Hibiscus chlorotic ringspot virus coat protein inhibits trans-acting small interfering RNA biogenesis in Arabidopsis

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Received 17 March 2008
Accepted 27 May 2008

Many plant and animal viruses have evolved suppressor proteins to block host RNA silencing at various stages of the RNA silencing pathways. Hibiscus chlorotic ringspot virus (HCRSV) coat protein (CP) is capable of suppressing the transiently expressed sense-RNA-induced post-transcriptional gene silencing (PTGS) in Nicotiana benthamiana. Here, constitutively expressed HCRSV CP from transgenic Arabidopsis was found to be able to rescue expression of the silenced GUS transgene. The HCRSV CP-transgenic Arabidopsis (line CP6) displayed several developmental abnormalities: elongated, downwardly curled leaves and a lack of coordination between stamen and carpel, resulting in reduced seed set. These abnormalities are similar to those observed in mutations of the genes of Arabidopsis RNA-dependent polymerase 6 (rdr6), suppressor of gene silencing 3 (sgs3), ZIPPY (zip) and dicer-like 4 (dcl4). The accumulation of microRNA (miRNA) miR173 remained stable; however, the downstream trans-acting small interfering RNA (ta-siRNA) siR255 was greatly reduced. Real-time PCR analysis showed that expression of the ta-siRNA-targeted At4g29770, At5g18040, PPR and ARF3 genes increased significantly, especially in the inflorescences. Genetic crossing of CP6 with an amplicon-silenced line (containing a potato virus X–green fluorescent protein transgene under the control of the 35S cauliflower mosaic virus promoter) suggested that HCRSV CP probably interfered with gene silencing at a step after RDR6. The reduced accumulation of ta-siRNA might result from the interference of HCRSV CP with Dicer-like protein(s), responsible for the generation of dsRNA in ta-siRNA biogenesis.

INTRODUCTION

Small-RNA-mediated silencing pathways play important roles in a variety of processes, including defence against viruses. Viruses in turn evolve mechanisms to interfere with the host silencing responses for successful infection, which usually results in differential developmental defects. The molecular basis of gene-silencing-related abnormal development is mediated by a group of small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Bartel, 2004; Bartel & Bartel, 2003; Jones-Rhoades et al., 2006). The miRNAs are a class of small RNAs similar to siRNAs that are key components of the complex networks of gene regulatory pathways. As most plant miRNAs contain near-perfect complementarities with target sequences, they are thought to function like siRNAs in guiding target RNA for cleavage (Rhoades et al., 2002). In Arabidopsis thaliana, there are four Dicer-like proteins (DCLs) that are responsible for the production of miRNAs and siRNAs of different sizes. All four DCLs act in combination to help mediate plant responses to diverse viral infections (Blevins et al., 2006; Deleris et al., 2006).

The plant miRNAs target a series of genes/gene families that are important for normal plant development (Kidner & Martienssen, 2005). Interference in miRNA biogenesis or miRNA–target interactions lead to developmental abnormalities (Bartel & Bartel, 2003; Dugas & Bartel, 2004). One group of miRNA targets comprises precursors of a subset...
of endogenous small RNAs referred to as trans-acting siRNAs (ta-siRNAs), which are able to direct the cleavage of non-identical transcripts. The biogenesis of ta-siRNAs requires components of both the miRNA and siRNA pathways in two consecutive phases: after the AGO1–DCL1–HEN1–HYL1-dependent miRNA-guided cleavage of the ta-siRNA precursor RNA (TAS), the 5′ or 3′ cleavage products are converted into dsRNA in an RDR6–SGS3-dependent manner; the dsRNAs are then cleaved by DCL4 to generate ta-siRNAs (Allen et al., 2005; Xie et al., 2005). The biogenesis pathway of ta-siRNA establishes a link between the miRNA and siRNA pathways (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). ta-siRNAs are functionally similar to miRNAs in that they regulate the expression of target genes to which they have limited sequence similarity, thus maintaining normal plant development (Yoshikawa et al., 2005). Six loci have been reported to generate ta-siRNAs in Arabidopsis: TAS1a, TAS1b, TAS1c and TAS2 transcripts are targeted by miR173, locus TAS3 is targeted by miR390 (Allen et al., 2005; Xie et al., 2005) and locus TAS4 was identified recently by scanning the Arabidopsis genome for phased clusters of 21 nt and the TAS4 transcript is targeted by miR828 for its phased maturation (Rajagopal et al., 2006).

