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Tomato Chlorotic Mottle Virus Is a Target of RNA Silencing but the Presence of Specific Short Interfering RNAs Does Not Guarantee Resistance in Transgenic Plants[▽]

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Tomato chlorotic mottle virus (ToCMoV) is a begomovirus found widespread in tomato fields in Brazil. ToCMoV isolate BA-Se1 (ToCMoV–[BA-Se1]) was shown to trigger the plant RNA silencing surveillance in different host plants and, coinciding with a decrease in viral DNA levels, small interfering RNAs (siRNAs) specific to ToCMoV-[BA-Se1] accumulated in infected plants. Although not homogeneously distributed, the siRNA population in both infected *Nicotiana benthamiana* and tomato plants represented the entire DNA-A and DNA-B genomes. We determined that in *N. benthamiana*, the primary targets corresponded to the 5′ end of AC1 and the embedded AC4, the intergenic region and 5′ end of AV1 and overlapping central part of AC5. Subsequently, transgenic *N. benthamiana* plants were generated that were preprogrammed to express double-stranded RNA corresponding to this most targeted portion of the virus genome by using an intron-hairpin construct. These plants were shown to indeed produce ToCMoV-specific siRNAs. When challenge inoculated, most transgenic lines showed significant delays in symptom development, and two lines had immune plants. Interestingly, the levels of transgene-produced siRNAs were similar in resistant and susceptible siblings of the same line. This indicates that, in contrast to RNA viruses, the mere presence of transgene siRNAs corresponding to DNA virus sequences does not guarantee virus resistance and that other factors may play a role in determining RNA-mediated resistance to DNA viruses.

The geminiviruses infect a broad range of plants and cause important crop losses worldwide (44, 75). They are named after their typical twinned icosahedral capsids and carry single-stranded circular DNA genomes. The genomic DNA replicates in infected cell nuclei through double-stranded DNA intermediates, using a combination of rolling circle and recombination-dependent replication (29, 32, 35, 53). The family *Geminiviridae* is divided into four genera based on the genome structure, insect vector, and host plant. Members of the largest genus, *Begomovirus*, are all transmitted by the whitefly *Bemisia tabaci* (Gennadius) and infect dicotyledonous plants. Most begomoviruses, including all New World begomoviruses, have their genome divided into two components, denoted DNA-A and DNA-B, that respectively encode genes involved in expression/replication/encapsidation and movement. In both components, the viral strand and its complement encode open reading frames (ORFs), separated by an intergenic region (IR). This region includes the common region (CR), the only part of the genome highly identical between DNA-A and DNA-B of the same virus species. The IR harbors the origin of replication and promoter sequences for the transcription of viral genes (50). DNA-A encodes the AV1 (coat protein [CP]) in the viral sense and four genes in the complementary sense. AC1 (Rep) encodes the only protein strictly essential for virus replication (25, 50), AC2 (TrAp) is a transcriptional factor acting on the promoters of viral sense genes (64) and has been shown to act as a silencing suppressor for a number of begomoviruses (71, 73, 74, 76, 77), and AC3 (REn) is a replication-enhancing factor (45, 66). For the AC4 protein no function had been assigned until it was implicated as an RNA silencing suppressor for two cassava-infecting begomoviruses (73). DNA-B encodes two genes involved in virus movement. BV1 is the nuclear shuttle protein (NSP) engaged in viral DNA transport from the nucleus to the cytoplasm, whereas BC1 (MP) is responsible for cell-to-cell and systemic movement (27, 48, 60, 61). Geminivirus transcription is bidirectional and can be quite complex, frequently giving rise to convergent multiple polycistronic RNAs that overlap at their 3′ ends (50, 65, 71).

RNA silencing is an evolutionary conserved process that is active in a wide variety of eukaryotic organisms and can lead to the inhibition of transcription or translation of a target gene in a sequence-specific manner (6, 24). A key role in this process is played by short interfering RNA molecules (siRNAs) of 21 to 26 nucleotides (nt) (30) that are the result of the cleavage of longer double-stranded RNAs (dsRNAs) by Dicer (10). The strands of the siRNAs are unwound, and one of the strands is retained in the RNA-induced silencing complex (RISC) (31), where it guides the RISC to a complementary mRNA target (41, 69). Transcriptional silencing as a result of methylation of DNA sequences homologous to siRNAs has also been observed, suggesting siRNAs may be active in the nucleus (14, 39, 79). Biological functions of RNA silencing in plants include the

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TABLE 1. Primers used to amplify the ToCMoV-[BA-Se1]-derived fragments

Fragment	Primer	Primer sequence (5′–3′)[a]	PCR fragment size (bp)
A1	A1F	GGGGAGTGGCATATTTG	379
	A1R	GGGGCMTCMCKCTTAGRCAT	
A2	A2F	CCCGTCGACATGYCTAAGMGKGAKGCCCC	360
	A2R	GATTTCTGCAGTTDATRTTYTCRTCCATCCA	
A3	A3F	GCATCTGCAGGGATGGANGANAANATNAA	420
	A3R	CGCAACAGACAGACAATATG	
A4	A4F	TGCGAATCGAACAGCTCTAAT	440
	A4R	ATGCGCAATTCATCTT	
A5	A5F	GCGCATTGTGCTTTGTCCT	320
	A5R	GCATCTGCAGACNGGRAAGACRATGTGGGC	
A6	A6F	GCATCTGCAGGCCCACATYGTCTTYCCNGT	731
	A6R	TAGGATCCATGCCACTCCCCAGACATTTC	
B1	B1F	GGAGGAACAACCAACTGAGAA	392
	B1R	TAGGATCCATGGCCGCCACGTGTGT	
B2	B2F	CTCGTTCACACACGTGG	464
	B2R	CGACGCTTGTTACCATTGAA	
B3	B3F	ATGTATTTCAATGGTAACAAGCGTCG	435
	B3R	ATGTGTACAGACTGCCGGAA	
B4	B4F	CGGCAGTCTGTACACATTCG	346
	B4R	ATTATCCAATATAGTCAAGGTC	
B5	B5F	ACTGGTCGACGCGGCCGCAYCTBGAYTATDTYGG	464
	B5R	GTAGGTTATGGGTCATGGGA	
B6	B6F	CCTACATGAACGAAATCGATATCC	526
	B6R	ATGGTACCGCGGCCGCATGRRDTCTCAGYTDG	
RC	GWAttb1-AC1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGACGTCTGAGGAGCTCTTAG	938
	GWAttb2-AV1R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTAATACCGTTACCACGTGT	

[a] N = A, C, G, or T; M = A or C; Y = C or T; B = C, G, or T; D = A, G, or T; K = G or T; R = A or G.

regulation of endogenous gene expression, heterochromatin formation, repression of transposable elements, and defense against virus infection (8, 82).

