et al. 2006). PTGS and TGS are often correlated with the appearance of 21- to 22- and 24-nt siRNAs, respectively. Therefore, we looked into the relative abundance of different classes of siRNAs in the two varieties at 6 dpi, using high-resolution Northern blots. We could detect two classes of the virus-derived siRNA, one of 21

to 22 nt and the other of 24 nt in size. The coding regions of the viral genome induced accumulation of mostly the smaller class (21 to 22 nt) of siRNAs, while the majority of IR-specific siRNAs were 24 nt in size (Fig. 4A). Concentrations of gene-specific siRNAs were higher in the susceptible than in the resistant plants, and this could be correlated to the corresponding transcript levels in both the varieties. In contrast, accumulation of siRNAs corresponding to both the IR of DNA A and DNA B was much higher in the resistant plants. Appearance of both the 21- to 22- and 24-nt siRNAs against the viral genes suggested that, in addition to PTGS, TGS-mediated mechanism also work for the viral gene silencing. It has been demonstrated with the *Pepper golden mosaic virus* (PepGMV) that DNA methylation also occurred in the coding region of coat protein (Rodriguez-Negrete et al. 2009).

Distribution of methylated cytosines in IR.

Accumulation of the 24-nt siRNA is considered as a hallmark of transcriptional gene silencing. Previous studies have reported that a higher proportion of viral DNA was methylated in the plant tissues showing symptom recovery (Rodriguez-Negrete et al. 2009). Therefore, bisulfite sequencing was performed to study the correlation between the presence of 24-nt siRNA and DNA methylation at the IR. To identify the methylated cytosines, both the methylated and nonmethylated virion strands corresponding to the IR of DNA A were amplified after bisulfite treatment of the DNA isolated from the inoculated plant tissues at 4 dpi. The IR of DNA B could not be amplified from the resistant samples, even after repeated effort, possibly because of a very low level of DNA B replication in that variety. The IR of DNA A could not be amplified after 4 dpi in the resistant variety. Clones from each of the samples ($n = 20$) were sequenced. Of the sequenced clones from the resistant samples, 70% showed methylation at any of the cytosines, in contrast to only 30% in the case of the susceptible samples. Sequence analysis showed that the cytosines were methylated both in the symmetric (CG/CNG) and asymmetric (CHH) positions. Out of the 65 cytosines present in the DNA A IR, a total of 22 have been methylated in different molecules in the resistant variety, while only eight sites were methylated in the DNA molecules amplified from the susceptible variety (Fig. 5B). This difference was due to methylation of more cytosine residues in the CNG and CHH contexts (Fig. 5C). While looking at the positions of DNA methylation, the resistant sample showed an even distribution of methylated cytosines along the IR, as opposed to the methylation pattern shown by the susceptible sample. Viral DNA isolated from the susceptible samples showed three methylation sites at the extreme left and right parts of the IR and two sites just after the stem-loop region (Fig. 5A). In the resistant variety, one methylation site was found just after the conserved nonanucleotide, which functions as origin of replication. The part of the DNA A IR that functions as promoter for the rightward transcription units also showed a relatively higher methylation frequency in the resistant variety (Fig. 5A).

DISCUSSION

In this report, we conducted a comparative study between two contrasting varieties of soybean in the context of RNA interference against a geminivirus that causes a nonrecovery type of infection. We used two soybean lines, one susceptible and one resistant to MYMIV infection, and followed the virus-specific siRNA accumulation and viral DNA methylation after inoculation with MYMIV agroinoculation construct. The contrasting siRNA distribution patterns along the viral genomic components between the susceptible and resistant varieties could result from

different reasons. For the transcribed regions, kinetics and accumulation levels of viral transcripts are different in two varieties. The virus produces more early and late viral transcripts in the susceptible variety. Accordingly, more siRNAs against the

early and the late viral transcripts were produced in this variety. These siRNAs were not evenly distributed along the coding regions. This phenomenon has also been reported in cases of infection with *African cassava mosaic virus* (ACMV), *East*