Several virus-encoded gene-silencing suppressors have been reported to cause developmental defects when expressed constitutively in Arabidopsis as transgenes (Chapman et al., 2004; Chen et al., 2004; Dunoyer et al., 2004). Transgenic Arabidopsis containing tobacco etch virus HC-Pro, turnip yellow mosaic virus (TYMV) p69, beet yellows virus p21 and tomato bushy stunt virus p19 proteins displayed differential leaf and rosette development, and obvious flower phenotypes and infertility were also observed (Chapman et al., 2004; Chen et al., 2004). Some of these proteins, such as HC-Pro and p69, affect the level of key gene-silencing components such as DCL1 in the transgenic plants, which leads to the extensive deviation of a number of miRNAs (Mallory et al., 2002; Xie et al., 2003; Chen et al., 2004; Dunoyer et al., 2004). The molecular mechanisms of silencing suppression have been reported for some silencing suppressors; p19, p21 and HC-Pro may function similarly by forming head-to-tail homodimers that sequester siRNA duplexes and prevent them from entering the RNA-induced silencing complex (RISC) (Vargason et al., 2003; Ye et al., 2003; Ye et al., 2005). Cucumber mosaic virus 2b protein is reported to interact directly with AGO1 (the core component of RISC) and block AGO1 cleavage activity to inhibit miRNA pathways and attenuate RNA silencing (Zhang et al., 2006). Tomato yellow leaf curl virus V2 protein suppresses gene silencing through its interaction with the host SGS3 protein (Glick et al., 2008).

Hibiscus chlorotic ringspot virus (HCRSV) is a positive-sense, single-stranded RNA virus in the genus Carmovirus (Huang et al., 2000). The coat protein (CP) of HCRSV has been identified as a strong gene-silencing suppressor, which suppresses transiently expressed sense-transgene-induced PTGS, and a complete CP is required for the effective suppression function (Meng et al., 2006). The suppression function of CP is also correlated with the host-induced avirulence of HCRSV during virus evolution (Meng et al., 2006). Turnip crinkle virus (TCV) is also a member of the genus Carmovirus. The CPs of TCV and HCRSV share approximately 30% amino acid sequence identity. TCV CP induces few or no developmental defects in Arabidopsis and does not interfere with the accumulation of miRNAs (Chapman et al., 2004; Dunoyer et al., 2004). TCV CP is reported to interfere with the ta-siRNA pathway and suppress DCL4 function (Qu et al., 2003; Deleris et al., 2006; Meng et al., 2006). TCV silencing is mediated by DCL2 and DCL4, with DCL2 providing redundant siRNA-processing functions when DCL4 is suppressed by TCV CP (Deleris et al., 2006). In this study, we investigated whether HCRSV CP acts on the ta-siRNA pathway and tried to determine its suppression mechanism. We showed that HCRSV CP suppresses gene silencing when it is constitutively expressed in Arabidopsis. The CP also affected normal plant development by interfering with the accumulation of siRNAs, miRNAs and ta-siRNAs.

METHODS

Plant materials. Line L1 carried a silenced β-glucuronidase (GUS) transgene in an A. thaliana ecotype Col-0 background (Elmayan & Vaucheret, 1998; Mourrain et al., 2000). Lines A and G carried a silenced potato virus X (PVX)—green fluorescent protein (GFP) transgene under the control of the 35S cauliflower mosaic virus (CaMV) promoter (35S–PVX::GFP) and a 35S–GFP transgene in the background of ecotype C24, respectively. The transformants were selected in vitro on medium supplemented with 10 mg/l phosphinothricin (Melford) 1− or 50 mg/l kanamycin 1−, respectively (Dalmay et al., 2000a). Line G×A was a cross between lines G and A. The transgenic plants were selected using 50 mg/l kanamycin 1− (Dalmay et al., 2000a). The L1 line was provided by Dr Hervé Vaucheret (Laboratoire de Biologie Cellulaire, INRA — Centre de Versailles, France) and lines G and G×A by Professor David Baulcombe (Department of Plant Sciences, University of Cambridge, UK). The mutant lines rdr6-11, sgs3-11 and zip-1 were obtained from the Arabidopsis Biological Resource Center (Ohio State University, OH, USA). Mutant line dda-2 was obtained from Dr James Carrington (Oregon State University, USA).