Plant cells infected with RNA viruses have been shown to produce virus-specific siRNAs, which were suggested to originate from the breakdown of dsRNA replicative forms or from secondary structures of the viral RNA (30, 43). DNA plant viruses such as caulimoviruses and geminiviruses are also targets of RNA silencing (2, 16, 40). In some cases this response can lead to the recovery of the plants from the virus symptoms (2, 16, 19); therefore, it was suggested that RNA silencing is a natural defense mechanism that protects plants from viral invasion (19, 55). Indeed, harnessing this RNA silencing by generating siRNA-producing transgenic plants has proven to be a potent means to engineer resistance to RNA viruses (7, 28, 54, 58). Also, for DNA viruses such as geminiviruses, biotechnological approaches using transgenic plants expressing sense and antisense RNA have been used successfully (26, 38).

Tomato chlorotic mottle virus is a typical New World begomovirus that is widespread in tomato fields in Brazil (56). We investigated here the RNA silencing response generated in plants against Tomato chlorotic mottle virus–[Bahia-Seabra1] (ToCMoV-[BA-Se1]) infection in terms of production and the origin of virus-specific siRNAs. Subsequently, the potential to generate begomovirus resistance in plants by using the most targeted sequences from the virus genome was explored.

MATERIALS AND METHODS

Plant inoculation. For virus inoculation, carborundum-dusted leaves of *Nicotiana benthamiana*, tomato (*Lycopersicon esculentum*), and petunia (*Petunia hybrida*) were rubbed with the extract of ToCMoV-[BA-Se1]-infected *N. benthamiana* plants prepared by grinding infected leaf tissue (1:10 [wt/vol]) in phosphate buffer (pH 8.0) containing 25 mM EDTA and 0.01% sodium sulfite. The inoculum source was obtained by bombarding *N. benthamiana* plants with ToCMoV-[BA-Se1] DNA-A and DNA-B infectious clones (57) and kept by subsequent mechanical inoculation of healthy plants.

Preparation of ToCMoV-[BA-Se1]-specific PCR fragments and in vitro transcripts. The ToCMoV-[BA-Se1] DNA-A and DNA-B components were PCR amplified in a series of six fragments for each component (A1 to A6 and B1 to B6) using the appropriate primer pairs (Table 1) and full-length infectious clones as templates.

ToCMoV-[BA-Se1]-derived PCR fragments A3, A4, and A6 (Table 1) were cloned into the pGEMT-Easy vector (Promega), and viral and complementary sense in vitro transcripts were prepared by T7 or SP6 RNA polymerases using the m-Message m-Machine kit (Ambion).

The PCR fragments or transcripts were separated on native agarose gel or a denaturing formaldehyde gel, respectively. The gels were blotted onto positively charged nylon membrane (Hybond N^+; Amersham) and probed with ^{32}P-labeled purified (67) low-molecular-mass RNA isolated from infected *N. benthamiana* or tomato plants.

Extraction of total nucleic acids and isolation of low-molecular-mass RNA. The extraction of total nucleic acid from plant leaves was done according to the method of Bucher et al. (12). Briefly, leaf material was ground in liquid nitrogen and mixed with extraction buffer (2% Sarkosyl–5 M NaCl). After phenol extraction, polysaccharide contaminants were precipitated by adding 3 M sodium acetate. The supernatant was ethanol precipitated and resuspended in Tris-EDTA (TE). To separate DNA and larger RNA molecules from low-molecular-mass RNAs, a polyethylene glycol precipitation step was performed (30). The pellet containing DNA and longer RNA was resuspended in TE. The supernatant containing the low-molecular-mass RNAs was precipitated with ethanol and resuspended in TE.

Preparation and labeling of siRNAs. For purification of small RNAs from infected and healthy *N. benthamiana* and tomato plants, about 30 μg of low-molecular-mass RNAs was fractionated in a 15% denaturing polyacrylamide gel containing 8 M urea. After staining with ethidium bromide, the region containing the small RNAs was excised from the gel, cut in small pieces, and incubated in 3 M NaCl overnight at 4°C to allow diffusion. After centrifugation, the supernatant was precipitated by ethanol (16). The small RNAs (approximately 1 μg) were dephosphorylated with alkaline phosphatase and labeled with ^{32}P by T4

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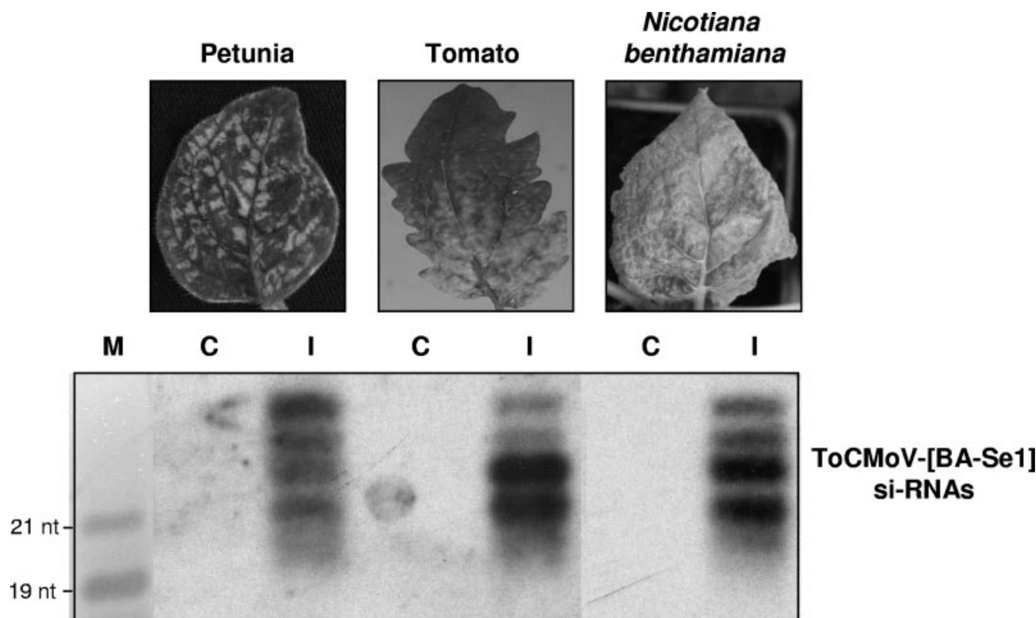

FIG. 1. Symptoms of ToCMoV-[BA-Se1] infection in petunia, tomato, and *N. benthamiana*, and Northern detection of virus-derived siRNAs in these hosts using a viral DNA-A specific probe. Low molecular RNAs were extracted from virus-infected plant (I) or mock inoculated control (C) plants. Synthetic siRNA molecules were used as size references (M).