Fig. 3. Distribution pattern of virus-derived siRNA along the intergenic region (IR) of DNA A and DNA B components of *Mungbean yellow mosaic India virus* (MYMIV) in JS335 and PK416. A total of 10 polymerase chain reaction products covering the entire DNA A and DNA B IR were blotted to nylon membrane and were hybridized with 5' end-labeled siRNA isolated from pCAMBIA-A2B2 and mock (pCAMBIA 1305.1)-inoculated stem nodes of JS335 and PK416 after 6 days postinoculation (dpi). Hybridization signals were quantified using Quantity One software and were plotted in a bar diagram. The relative percent intensities of the signals were estimated, and the highest intensity value was scored as 100%. **A.** Autoradiograms showing DNA A IR (IR-A)-specific siRNA accumulation in JS335 (i) and PK416 (ii). A bar diagram shows relative percent accumulation of IR-A-specific siRNA in JS335 (iii) and PK416 (iv). **B.** Autoradiogram shows the DNA B IR (IR-B)-specific siRNA accumulation in JS335 (i) and in PK416 (ii). A bar diagram shows relative percent accumulation of IR-B-specific siRNAs in JS335 (iii) and PK416 (iv). As control, blots were deprobed and hybridized with siRNAs isolated from the mock-inoculated JS335 and PK416. Ethidium bromide-stained DNA gels are shown for equivalent loading.

African cassava mosaic Cameroon virus, and *PepGMV*. Accumulation and distribution patterns of the siRNA vary with viruses and their interactions with the host plants (Chellappan et al. 2004; Rodriguez-Negrete et al. 2009). In our experiments, we found three regions in DNA A were the preferential targets of siRNA accumulation. Interestingly, all three of these regions encode overlapping open reading frames. Two of them cover the overlapping regions between AC2 and AC3 (TrAP and REn), and AC1 (Rep) and AC4 encode the Rep-REn interaction domain and TrAP DNA-binding domain, which are important for viral replication. This might be one of the reasons that these sequences seem to be the preferred targets. Alternatively, as discussed previously, these sequence segments might be the preferred targets for the dicers, and these siRNAs cause more cleavage to these transcripts to produce more siRNA from the same region or might act as primers to synthesize the complementary strands to amplify silencing signals (Chellappan et al. 2004). In the susceptible variety, DNA B-specific siRNAs were mostly targeted to the NSP transcript, as in the case of ACMV infection. However, EACMCV and *PepGMV* infections produce more siRNAs against the movement protein transcript (Chellappan et al. 2004; Rodriguez-Negrete et al. 2009).

The distribution patterns of siRNAs along the IR are surprisingly different in the susceptible versus the resistant variety. In the susceptible variety, the whole IR was the target of RNA interference, although the stem-loop region was the major target. Contrastingly, the stem-loop regions within the IR of both DNA A and DNA B were the only detectable targets in the resistant variety. Comparison of siRNA accumulation also showed that the resistant variety produced more IR-specific siRNA, of which the major proportion was of 24 nt in size. This indicated that

transcriptional gene silencing of the IR was more profound in the resistant variety. The higher frequency of IR DNA methylation in the resistant variety corroborated this observation. However, the IR-specific DNA methylation patterns did not correlate with the distribution of siRNAs along this region in both the varieties. This suggested that RNA interference independent of the known mechanisms might also contribute to silencing in this region. Nonetheless, the enhanced IR-specific siRNA accumulation and DNA methylation correspond to the undetectable viral DNA replication in the resistant variety after 4 dpi (Supplementary Fig. 1).

Two natural sources of resistance against the yellow mosaic disease were used in soybean breeding programs in India. One was a cultivated variety (*Glycine max* PI171443/UPSM534) from China and the other was a wild soybean (*Glycine soja*). Inheritance studies suggested that the resistance in PI171443, one of the parents of PK416, was governed by two recessive loci *rym1* and *rym2* (Singh and Malick 1978). In this report, we tried to delineate the mechanism of resistance through RNA interference in one of the natural sources. Our results demonstrated that the genomic regions of MYMIV containing the origin of replication were profoundly targeted for transcriptional gene silencing in the resistant variety.

MATERIALS AND METHODS

Plant inoculation and tissue harvest.

Soybean (*Glycine max*) seeds were germinated at 26°C in the glass house with a 16-h light and 8-h dark photoperiod. Soybean plants at the two-leaf stage were agroinoculated at the stem nodes with pCAMBIA-A2B2, as described previously

Fig. 4. Sizes and relative quantities of the virus-specific siRNAs produced by the soybean plants after inoculation with *Mungbean yellow mosaic India virus* (MYMIV) construct. **A**, Low molecular weight RNA isolated from the inoculated stem nodes of JS335 and PK416 were separated on a 15% polyacrylamide and 7 M urea gel and were blotted to a nylon membrane. Membranes were hybridized with radiolabeled probes derived from the DNA A intergenic region (IR-A), DNA B IR (IR-B), coat protein (AV1), TrAP (AC2), Rep (AC1), and NSP (BV1). Synthetic oligonucleotides were used as standard markers. Ethidium bromide-stained gel is shown for equivalent loading. **B**, A bar diagram shows relative signal intensities of the different classes of siRNAs. The lowest intensity value was scored as one, and the rest of the values were normalized accordingly. The white bars designate the 24-nt siRNAs, while the 21-nt siRNAs are designated by the black bars.