Generation of CP- and ΔCP-transgenic Arabidopsis. The CP coding sequence of HCRSV (Huang et al., 2000) was PCR amplified with a 5′ leader (5′-AAGGAGATATAACA-3′) and cloned into pBI212 by replacing the GUS sequence between the 35S promoter and the nopaline synthase terminator to yield pBICP (Fig. 1a). The third amino acid codon (CAG) of CP was mutated to TAG and the start codon of the overlapping p25 was changed to GTG in pBICP (Fig. 1a). The CP and L1, A or G×A lines were carried out as described previously (Chen et al., 2004). After crossing, the introduced...
sequences and the effects of CP and ΔCP on the accumulation of transgene and amplicon-specific mRNA and siRNAs were detected by Northern blotting.

**Isolation of total RNA and detection of high- and low-molecular-mass RNAs (siRNAs, miRNAs and ta-siRNAs).** Isolation of RNAs was carried out as described previously (Li et al., 2002). To detect the high-molecular-mass RNAs, 5 μg total RNA was run on a formaldehyde denaturing gel and transferred to a Hybond-N membrane, followed by detection with a digoxigenin (DIG)-labelled RNA probe. To detect siRNAs and miRNAs, 50 μg total RNA was run on a 15 % polyacrylamide sequencing gel and detected by 32P-end-labelled DNA oligonucleotides. The probe used for the detection of miR173 was 5'-GTGATTTCTCTCTGCAAGCGA-3' and for the detection of ta-siR255 was 5'-TACGCTATGTTGGACTTAGA-3'.

**Phenotypic analysis of CP-transgenic Arabidopsis.** For phenotypic analysis, wild Arabidopsis and mutants were grown in 24-well plant growth trays under conditions of 16 h light/8 h darkness at 22 °C. High humidity was maintained during germination and the early seedling stage by covering trays with transparent plastic lids. Growth and developmental parameters were analysed by using at least 30 homologous seeds from each line.

**Real-time PCR analysis of CP-transgenic Arabidopsis.** Total RNA for real-time PCR was extracted from leaves and inflorescences using Trizol reagent (Sigma). Real-time PCR was carried out using a SYBR Green two-step real-time PCR kit (Applied Biosystems). cDNA was synthesized using random hexamers. The genes tested and the primers used are available in Supplementary Table S1 (available with JGV Online). The real-time PCR primers were designed using Primer Express v2.0 (Applied Biosystems). The primers for the TAS target genes were designed to flank the siRNA-directed cleavage sites. The samples were run on an ABI Prism 7000 Sequence Detection System. Thermal cycling conditions were one cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Following amplification, a dissociation stage was performed to check for non-specific PCR product. The 18S rRNA gene was used as the endogenous control and RNAs from ΔCP20 were used as a calibrator sample. The relative abundance of the genes (fold increase) in CP6 compared with ΔCP20 was calculated using the 2^(-ΔΔCt) method (Applied Biosystems, 2008).

**RESULTS**

**HCRSV CP inhibits PTGS induced by a sense-RNA transgene**

pBICP but not pBIΔCP has been confirmed previously to function as a PTGS suppressor using Agrobacterium-mediated transient assays in *Nicotiana benthamiana* (Meng et al., 2006). pBICP and pBIΔCP were introduced as transgenes into Arabidopsis (ecotype Col-0). Northern and Western blot analyses showed that the HCRSV CP mRNA (Fig. 1b, lanes 2 and 3) and protein (Fig. 1c, lanes 2 and 3) both accumulated at high levels in the CP-transgenic Arabidopsis, indicating that the HCRSV CP gene was transcribed and translated in the transgenic plants. In contrast, ΔCP-transgenic Arabidopsis did not express the CP, although the mRNA was transcribed at a relatively high level (Fig. 1b and c, lane 4). Lines CP6 and ΔCP20 were chosen for further studies.