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polynucleotide kinase using [γ-^{32}P]ATP according to the manufacturer's instructions (Invitrogen).

Construction of plant expression vectors. To evaluate the potential of RNA silencing for the control of ToCMoV-[BA-Se1], an intron-hairpin construct was generated containing the virus sequences that were most highly targeted by RNA silencing during virus infection in *N. benthamiana*. A fragment of 938 bp (denoted RC) was amplified by PCR from ToCMoV-[BA-Se1] DNA-A with *Pfu* DNA polymerase using the primers GWAttb1-AC1F and GWAttb2-AV1R (Table 1). The RC fragment consists of 300 nt of the 5′ end of the AV1 gene (including the end of the overlapping AC5 ORF) the entire common region and 300 nt of the 5′ end of the AC1 gene (including a part of the nested AC4 gene). The recombinant plasmids were obtained by using the GATEWAY system (Invitrogen) according to the manufacturer's instructions. The RC fragment was introduced by BP recombination into pDonr 207 and subsequently into the destination binary vector pK7GWIWG2 (37) by LR recombination by which it was inserted downstream of a cauliflower mosaic virus 35S promoter in an inverted repeat array, separated by an intron, producing the binary plant expression vector pIR-RC. After we confirmed the presence of the sense and antisense arms of the inverted repeat and the orientation of the intron by restriction enzyme digestion, pIR-RC was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Plant transformation and evaluation. Leaf discs of *Nicotiana benthamiana* plants were transformed by using standard protocols (70). Kanamycin-resistant regenerated shoots were rooted and transferred to soil. Transgene integration in the R_0 plants was verified by PCR. PCR-positive plants were self-fertilized, and the progenies of these plants (T1) were sown on selective Murashige and Skoog medium containing kanamycin (100 mg/liter). Green seedlings with well developed roots were potted and evaluated for virus resistance.

Eleven transgenic lines were tested for virus resistance. Ten seedlings from each transgenic line were mechanically inoculated as described above. Wild-type *N. benthamiana* plants were inoculated as positive controls. The plants were monitored regularly and scored for symptoms. Virus infection was confirmed by squash or tissue blot analysis at 20 days postinoculation (dpi) and 45 dpi using PCR fragment A3 (Table 1) labeled by random priming with ^{33}P as a probe.

Accumulation of viral DNA forms was detected by Southern blot analysis. Approximately 3 μg of total DNA was fractionated on a 1% agarose gel and blotted onto nylon membrane. Viral DNA was detected by Southern hybridization with ^{33}P-labeled whole ToCMoV-[BA-Se1] DNA-A and DNA-B as probes.

Northern and Southern blot analysis. For Northern blotting, 12 μg of low-molecular-mass RNA was separated on a 15% denaturing polyacrylamide gel and probed with digoxigenin-labeled A4 fragment (Table 1) for low-molecular-weight RNA molecules isolated from infected *N. benthamiana*, petunia, and tomato plants or random-primed [^{33}P]dCTP-labeled RC fragment (Table 1) for transgenic plants. For size reference, synthetic siRNA molecules (Eurogentec, Seraing, Belgium) were used.

A total of 15 μg of total RNA extracted from transgenic plants at 0 dpi was separated on a 1% denaturing formaldehyde-agarose gel and hybridized with a 35S terminator-derived digoxigenin-labeled probe and a [^{33}P]dCTP-labeled RC fragment. For Southern blotting, 10 μg of genomic DNA from transgenic plants was digested with HindIII, separated on a 1% agarose gel, blotted onto a nylon membrane, and probed with ^{33}P-labeled RC fragment. Hybridization using Church's buffer (59) was carried out at 48 and 65°C for Northern or Southern blotting, respectively.

RESULTS

ToCMoV-[BA-Se1] infection triggers RNA silencing in plants. Although in nature ToCMoV-[BA-Se1] has thus far been found only in tomato plants, experimentally this begomovirus infects a range of different host plants, including *N. benthamiana* and petunia. Mechanically inoculated tomato plants show chlorotic spots, vein chlorosis, and crumpling, followed by yellow mottling starting about 14 dpi (Fig. 1). *N. benthamiana* plants develop vein chlorosis, crumpling and epinasty starting at 7 to 10 dpi, followed by systemic mottling and petunia exhibited vein chlorosis, chlorotic spots, concentric rings, and chlorotic mottling (Fig. 1).

Northern blot analysis of low-molecular-mass RNAs extracted from systemically infected leaves from two plants using a ToCMoV-[BA-Se1] DNA-A-derived probe showed the accumulation of virus-specific siRNAs of between 21 and 24 nt. The presence of virus-specific siRNAs indicates that ToCMoV-[BA-Se1] infection activates the RNA silencing machinery, and ToCMoV-[BA-Se1] mRNAs were targeted by RNA silencing in all three plant species tested (Fig. 1). Although over time the accumulation of virus-specific

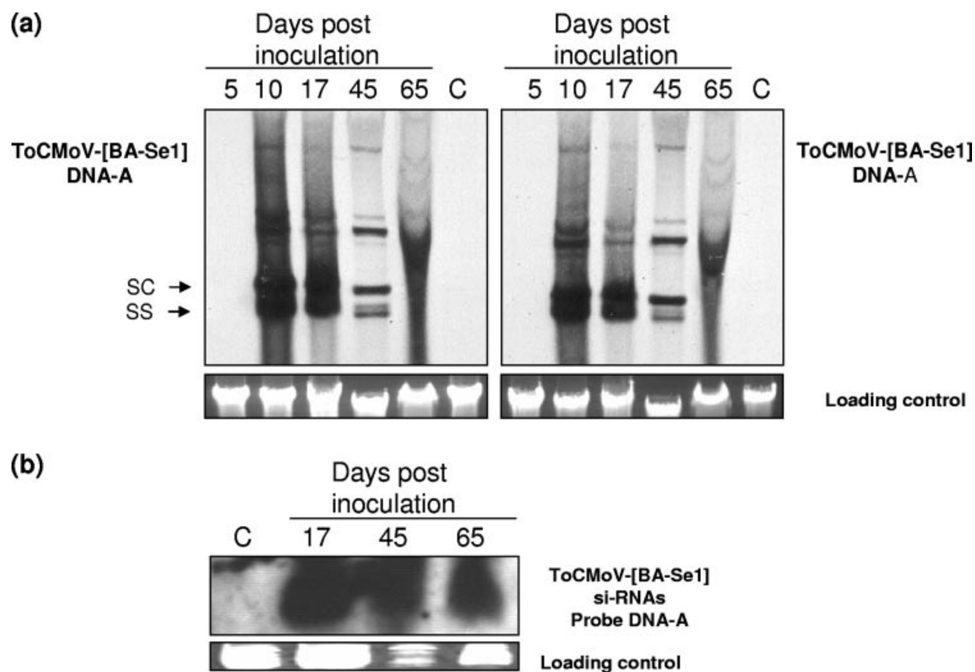

FIG. 2. Time course analysis of ToCMoV-[BA-Se1] DNA and virus-derived siRNA accumulation in infected *N. benthamiana* plants. (a) Viral DNA was extracted at different days postinoculation and detected by Southern hybridization using either DNA-A (left panel) or DNA-B-derived probe (right panel). ToCMoV-[BA-Se1] single-stranded (SS) and supercoiled double-stranded (SC) DNA forms are indicated. (b) Northern blot of ToCMoV-[BA-Se1]-specific siRNAs at different time points after inoculation. C, mock-inoculated control.