(Yadav et al. 2009). *Agrobacterium tumefaciens* harboring only pCambia 1305.1 was used for mock inoculation. Infected nodal tissues were collected at different timepoints after inoculation for nucleic acid isolation.

Total RNA isolation and LMW RNA extraction.

Total RNA from agroinoculated plant tissues was isolated using Tri reagent (Sigma, St. Louis), following the manufac-

Fig. 5. Distribution of methylated cytosines along the intergenic region of *Mungbean yellow mosaic India virus* (MYMIV) DNA A (IR-A). Total DNA isolated from the inoculated stem nodes of JS335 and PK416 was treated with bisulfite and the IR-A-specific DNA fragment was amplified by polymerase chain reaction using virion strand-specific primers. The amplified DNAs were cloned and sequenced. **A**, MYMIV IR-A sequence shows the positions of methylation in JS335 and PK416. Locations of various conserved domains in the IR-A are shown, and the methylated cytosines are highlighted in gray. **B**, Comparative distributions of methylated cytosines along the IR-A in JS335 and PK416 are shown as bars. Each bar represents the position and frequency of methylated cytosine. **C**, Histogram represents the percentage of methylated cytosine in the different sequence contexts (with respect to total C/CG/CNG/CHH) in JS335 and PK416.

turer's instructions. For extraction of LMW RNA, total RNA was added to polyethylene glycol (molecular weight 8,000) and sodium chloride to the final concentrations of 5% and 500 mM, respectively. Samples were incubated on ice for 30 min and were centrifuged at $7,900 \times g$ for 20 min at 4°C. Supernatant containing the LMW RNA was transferred to new tubes. To precipitate LMW RNA, $1/10$ volume of 3 M sodium acetate, and 3 volumes of cold ethanol were added to the supernatant and were incubated at -20°C for 4 h. The precipitated RNA was collected by centrifugation at $19,100 \times g$ for 30 min. The RNA pellet was washed twice with 80% ethanol to remove salt. The pellet was air dried and dissolved in nuclease free water.

RT-PCR.

Total RNA was isolated as mentioned previously and was digested with DNase I enzyme to remove DNA contamination. The first-strand cDNA was synthesized with 100 ng of total RNA, random primers, 10 mM each dNTP, buffer, and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) by incubating at 50°C for 1 h. First-strand cDNA (1 µl) was used as the template for PCR amplification of the viral transcripts with the gene-specific primers for AC1 (AC1f: 5'-A TGCCAAGGGATGGTCGTTTTCG-3', AC1r: 5'-TCAATTC GAGAGCGTCGAATTG-3'), AC2 (AC2f: 5'-ATGAAGAGAA CAATGCTGCGCTTAAAC-3', AC2r: 5'-TTACGGAAGATC GATAAGATCATCCC-3'), AV1 (AV1f: 5'-ATGCCAAAGCG GACCTTCGATACTG-3', AV1r: 5'-TTAATTCATATCGAAT CG-3'), BC1 (BC1f: 5'-ATGGAGAATTATTCAGGAGCAGT CG-3', BC1r: 5'-TTACAACCTGTTTGTTCAC-3') and BV1 (BV1f: 5'-ATGTTTAACCGGAATTATCG-3', BV1r: 5'-CTT AACTTTATGCAACCTATT-3'). Actin (GenBank: VO0450) was used as the internal control, using the primers ACTf (5'-TATTGAACCCCTTGTTCGAC-3') and ACTr (5'-CTGTG GACAATGGATGGGCC-3'). To ensure that the PCR products were derived from the mRNA only, parallel reactions lacking reverse transcriptase were also performed.

Detection of virus specific siRNA.