To test whether the constitutively expressed CP was able to suppress PTGS induced by the sense-RNA transgene, lines pBICP but not pBIΔCP has been confirmed previously to function as a PTGS suppressor using Agrobacterium-mediated transient assays in *Nicotiana benthamiana* (Meng et al., 2006). pBICP and pBIΔCP were introduced as transgenes into Arabidopsis (ecotype Col-0). Northern and Western blot analyses showed that the HCRSV CP mRNA (Fig. 1b, lanes 2 and 3) and protein (Fig. 1c, lanes 2 and 3) both accumulated at high levels in the CP-transgenic Arabidopsis, indicating that the HCRSV CP gene was transcribed and translated in the transgenic plants. In contrast, ΔCP-transgenic Arabidopsis did not express the CP, although the mRNA was transcribed at a relatively high level (Fig. 1b and c, lane 4). Lines CP6 and ΔCP20 were chosen for further studies.

To test whether the constitutively expressed CP was able to suppress PTGS induced by the sense-RNA transgene, lines
CP6 and ΔCP20 were crossed with line L1 in which GUS expression was silenced. GUS staining and Northern blot analysis showed that high levels of GUS activity (Fig. 1d) and GUS mRNA (Fig. 1e, upper panel, lane 3) were detected in the F1 progeny of the L1 × CP6 cross. In contrast, 35S–GUS remained silenced in the F1 progeny of L1 × ΔCP20 (Fig. 1d) as well as L1 × Col-0 (data not shown). Furthermore, the GUS-specific siRNAs accumulated to high levels in L1 plants and L1 × ΔCP20 plants, but was undetectable in L1 × CP6 plants (Fig. 1e, third panel, lanes 2–4). These results indicated that the silencing suppression activity required the expression of CP, as shown in CP6. Transcription of CP mRNA alone, as shown in ΔCP20, did not suppress PTGS.

Notably, these RNA analyses showed that suppression of GUS RNA silencing in CP6 plants was as effective as in the rdr6 mutant, which contains a defective cellular RNA-dependent RNA polymerase (Fig. 1e, lane 1). In the rdr6 mutant, high levels of GUS mRNA and no GUS siRNA were detected. This is due to the GUS transgene failing to convert to dsRNA, resulting in PTGS not being induced. This is in agreement with the findings of Mourrain et al. (2000). Rescued GUS expression (Fig. 1d) and the lack of GUS-specific siRNAs in the progeny of L1 × CP6 plants (Fig. 1e, third panel, lanes 3) further indicated that the constitutively expressed CP interferes with PTGS at the early initiation stage. This result supports the suggestion that HCRSV CP actively suppresses RNA silencing targeted against transienly expressed 35S-controlled GFP transcripts in GFP-transgenic N. benthamiana (Meng et al., 2006).

**Arabidopsis** expressing the HCRSV CP transgene shows developmental defects resembling those of mutant lines *rdr6-11* and *dcl4-2*

Many gene-silencing suppressors have been implicated in abnormal plant development by interfering with the miRNA/siRNA pathways (Chapman et al., 2004; Chen et al., 2004; Dunoyer et al., 2004; Zhang et al., 2006). To test whether the carmovirus HCRSV CP would affect normal plant development, lines CP6 and ΔCP20 were used for phenotypic analysis. The wt line Col-0 was included as a negative control. The mutant lines *rdr6-11*, *sgs3-11*, *zip-1* and *dcl4-2* were also included as controls. RNA-dependent polymerase 6 (RDR6) and suppressor of gene silencing 3 (SGS3) are required for PTGS (Dalmay et al., 2000b; Mourrain et al., 2000; Qu et al., 2005; Schwach et al., 2005) and the production of ta-siRNAs in *Arabidopsis* (Peragine et al., 2004). RDR6 has also been implicated in natural virus resistance (Qu et al., 2005). ZIP encodes ARGONAUTE7 (AGO7) whose primary function is in the regulation of developmental timing (Hunter et al., 2003). DCL4 produces 21 nt RDR6-dependent ta-siRNAs and has been reported to participate in siRNA production following viral infections (Allen et al., 2005; Gascioli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005; Bouché et al., 2006; Deleris et al., 2006).