siRNAs appeared to be relatively stable in *N. benthamiana*, the amount of viral DNA decreased dramatically. This may indicate that targeting ToCMoV-[BA-Se1] mRNAs interferes with viral replication and viral DNA accumulation, since both replicative dsDNA and single-stranded DNA (ssDNA) forms are reduced (Fig. 2). At 65 dpi, it was difficult to visualize viral DNA forms; this is probably also due to the senescent stage of the plant and presence of compounds that interfere with the gel electrophoresis, thus making it difficult to compare with the DNA amounts at 45 dpi (Fig. 2).

siRNAs representing the entire genome of ToCMoV-[BA-Se1] are generated in infected plants. To determine whether all genes and noncoding sequences in the bipartite genome are targeted by virus-induced silencing, PCR-amplified fragments covering the entire genome were prepared. Using appropriate primers, a series of six fragments was derived from each viral component (Fig. 3). Blotted fragments were probed with labeled small RNA molecules purified from ToCMoV-[BA-Se1]-infected *N. benthamiana* or tomato plants (Fig. 3). These analyses revealed that siRNA populations isolated either from infected *N. benthamiana* or tomato plants represented the whole bipartite genome. Interestingly, these included both the coding and the intergenic regions. For the DNA-A, the fragments A6 (5′ end of the AC1 gene and the entire embedded AC4 gene), A1 (AC1 leader sequence, the intergenic region including the common region, the AV1 leader sequence, and the overlapping 3′ end of AC5), and A2 (5′ end of AV1 and overlapping middle part of AC5) seemed to be the more abundantly targeted portions, whereas segment A5 (3′ end of the AC1 gene) was under-represented in the siRNA population from *N. benthamiana* (Fig. 3a). Labeled siRNAs isolated from tomato hybridized predominantly to segments A6 and A4 (AC2 and AC3 sequences), whereas the differences among the rest of the segments were less pronounced (Fig. 3a). For the B component, the siRNAs isolated from both plants seem to accumulate in high amounts and were spread about equally over the entire viral genomic sequence. Corrected for the amounts of PCR fragment loaded on the gel (Fig. 3b), the B2 and B1 fragments also seem to bind considerable amounts of siRNA. The identification of siRNAs matching to the intergenic region might be due to the size of the PCR fragments used in the experiments. Since the PCR fragments used include stretches of surrounding transcribed regions, this does not necessarily indicate the targeting of the promoter and origin of replication. A finer mapping of this region using smaller PCR fragments would be required to indicate if the promoter and origin of replication sequences are actually targeted by the RNA silencing machinery. As expected, strong hybridization signals for all fragments were obtained when the same blot was probed with either ToCMoV-[BA-Se1] DNA-A or DNA-B (Fig. 3). No signal was present when the blots were reprobed with labeled low-molecular-weight RNAs from noninfected plants (Fig. 3).

ToCMoV-[BA-Se1]-specific siRNAs originate from diverse dsRNA sources. Geminivirus transcripts in opposite orientations are known to overlap, producing dsRNA at the 3′ ends of the AV1 and AC3 genes (16). However, it is not known whether these overlapping transcripts can be elongated to some extent by the host RNA-dependent RNA polymerase reported to be involved in RNA silencing (20). To gain further information on this question, we have identified the polarity of

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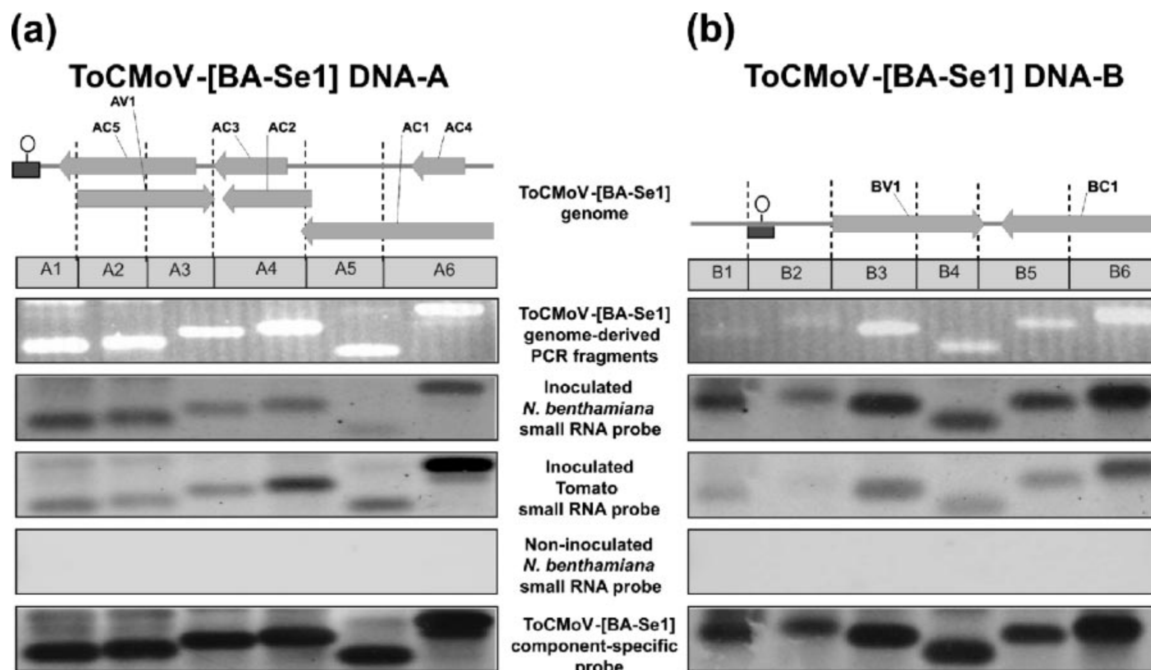

FIG. 3. Origin and distribution of siRNAs from ToCMoV-[BA-Se1]-infected *N. benthamiana* and tomato plants. The bipartite genome of ToCMoV-[BA-Se1], consisting of DNA-A (a) and DNA-B (b), is schematically depicted. PCR fragments covering the viral genome were obtained and probed with 5′-labeled small RNAs extracted from inoculated *N. benthamiana* and tomato plants. As controls, the same blots were also hybridized with 5′-labeled small RNAs extracted from noninoculated *N. benthamiana* and with probes derived from the complete DNA-A or DNA-B components of ToCMoV-[BA-Se1].