Virus-specific siRNAs were detected by Northern blotting. LMW RNA (20 µg) isolated from the pCambia-A2B2 and mock-inoculated soybean plants were denatured at 65°C in loading dye and were separated on a 15% polyacrylamide (19:1) and 7 M urea gel. Separated RNAs were transferred to nylon membrane according to a procedure described by Sambrook and Russell (2001). Different MYMIV-derived DNA fragments were used as probes in the study. The full-length DNA A probe was amplified by using 5'-GGATCCATTGTTGAACGA CTTTCC-3' and 5'-GGATCCCACATTGTTAGTGGGTTTC-3' primers. Viral genome-derived A1, A2, A6, A9, B1, and B9 DNA fragments were used as probes corresponding to the IR of DNA A (IR-A), coat protein (AV1), transcription activator protein (AC2), replication protein (AC1), nuclear shuttle protein (BV1), and IR of DNA B (IR-B), respectively. Probes were radiolabeled with [α^{32} P] deoxycytidine triphosphate (dCTP) using Megaprime DNA labeling system (Amersham Biosciences, Piscataway, NJ, U.S.A.). Hybridization with the radiolabeled probes was performed overnight at 42°C. Ethidium bromide-stained 21- and 24-nt long synthetic oligonucleotides were used as standard size markers.

Preparation of MYMIV-specific PCR fragments.

Primers (20 sets) were designed for amplification of 10 fragments (275 bp each) of DNA A and 10 fragments of DNA B (267 bp each) to cover the entire genomic length of MYMIV. Similarly, for amplification of the IR of DNA A (IR-A) and DNA B (IR-B), 10 sets of primers were used to amplify five

fragments (55 bp each) corresponding to IR-A and five fragments (53 or 54 bp) corresponding to IR-B. Sequence of all the primers and their positions in the viral genome are listed in Supplementary Table 1. PCR was carried out using the viral genomic DNA clone as the template and *Taq* DNA polymerase (Sigma).

Isolation, labeling of siRNA, and reverse Northern blot analysis.

Small-RNAs were isolated and radiolabeled following the methods described previously (Rodriguez-Negrete et al. 2009). Briefly, LMW RNAs were isolated from pCambia-A2B2 and mock-inoculated stem nodes of JS335 and PK416. About 100 µg of LMW RNA were separated on 15% denaturing polyacrylamide gel. The region corresponding to 21 and 24 nt was sliced out from the gel and was crushed in a centrifuge tube having elution buffer (80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl). Tubes were kept overnight at shaking conditions for optimum recovery of RNA. Eluted siRNA was transferred to a new tube and 1 volume of isopropanol and 1 µl of 20 mg/ml glycogen were added for precipitation. The pellet was dissolved in 400 µl of 20 mM Tris-HCl, pH 7.5, and 300 mM sodium acetate. siRNA in the solution was again precipitated by adding 3 volumes of cold ethanol, followed by centrifugation at $19,100 \times g$ for 20 min. Precipitated siRNA was air dried and dissolved in nuclease free water. For radiolabeling, 1 µg of siRNA was first dephosphorylated with shrimp alkaline phosphatase (Roche, Indianapolis, IN, U.S.A.) and was labeled with [γ^{32} P] ATP in the presence of T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.). The blots containing DNA fragments were hybridized with labeled siRNA at 42°C overnight. The images were scanned using phosphorimager, and signals were quantified by using the image analysis software (Quantity One; Bio-Rad, Hercules, CA, U.S.A.).

Bisulfite sequencing.

Bisulfite sequencing procedure was carried out as described previously (Raja et al. 2008; Rodriguez-Negrete et al. 2009). Total DNA isolated from infected tissues was linearized with Not I endonuclease, followed by proteinase K digestion. Bisulfite conversion of genomic DNA was carried out using EZ DNA methylation-gold kit (Zymo Research, Irvine, CA, U.S.A.) following manufacturer's instructions. Virion strand-specific primers were designed from IR of DNA A (MethCRAF: 5'-GGGATATGTCAAGAAATAGTTTTTTG-3', MethCRAR: 5'-AACTTGCTGCAAATACTTTATT-3') to amplify both the methylated and nonmethylated viral DNA. The amplified products were cloned into pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.) following manufacturer's instructions. Cloned DNA fragments were sequenced to identify converted and nonconverted cytosines. To check the efficiency of the method and degree of conversion of nonmethylated cytosines, plasmid containing viral DNA A was mixed with the noninfected soybean genomic DNA and was used as control.