In wt Col-0 *Arabidopsis*, the first two rosette leaves appeared round and relatively flat (Fig. 2a, row 2). In contrast, line CP6 showed elongated, downward curled leaves, which resembled the leaf phenotypes of the *rdr6-11*, *sgs3-11*, *zip-1* and *dcl4-2* mutants (Fig. 2a, rows 2 and 4). Transgenic *Arabidopsis* line ΔCP20 harbouring ΔCP showed the same developmental phenotype as wt Col-0.
(Fig. 2a, row 2). Compared with the wt Col-0 and ΔCP20, line CP6 showed early flowering by 2–3 days and reduced fertilization. Further analysis revealed that the carpels of CP6 flowers were elongated, which caused a lack of coordination between stamen and carpel (Fig. 2a, row 1), resulting in reduced siliques (data not shown). In the rdr6-11, sgs3-11, zip-1 and dcl4-2 mutants, the carpels also showed an incompatible elongation with stamens (Fig. 2a, row 3). The sepals of CP6 flowers appeared normal under the light microscope compared with wt (data not shown). The pollens and the stigmata of CP6 were normally developed (no obvious differences compared with those of ΔCP20) when observed under the scanning electron microscope (Fig. 2b, columns 1 and 2). However, without artificial pollination, only ΔCP20 stigmata were fertilized (Fig. 2b, column 3). Manual self-crossing of the pollens to the stigmata of CP6 resulted in similar fertilization rates compared with those of wt Col-0. Another CP overexpression line, CP8 (Fig. 1c, lane 3), exhibited the same phenotype as CP6 (data not shown). However, a closely related gene-silencing suppressor, TCV CP, has been shown to develop only mild (Chapman et al., 2004) or no (Dunoyer et al., 2004) developmental defects compared with wt. Comparison of TCV- and HCRSV-encoded suppressors indicates that differences in the sequence and structure of suppressors have substantial impacts on plant development. Changes in small-RNA accumulation and the level of suppressor protein expressed in the transgenic plants may account for the different phenotypes induced by the two CPs of HCRSV and TCV.

**HCRSV CP increases the accumulation of miR171 and miR172 but not miR173**

As the miRNA pathway shares some key components with the siRNA pathway, the suppressors may affect the accumulation of some miRNAs that are important for normal plant development. To test whether HCRSV CP could influence the biogenesis and accumulation of miRNAs, total RNAs were isolated from rosettes and flowers, and three miRNAs were detected by Northern blot hybridization. The mutant line rdr6-11 was included as a control.

miR171 is perfectly complementary to three SCARECROW-like (SCL) genes, which encode transcription factors that control a wide range of developmental processes, including radical patterning in roots and hormone signalling. It downregulates its target by mRNA cleavage in a similar way to siRNA (Llave et al., 2002; Reinhart et al., 2002). miR172, however, downregulates APETALA2 (AP2) and other AP2-like genes to promote flowering by translational repression (Aukerman et al., 2003; Chen, 2003). miR173 guides the phased maturation of ta-siRNAs, including siR255 (Allen et al., 2005; Xie et al., 2005). All miRNAs showed greater abundance in flowers than in leaves. Both miR171 and miR172 increased in CP6 flowers (Fig. 3a, lanes 2 and 5), compared with the wt control (Fig. 3a, lanes 1 and 4) and the rdr6-11 plants (Fig. 3a, lanes 3 and 6), which showed similar phenotypes of leaf curling and incompatible stamen and carpel elongation to the CP6 plants. The level of miR173 detected was comparable in CP6 and Col-0 (Fig. 3a, lanes 1 and 2, and 4 and 5, respectively).
HCRSV CP reduces siR255 accumulation

Ta-siRNAs resemble miRNAs in plants, acting in trans to direct cleavage of target mRNAs. As CP6 resembles mutants in the trans-acting pathway, we tested whether HCRSV CP interferes with the ta-siRNA pathway. The accumulation of siR255 was chosen as a marker for ta-siRNAs. siR255 is one of the cleavage products of TAS1a, TAS1b and TAS1c, and targets At4g29770 (unclassified) and At5g18040 (unclassified), respectively. Northern blot analysis of RNAs extracted from inflorescences showed that the accumulation of siR255 was significantly reduced in line CP6 (Fig. 3b, lane 2) compared with wt Col-0 (Fig. 3b, lane 1). In the rdr6-1 cell line, siR255 was not detected (Fig. 3b, lane 3). Similar results have been reported for TCV CP in which the accumulation of siR255 was downregulated in TCV-inoculated plants or TCV CP-transgenic Arabidopsis (Bouché et al., 2006; Deleris et al., 2006).