ToCMoV-[BA-Se1]-specific siRNAs derived from DNA-A segments A3 and A4 that are known to overlap, as well as the more distal segment A6. In vitro transcripts in both viral and complementary sense were produced and probed with labeled low-molecular-mass RNAs from infected tomato plants. As anticipated, an approximately equal ratio between viral and complementary strand-derived siRNA was obtained for A3 and A4 (Fig. 4), indicating that RdRp may play a role in elongating the overlapping transcripts of AV1 and AC3/AC2. However, for the segment A6 a much higher proportion of siRNAs hybridized to the viral sense transcript (Fig. 4), demonstrating that they are mainly—but not exclusively—derived from the complementary sense. This may indicate that they are primarily derived from the secondary structure of the AC1/AC4 mRNA.

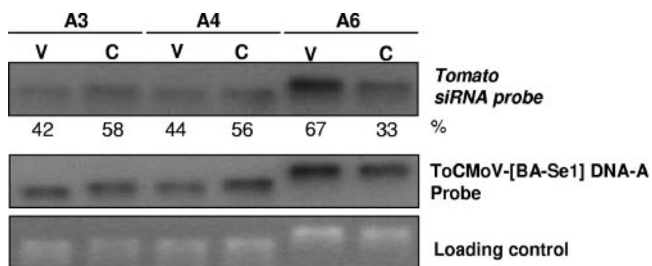

FIG. 4. Polarity of siRNAs accumulating in infected tomato plants corresponding to different ToCMoV-[BA-Se1] DNA-A-derived transcripts. Fragments A3, A4, and A6 were transcribed and 100 μg of transcripts in viral (V) and complementary sense (C) were blotted and probed with 5′-labeled siRNAs extracted from ToCMoV-[BA-Se1]-infected tomato plants. The blot was quantified by using Gene Tools software, and the numbers below each lane corresponds to the relative amounts of siRNAs for the respective polarity of a fragment. As a control, the same blot was hybridized with a ToCMoV-[BA-Se1] DNA-A-labeled probe.

Enhancing the plant RNA silencing system to increase virus resistance. From the experiments described above it could be concluded that as a response to infection with ToCMoV-[BA-Se1] the plant produces virus-specific siRNAs, the occurrence of these siRNAs coinciding with decreased viral DNA accumulation.

To enhance the plant's antiviral RNA silencing potential, transgenic *N. benthamiana* plants were produced that expressed a virus-derived intron-hairpin construct. Thus, siRNAs would be produced prior to virus infection. This strategy has been demonstrated to be highly efficient and has been widely used to obtain host resistance against RNA viruses (18, 63, 78, 81).

Segments from the ToCMoV-[BA-Se1] DNA-A that were shown to be most abundantly targeted by RNA silencing in *N. benthamiana* (Fig. 3) were cloned into the intron-hairpin binary destination vector pK7GWIWG2 (37) by using the GATEWAY technology. The inverted repeat construct, pIR-RC, comprised the 300 nt at the 5′ end of the AC1 gene (and part of the nested AC4 gene), the entire common region, and the 300-nt 5′ end of the AV1 gene (including the 3′ end of the AC5 ORF) (Fig. 5).

This construct was used for plant transformation, resulting in 48 independent transgenic *N. benthamiana* lines. Proper insertion of the entire transgene sequence was confirmed by PCR analysis (not shown). All plants developed a normal phenotype.

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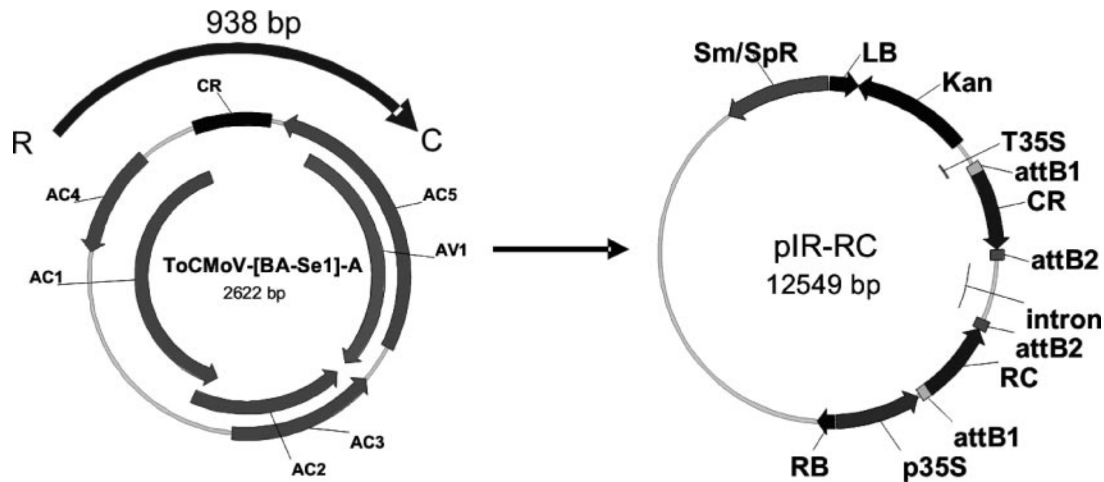

FIG. 5. Genetic map of ToCMoV-[BA-Se1] DNA-A showing the origin of the fragment amplified and cloned into the plant expression vector pIR-RC used for *N. benthamiana* transformation.

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Performance of transgenic plants challenged with virus. Initial inoculation experiments were performed with the T1 progeny of 11 transgenic *N. benthamiana* lines. Northern blot analysis of low-molecular-weight RNA extracted from leaves from noninoculated transgenic plants of three lines hybridized with a probe derived from the transgene, indicating that the transgene had been transcribed and converted into siRNAs (not shown). Ten plants of each line were inoculated with ToCMoV-[BA-Se1] at the five- to six-leaf stage. In wild-type plants used as controls, symptoms of crumpling and chlorotic veins started to appear at 7 to 8 dpi. At 14 dpi, all control plants were showing systemic symptoms. Most transgenic lines showed a delay in symptom expression of at least 8 days compared to wild-type plants. Later, however, many transgenic plants started to show symptoms. By 45 dpi, five lines still had plants without symptoms, although virus could be detected in some of the symptomless plants by dot blot analysis (not shown).