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LITERATURE CITED

Askbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., Zhang, P., Gruissem, W., Meins, F., Jr., Hohn, T.,

- and Pooggin, M. M. 2006. Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic Acids Res.* 34:462-471.
- Baulcombe, D. 2004. RNA silencing in plants. *Nature* 431:356-363.
- Bisaro, D. M. 2006. Silencing suppression by geminivirus proteins. *Virology* 344:158-168.
- Blevins, T., Rajeswaran, R., Shivaprasad, P. V., Beknazariants, D., Si-Ammour, A., Park, H., Vazquez, F., Robertson, D., Meins Jr., F., Hohn, T., and Pooggin, M. M. 2006. Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res.* 34:6233-6244.
- Boulton, M. I. 2003. Geminiviruses: Major threats to world agriculture. *Ann. Appl. Biol.* 142: 143.
- Brodersen, P., and Voinnet, O. 2006. The diversity of RNA silencing pathways in plants. *Trends Genet.* 22:268-280.
- Carr, J. P., Lewsey, M. G., and Palukaitis, P. 2010. Signaling in induced resistance. *Adv. Virus Res.* 76:57-121.
- Chellappan, P., Vanitharani, R., Pita, J., and Fauquet, C. M. 2004. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* 78:7465-7477.
- Daxinger, L., Kanno, T., Bucher, E., van der Widen, J., Naumann, U., Matzke, A. J. M., and Matzke, M. 2009. A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. *EMBO (Eur. Mol. Biol. Organ.) J.* 28:48-57.
- Dogar, A. M. 2006. RNAi dependent epigenetic marks on a geminivirus promoter. *Virology J.* 3:5.
- Gutierrez, C. 1999. Geminivirus DNA replication. *Cell Mol. Life Sci.* 56:313-329.
- Hanley-Bowdoin, L., Settledge, S. B., Orozco, B. M., Nagar, S., and Robertson, D. 2000. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit. Rev. Biochem. Mol. Biol.* 35:105-140.
- Jeske, H. 2009. Geminiviruses. *Curr. Top. Microbiol. Immunol.* 331:185-226.
- Jeske, H., Lutgemeier, M., and Preiss, W. 2001. DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *EMBO (Eur. Mol. Biol. Organ.) J.* 20:6158-6167.
- Llave, C. 2010. Virus-derived small interfering RNAs at the core of plant-virus interactions. *Trends Plant Sci.* 15:701-707.
- Mathieu, O., and Bender, J. 2004. RNA-directed DNA methylation. *J. Cell Sci.* 117:4881-4888.
- Nash, T. E., Dallas, M. B., Reyes, M. I., Buhrman, G. K., Ascencio-Ibanez, J. T., and Hanley-Bowdoin, L. 2011. Functional analysis of a novel motif conserved across geminivirus Rep proteins. *J. Virol.* 85:1182-1192.
- Preiss, W., and Jeske, H. 2003. Multitasking in replication is common among geminiviruses. *J. Virol.* 77:2972-2980.
- Raja, P., Sanville, B. C., Buchmann, R. C., and Bisaro, D. M. 2008. Viral genome methylation as an epigenetic defense against geminiviruses. *J. Virol.* 82:8997-9007.
- Rodriguez-Negrete, E. A., Carrillo-Tripp, J., and Rivera-Bustamante, R. F. 2009. RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J. Virol.* 83:1332-1340.
- Sambrook, J., and Russell, D. W. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Shivaprasad, P. V., Akbergenov, R., Trinks, D., Rajeswaran, R., Veluthambi, K., Hohn, T., and Pooggin, M. M. 2005. Promoters, transcripts, and regulatory proteins of Mungbean yellow mosaic geminivirus. *J. Virol.* 79:8149-8163.
- Singh, B. B., and Malick, A. S. 1978. Inheritance of resistance to yellow mosaic in soybean. *Indian J. Genet. Plant Breed.* 38:258-261.
- Usharani, K. S., Periasamy, M., and Malathi, V. G. 2006. Studies on the activity of a bidirectional promoter of Mungbean yellow mosaic India virus by agroinfiltration. *Virus Res.* 119:154-162.
- Vanitharani, R., Chellappan, P., and Fauquet, C. M. 2004. Geminiviruses and RNA silencing. *Trends Plant Sci.* 10:144-151.
- Varma, A., and Malathi, V. G. 2003. Emerging geminivirus problems: A serious threat to crop production. *Ann. Appl. Biol.* 142:145-164.
- Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E., and Carrington, J. C. 2004. Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* 2:642-652. Published online.
- Yadav, R. K., Shukla, R. K., and Chattopadhyay, D. 2009. Soybean cultivar resistant to Mungbean yellow mosaic India virus infection induces viral RNA degradation earlier than the susceptible cultivar. *Virus Res.* 144:89-95.