HCRSV CP upregulates ta-siRNA target genes

In the event of reduced ta-siRNA biogenesis, expression of their target genes should increase correspondingly. To test this hypothesis, the relative concentrations of various ta-siRNA target mRNAs from leaves and inflorescences of CP6 and ΔCP20 were compared by real-time RT-PCR. The primers for TAS target genes were designed to flank the siRNA cutting sites. 18S rRNA was used as an endogenous control to normalize the RNA input for each sample. Altogether, four tested ta-siRNA targets, TAS1a (At4g29770), TAS1b (At5g18040), TAS2 PPR and TAS3 ARF3, were all upregulated, especially in the inflorescences of CP6 (Fig. 4a), supporting the proposal that upstream ta-siRNA biogenesis was inhibited. Among the four genes tested, TAS1b and TAS3 ARF3 accumulated to a relatively higher level than the other two genes in CP6 leaves (Fig. 4a). As normal regulation of TAS3 target genes by TAS3 ta-siRNAs is required for proper leaf development (Gasciolli et al., 2005; Adenot et al., 2006), the enhanced accumulation of ARF genes may explain the abnormal leaf phenotypes in CP6.

Other genes that are involved in gene-silencing-related pathways were also tested, including DCL1, DCL2, DCL3, DCL4, DRB4, RDR6 and AGO1. Most of the genes tested increased in the CP6 line, especially in the inflorescences (Fig. 4b). Among these genes, DCL1, which is important for miRNA and ta-siRNA biogenesis, increased by 1.8-fold, DCL2 and DCL3 both increased by around 1.5-fold in inflorescences, and DCL4, which is crucial for ta-siRNA biogenesis, increased by 1.3-fold in leaves. DRB4, which interacts with DCL4 in vivo and functions in the ta-siRNA pathway, remained unchanged in both leaves and inflorescences. RDR6 remained unchanged in leaves but increased by more than 1.5-fold in the inflorescences. AGO1 selectively recruits miRNAs and siRNAs into RISC (Baumberger & Baulcombe, 2005) and was enhanced 2.5-fold in the inflorescences.

HCRSV CP may interfere with RNA silencing at a step after RDR6

RDR6 plays a crucial role in gene-silencing-related pathways by converting ssRNA into dsRNA. To determine whether HCRSV CP interferes with RDR6, CP6 and ΔCP20 were each crossed with the A. thaliana lines A and G × A (Dalmary et al., 2000a). In line A (Amp243, which carries the 35S-PVX::GFP amplicon), initiation of PTGS is dependent on the weak replication of PVX::GFP (Dalmary et al., 2000a, b), corresponding to the genetically determined PTGS initiation pathway. In line G × A
(homologous line resulting from crossing of line A and the GFP-overexpression line GFP142), PTGS is initiated by the replicating PVX::GFP and maintained by the 35S–GFP transgene (Dalmay et al., 2000b); host RDR6 is required for the strong PTGS in G × A (Dalmay et al., 2000b).

The F1 plants of A × CP6 and (G × A) × CP6 were both of similar size and the GFP fluorescence was weak; no apparent differences were observed compared with the wild-type and ΔCP20-crossed controls (data not shown) under a UV (312 nm) lamp (VL-6L; Vilber Lourmat). By RNA blot analysis, we detected a significant increase in the accumulation of PVX::GFP genomic and subgenomic RNAs in both A and G × A plants (Fig. 5, upper panel, lanes 2 and 5) after they were crossed with line CP6, but not with ΔCP20 or wt (Fig. 5, upper panel, lanes 1, 3, 4 and 6). Higher levels of GFP siRNAs were also detected in the progeny of both A × CP6 and (G × A) × CP6 crosses (Fig. 5, third panel, lanes 2 and 5) compared with those in the control plants (Fig. 5, third panel, lanes 1, 3, 4, and 6), suggesting that HCRSV CP is able to rescue the genetically silenced 35S–PVX::GFP in line A and the epigenetically silenced 35S–GFP and 35S–PVX::GFP in line G × A. Similar results have been reported for the TYMV silencing suppressor p69 and CaMV p6 suppressor (Chen et al., 2004; Love et al., 2007). The increase in both PVX::GFP viral RNA and GFP siRNA can be explained, as HCRSV CP is unable to inhibit degradation of pre-existing dsRNAs, whilst viral replication produces a continuous supply of dsRNAs. These results are different from the inverse correlation of GUS mRNA and siRNA in the L1 × CP6 cross (Fig. 1e, lane 3).