The challenge experiment with the lines RC-8.1, RC-11.1, RC-19.3, RC-20.1, and RC-24.2, showing enhanced resistance, was repeated, and similar results were obtained. At 12 dpi, all inoculated nontransgenic control plants exhibited systemic symptoms, whereas the onset of the disease was delayed for the transgenic plants (Fig. 6a). At 20 dpi a large number of transgenic plants were still symptomless, although viral DNA could be detected in some on them (Fig. 6b). The symptoms displayed by infected susceptible transgenic plants were very similar to those displayed by wild-type plants, although stunting was often less pronounced (Fig. 6c). At 45 dpi, the best-performing lines were RC-24.2, with 50% of the plants resistant to virus infection (no symptoms and no virus), and RC-19.3, with 50% of the plants symptomless, including 30% entirely virus-free plants (Fig. 6a and b).

siRNA levels in transgenic plants do not correlate with resistance. A more detailed analysis of symptomatic and symptomless siblings of lines RC-24.2 and RC-19.3, as judged by visual observation at 45 dpi, was performed, including Northern analysis of transcripts and siRNA at three time points during the infection (0, 20, and 45 dpi). Southern analysis was performed to estimate the transgene copy number and viral DNA accumulation at 45 dpi. Southern blot analysis showed that siblings within a transgenic line had the same number and pattern of integrated copies, one in the case of RC-19.3 and two in the case of RC-24.2 (not shown). Transgene-specific siRNA was readily detected in all transgenic plants before virus inoculation (0 dpi) by Northern analysis (Fig. 7a). Except for plant 17 of line RC-19.3, which clearly contained a higher amount of siRNA, all of the plants assayed displayed similar siRNA levels. The generation of siRNA from the double-stranded transgenic mRNA in the plants seemed to be highly efficient, since transgene transcripts could not be detected by Northern analysis using either a probe corresponding to the 35S terminator or a transgene-derived probe. However, transcripts could be amplified by reverse transcription-PCR after DNase treatment of the sample (data not shown).

The amount of siRNAs that hybridize with the probe (fragment RC) increased greatly at 20 dpi (Fig. 7a). The origin of the siRNAs can be from either the transgene or the virus, but the fact that no virus was detected in several plants that nonetheless have elevated siRNA levels suggests that the siRNAs arise from the transgene. At this time point only transgenic plant RC-24.2-11 and wild-type inoculated controls were virus infected as determined by the systemic symptoms and PCR using a virus-specific primer pair outside the transgene sequence (not shown).

Figure 7a shows that there was no evident difference in the levels of transgene-derived siRNA among the analyzed transgenic plants, despite some of the plants showing symptoms, whereas others proved to be virus-free. At 45 dpi, the accumulation of siRNA corresponding to the RC sequence was still very high in all transgenic plants tested. A slightly higher amount of siRNA was present in resistant and immune plants in both transgenic lines compared to a susceptible sibling of the same line (Fig. 7a). Interestingly, a larger RNA species was visible in resistant and immune plants, especially in plants of line RC-19.3 (Fig. 7a).

Southern blot analysis at 45 dpi showed that plants RC-

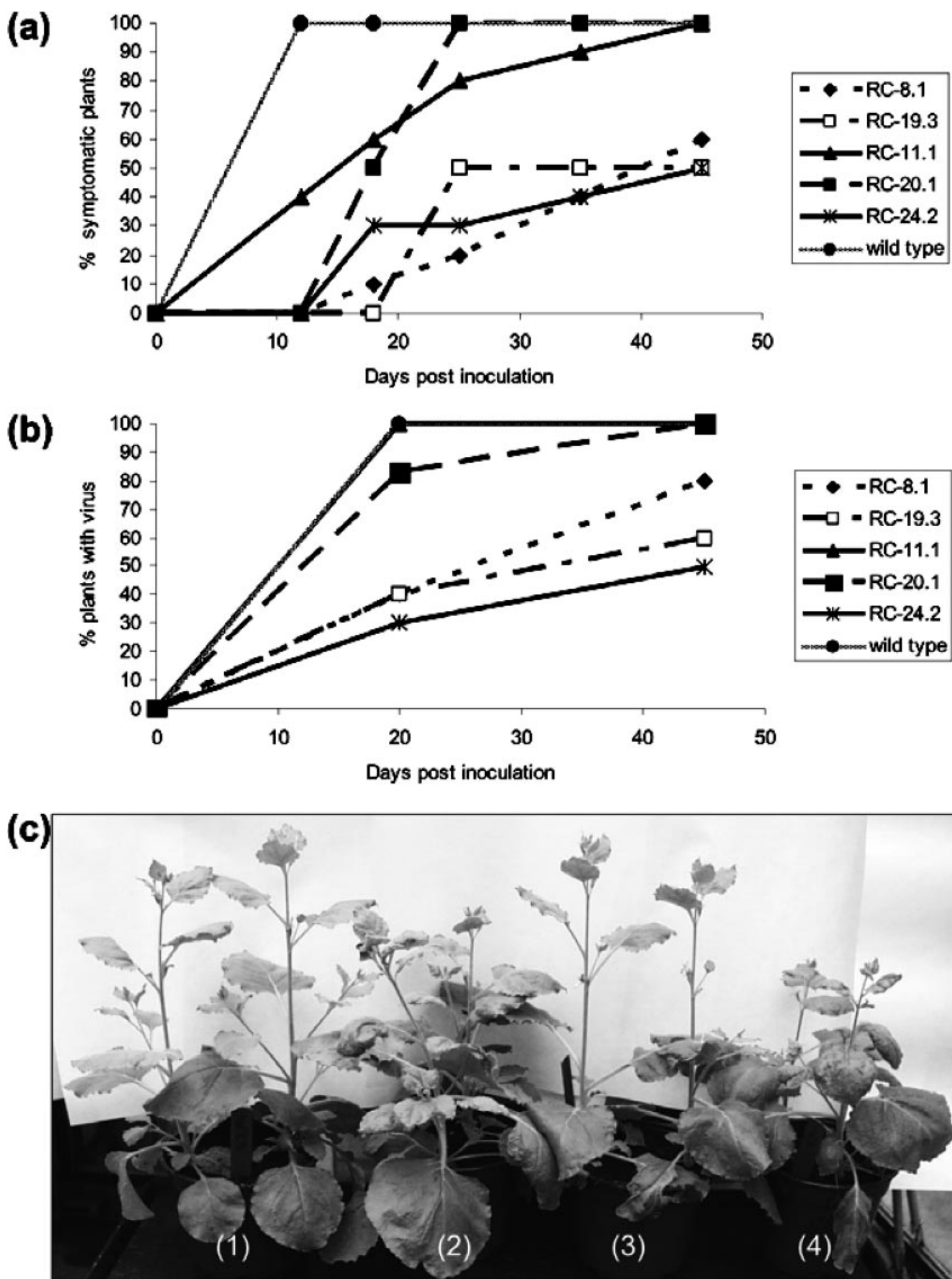

FIG. 6. Systemic symptoms and virus accumulation in transgenic *N. benthamiana* plant lines challenge-inoculated with ToCMoV-[BA-Se1]. (a) Percentage of plants with symptoms evaluated visually in relation to time postinoculation (dpi). (b) Percentage of plants containing virus at 20 and 45 dpi as evaluated by tissue blot hybridization. (c) Symptoms on plants from transgenic line RC-19.3 at 20 dpi: plants 1, resistant inoculated transgenic plants; plants 2, susceptible inoculated transgenic plants; plants 3, transgenic mock-inoculated controls; and plants 4, wild-type inoculated controls.