In the CP6 × (G × A) line, the GFP mRNA level (Fig. 5, upper panel, lane 5) was much weaker than the PVX RNAs. In contrast, the RDR6 mutant line rdr6(G × A) accumulates much more GFP mRNAs than PVX RNAs because the RDR6-dependent PTGS is disabled in rdr6(G × A) (Dalmay et al., 2000b). Comparing the GFP mRNA and siRNA accumulation in CP6 × (G × A) and rdr6(G × A) suggests that HCRSV CP probably interferes with gene silencing at a step after RDR6; HCRSV CP and RDR6 might act at different steps of the same pathway.

**DISCUSSION**

HCRSV CP has been shown to be a strong gene-silencing suppressor, and the reduced suppression function of the CP after serial passages may be correlated with the avirulence of HCRSV in Hibiscus (Meng et al., 2006). Most of the suppressors identified are pathogenicity determinants, and many of them have been reported to show developmental defects when expressed as transgenes in Arabidopsis. These defects are linked with the interference of endogenous small-RNA pathway(s). In this paper, we investigated the effects of HCRSV CP on the accumulation of selected miRNAs, ta-siRNAs and genes related to gene-silencing pathways and plant development.

Different silencing-suppression assays may result in different interpretations of suppression mechanisms, and stable expression assays are mostly free of complications (Roth et al., 2004). HCRSV CP-transgenic Arabidopsis was created to test the constitutively expressed CP on suppression of gene silencing. Genetic crosses of HCRSV CP-transgenic Arabidopsis with the GUS-silenced L1 line restored GUS expression, confirming the function of HCRSV CP as a silencing suppressor in the Arabidopsis system.

The Arabidopsis CP-transgenic line CP6 showed several developmental abnormalities (Fig. 2a), similar to those of RDR6, SGS3, ZIP and DCL4 mutants (Peragine et al., 2004). CP8 showed the same developmental defects as CP6. In contrast, another CP-transgenic line with marginal levels of CP expression showed no difference in phenotype when compared with wt (data not shown), suggesting that the severity of the abnormalities was correlated with the level of CP expression in the transgenic plants. This is in agreement with the observation in turnip mosaic virus HC-Pro- and TYMV p69-transgenic Arabidopsis (Chen et al., 2004; Dunoyer et al., 2004). Developmental phenotypes in Arabidopsis have been correlated with strong gene-silencing suppressor activity (Chapman et al., 2004). However, the molecular basis may be more complex. By comparing the
results of this study with those of TCV CP, we have clearly demonstrated that such a correlation is not universally applicable to all virus suppressors. TCV belongs to the same genus as HCRSV. TCV CP is a strong suppressor that completely abolishes siRNA accumulation in N. benthamiana (Qu et al., 2003). Transgenic Arabidopsis expressing TCV CP shows no significant developmental defects and miRNA accumulation remains unchanged (Chapman et al., 2004; Dunoyer et al., 2004). However, the accumulation of miR171 and miR172 increased in the HCRSV CP6 inflorescences (Fig. 3a). Arabidopsis expressing CaMV 2b protein from a severe strain (FNY) was reported to cause significant accumulation of miRNAs and to display obvious developmental defects (Zhang et al., 2006). However, CaMV 2b from mild strains (LS and Q) was reported to have a mild effect on miRNA-guided functions and plant development (Chapman et al., 2004; Lewsey et al., 2007).

Compared with wt Col-0, the accumulation of miR171 and miR172 was enhanced in the CP6 line, especially in the flowers (Fig. 3a). The downregulation of SCL genes by miR171 may contribute to the phenotype of CP6, as SCL genes control a wide range of developmental processes, such as hormone signalling (Llave et al., 2002; Reinhart et al., 2002). The enhanced accumulation of miR172 may change the expression pattern of AP2, which leads to morphological changes in flowers. The reduction in AP2 gene expression in the carpel region may reduce inhibition of C gene expression by the A gene, which may contribute to the abnormal growth of carpels and stamens. The AP2 mutant showed transformation of sepals into carpels and petals into stamens (Drews et al., 1991). Therefore, the phenotype observed in CP6 may involve other genes (e.g. flower homeotic genes) that act in concert with miR172 for its abnormal development.