19.3-11 and RC-19.3-13 became infected and that viral DNA accumulation in RC-19.3-13 was comparable to that in the inoculated wild-type control. Plant RC-19.3-11 remained symptomless and yet became infected, albeit the viral DNA accumulation was extremely reduced (Fig. 7b). Plants RC-24.2-12, RC-24.2-13, and RC-24.2-17 remained symptomless, and viral DNA could not be detected by Southern blotting (Fig. 7b) or PCR (not shown).

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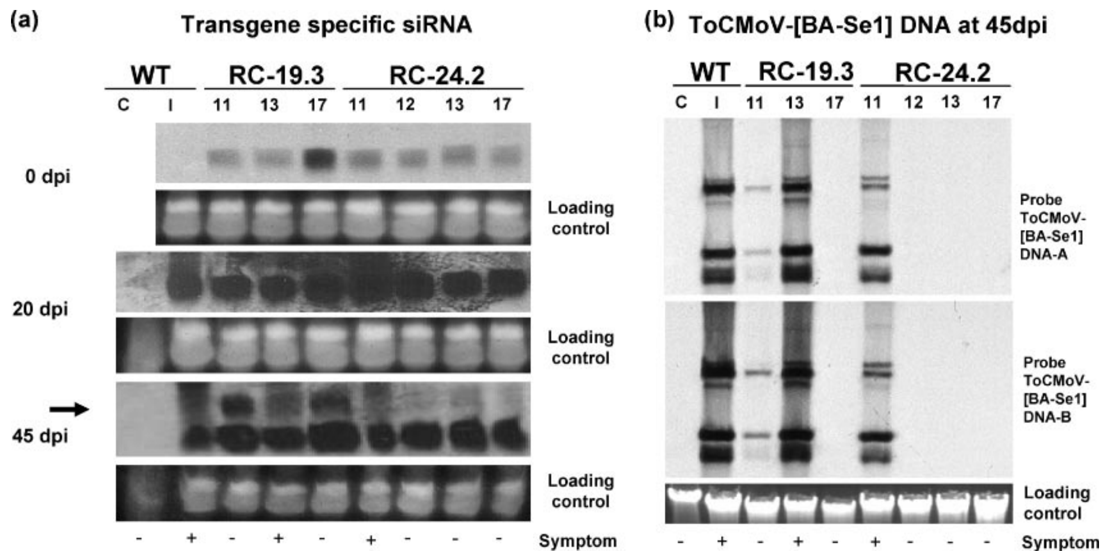

FIG. 7. Analysis of resistant and susceptible T1 siblings of *N. benthamiana* lines RC-19-3 and RC-24-2. (a) Presence of siRNAs corresponding to the RC probe at 0, 20, and 45 dpi; (b) accumulation of ToCMoV-[BA-Se1] DNA-A and DNA-B at 45 dpi. I, inoculated wild-type control; C, mock-inoculated wild-type control. The arrow indicates the higher-molecular-mass RNA species discussed in the text.

DISCUSSION

Upon virus infection, plants undergo complex metabolic changes to accommodate the host requirements for defense but also the needs of the virus for replication and movement. RNA silencing is an important defense response to virus infection in plants. In a normal infection by an RNA or DNA virus or by a viroid, it has been observed that the silencing machinery in the plant can be activated with specific siRNAs directed to the invading pathogen being produced (16, 23, 30, 40, 43, 49, 84).

In the present study it is shown that the begomovirus ToCMoV-[BA-Se1] also triggers the plant RNA silencing machinery in different host plants. Upon infection, a population of siRNAs specific to ToCMoV-[BA-Se1] is generated. The size distribution of the produced siRNAs is in good agreement with those published for another geminivirus in a recent study (1) and suggests that multiple Dicer-like proteins of the plants are involved in their generation (22). siRNA generation seems to interfere with virus replication and/or accumulation in *N. benthamiana* because, following the acute symptoms of chlorotic veins, crumpling and epinasty, the plant develops a rather ameliorated systemic symptom of mottling that is accompanied by a decrease in the amount of viral DNA. These results are similar to those obtained with *N. benthamiana* and cassava (*Manihot esculenta*) plants infected by African cassava mosaic virus–[Cameroon] (ACMV-[CM]) and Sri-Lankan cassava mosaic virus, which are regarded as recovery phenotype viruses. Also, in these viruses the remission of the symptoms is accompanied by an increase in virus-specific siRNAs and a decrease in both viral mRNA and DNA (16).

Recent studies on siRNA induced by virus infection by RNA or DNA viruses in different hosts plants (43, 67) have revealed that most viral genomes are not uniformly targeted. Although the siRNA population present in both *N. benthamiana* and tomato infected by ToCMoV-[BA-Se1] represented the entire DNA-A and DNA-B, it was not homogeneously distributed over the whole extent of the genome. Comparing the distribution of DNA-A-derived siRNA from *N. benthamiana* and tomato, we observed a divergence in the preference for different parts of the genome in different hosts. In *N. benthamiana* the highest levels of siRNA accumulation corresponded to fragments A6, A1, and A2. The precise reasons why the RNAs derived from these regions of the genome are preferentially targeted by the RNA silencing mechanism are not known. The abundance of the transcript, the secondary structure of the mRNA or the accessibility to Dicer may influence the targeting (10, 67). The transcript corresponding to AC1 is probably abundant early in the infection cycle (16). Although, the ToCMoV-[BA-Se1] AC1 transcript has not been mapped, several high-energy structures involving predicted stem-loops were reported for the ACMV-[CM] AC1 transcript (16). These structures were targeted by the RNA silencing machinery in plants infected by *Cymbidium ringspot virus* (43). Similar structures were predicted from the ToCMoV AC1 ORF by using Mfold (85). Since AC1 is the main protein involved in the viral DNA replication and paramount for virus replication (32), targeting AC1 mRNA would impact viral replication by reducing viral DNA accumulation over time in this host. AV1 codes for the CP, which is a late expressed gene, and the correspondent mRNA was shown to accumulate in high amounts (71). The CP is involved in ssDNA accumulation (5), and therefore downregulating CP production would result in a further decrease in ssDNA accumulation.

Similar to *N. benthamiana*, the majority of siRNAs in infected tomato is targeted to the 5′ end of the AC1 gene. However, also a large amount of siRNAs preferentially hybridized to the fragment corresponding to AC2 and the overlapping AC3 (fragment 4). Since AC2 is a transcription activator for the late genes AV1 and BV1, an indirect downregulation of these two genes would lead to reduced amounts of viral DNA accumulation and movement. The AC1 and AC2 sequences are also the preferred target of RNA silencing directed to

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different viruses involved in cassava mosaic disease (16, 17). The relative discrepancy on the major target sequences in different hosts infected by ToCMoV-[BA-Se1] might reflect different compositions of the silencing machinery in the two host plants, leading to different interactions with the virus, or may be due to differential transcription or accumulation of complementary sense RNAs, since for other begomoviruses different mRNAs have been mapped for complementary sense genes (32, 71). This is similar to East African cassava mosaic virus, which is also targeted differently in *N. benthamiana* and cassava (17).

An intriguing and unresolved issue is how geminiviruses, with no dsRNA step in their replication cycle, can induce RNA silencing in infected plants. Vanitharani et al. (72) suggested three possibilities including induction by overlapping transcripts in opposite direction, by abundant early transcripts converted into dsRNA by a host RNA-dependent RNA polymerase, or by strong secondary structures of transcripts perceived as dsRNA. We addressed these possibilities by looking at the polarity of the siRNAs from ToCMoV-[BA-Se1]-infected tomato. For siRNAs binding to fragments that include the region where transcripts are known to overlap, we found that siRNAs of viral and complementary sense are present in almost equal amounts. This is similar to the results of Chellappan et al. (16), who used strand-specific probes to detect the overlapping transcripts. However, for the 5′ end of AC1 a higher proportion (2:1) of complementary sense polarity was present, suggesting that the majority of the siRNAs from this region possibly originated from the AC1 mRNA fold-back structure directly being the substrate for a Dicer-like protein as described for *Cymbidium ringspot virus*, *Potato virus X*, and *Tobacco mosaic virus* (43). An alternative explanation would be the preferential incorporation of the complementary sense-strand into the RISC (34). This question would be unequivocally resolved by sequencing the siRNAs from the virus-infected plant (43).

RNA-mediated virus resistance has been used as a potent strategy to generate host resistance against RNA viruses (54, 58). Also, for the ssDNA geminiviruses, sense and antisense versions mainly involving the AC1 gene have been used, although with various success rates (3, 9, 21, 40, 83). The realization that RNA silencing was responsible for the RNA-mediated virus resistance mechanism (7) and the recognition that dsRNA and siRNAs played a major role in the process (30, 80) led to the introduction of intron-hairpin constructs which directly produce self-complementary dsRNAs that efficiently induce targeted gene silencing and virus resistance (63). Using this type of construct containing the common region of the begomovirus *Vigna mungo yellow mosaic virus* in a transient assay, Pooggin et al. (52) obtained recovery from virus infection.

Here, we have explored the natural RNA silencing response to begomovirus ToCMoV-[BA-Se1] infection in *N. benthamiana* by preprogramming transgenic plants to express dsRNA corresponding to the most targeted portions of the viral genome. This was achieved by using an intron-hairpin construct comprising the 300 nt of the 5′ end of the AV1 gene (including the end of the overlapping AC5 ORF), the entire common region, and 300 nt of the 5′ end of the AC1 gene (including a part of the nested AC4 gene). Indeed, transgenic plants were shown to contain transgene-specific siRNAs. In contrast to experiments with RNA viruses (18, 33, 42, 46) completely immune lines were not observed. Most transgenic lines, however, did show significant delays in symptom development, and two lines displayed highly resistant and even immune plants. In siblings of these lines, RC-19.3 and RC-24.2, transgene-specific siRNAs were readily detected, and the amount increased at least three- to sixfold after virus inoculation, although virus infection could not be detected by PCR, a feature also observed upon infection of resistant plants expressing tospovirus siRNAs with the corresponding virus (13). At 45 dpi, both lines had susceptible and resistant plants even though resistant plants at this stage contained a slightly higher amount of siRNAs. Interestingly, a larger species of small RNA was visible in resistant and immune plants of line RC-19.3. This fragment might represent an incompletely cleaved RNA product, but why it specifically occurs in resistant plants remains puzzling. Interestingly, Boutla et al. (11) reported that an RNA species of ~80 nt purified from GFP silenced plants was a very potent inducer of silencing when injected in GFP-expressing *Caenorhabditis elegans*, suggesting the presence of functional larger small RNA species. Indeed, such RNA species were also observed by the same researchers in cucumber mosaic virus resistant plants expressing IR transgenes (36; K. Kalantidis, unpublished data). Our results collectively indicate that the presence of transgene siRNAs corresponding to viral sequences prior to virus inoculation does not guarantee virus resistance. Noris et al. (47) reported similar results for *Tomato yellow leaf curl Sardinia virus*, where this virus was able to infect tomato plants expressing siRNAs specific to AC1. Both cases contrast with RNA silencing-mediated resistance to RNA viruses, where the presence of virus-specific siRNAs in transgenic plants strongly correlates with virus resistance (18, 33, 42, 46), indicating that RNA viruses are more susceptible to RNA silencing, possibly because both mRNAs and genomic RNAs can be the target of the silencing machinery.

When plants infected with *Vigna mungo yellow mosaic virus* were bombarded with a hairpin construct containing virus-specific common region sequences they can recover from infection (52). These authors suggested that the viral DNA is targeted and methylated possibly by an RNA-dependent DNA methylase, thus resulting in remission of the symptoms. In another study, a transgene encoding a *Tomato leaf curl virus* (62) promoter becomes methylated after virus infection, while the virus itself is not affected. We have not been able to prove whether or to what extent the transgene or the viral genomic DNA is affected by methylation.

We favor the suggestion by Noris et al. (47) that a threshold of siRNA expression exists below which the virus can bypass the resistance and build up a critical mass of virus in the primary infected cell from which it can subsequently spread over the plant, although recent results by Akbergenov et al. (1) may indicate that several distinct RNA silencing pathways may need to be triggered for effective resistance.

The results reported here, together with those published by others (1, 16, 17, 51, 71, 72, 73), add to the conclusion that there is a significant but highly complex relationship between begomoviruses and the RNA silencing plant defense process. Even though transgenic resistance associated with gene silencing can be achieved successfully (4, 15), it is dependent on the virus-host combination, possibly the strength of the RNA si-

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lencing suppressor of the virus (15), and environmental factors (17, 68).

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