Based on the phenotypic similarities of CP6 with those of ta-siRNA pathway mutants, interference in the ta-siRNA pathway was suspected. We observed that siR255 accumulation was greatly reduced in the CP6 line or not detectable in the rdr6-11 line (Fig. 3b). The reduced siR255 in CP6 may result from changes at three stages: (i) the level of miR173, which is responsible for the in-phase maturation of ta-siRNA siR255; (ii) the downstream RDR6–SGS3-dependent conversion of the miR173 cleavage product into dsRNAs; and (iii) the DCL4 cleavage step. It has been reported that DCL4 interferes with the biogenesis of ta-siRNAs including siR255 but not the level of miR173 (Yoshikawa et al., 2005; Bouche et al., 2006), or that it marginally increased the level of miR173 (Xie et al., 2005). miR173 was detected in the same sample used for siR255 in Northern blotting. The level of miR173 remained constant in line CP6 inflorescences compared with wt Col-0 (Fig. 3b, lanes 1 and 2). The constant miR173 level indicates that the reduced siR255 level in CP6 might be the direct consequence of reduced dsRNAs or inhibition of DCL4 by HCRSV CP.

siR255 is the cleavage product of TAS1a, TAS1b and TAS1c (Allen et al., 2005; Xie et al., 2005). Target gene analysis (Fig. 4a) in CP6 showed that the expression levels of the genes for TAS1a (At4g29770), TAS1b (At5g18040), TAS2 PPR and TAS3 ARF3 were all significantly increased. These data further support our hypothesis that HCRSV CP interferes with the ta-siRNA pathway. TAS3 specifies leaf polarity by targeting ARF3 and ARF4, which in turn downregulate the FILAMENTOUS FLOWER (FIL) gene (Garcia et al., 2006). Based on the developmental roles of TAS target genes, it is possible that these varied ta-siRNA targets may be crucial for the abnormal development in CP6.

In addition, the contribution of endogenous small RNAs, the expression of some other genes that are closely involved in silencing pathways may also be changed by the expression of HCRSV CP. Through real-time PCR analysis, the genes for the four DCLs and RDR6 and AGO1 were shown to be expressed at higher levels in CP6, especially in the inflorescences (Fig. 4b). The increased accumulation of these gene transcripts indicates that the overall antiviral silencing in CP6 might have been enhanced. The fact that DCL4 is expressed at about the same level or higher in the CP6 plants suggests that the CP might inhibit DCL4 directly, given that siR255 is reduced in CP6-transgenic Arabidopsis.

RDR6 is not only indispensable for transgene-induced gene silencing, it also plays a vital role in the biogenesis of ta-siRNAs and in the natural host antiviral response. HCRSV CP enhanced the level of viral RNA in lines A and G × A (Fig. 5). The replication of PVX overcomes viral dsRNA-mediated degradation and/or 35S-GFP (in the case of G × A)-induced silencing by the host plant. These results indicate that silencing in the genetically silenced GFP in line A and epigenetically silenced GFP in line G × A was reversed. Comparing the siRNA accumulation patterns in L1 × CP6 with A × CP6 and (G × A) × CP6, an inconsistent inverse correlation of PVX : : GFP mRNAs and GFP siRNAs was observed (Fig. 5, lanes 2 and 5). The increase in GFP siRNAs in the A × CP6 and (G × A) × CP6 crosses suggests that large amounts of GFP-containing RNA were produced, some of which were cleaved into GFP siRNAs, indicating that some Dicer and AGO1 proteins are still functioning, despite the presence of HCRSV CP suppressor. The presence of PVX and its replication provides a continuous source of dsRNAs, which accounts for the increased accumulation of GFP and PVX siRNAs in the A × CP6 and (G × A) × CP6 crosses. However, HCRSV CP may not interfere with the RDR6 function in (G × A) × CP6. The GFP mRNA accumulation pattern compared with the PVX genomic RNA and subgenomic RNA levels in (G × A) × CP6 was different from that of rdr6(G × A) (Dalmay et al., 2000b), suggesting a block in gene silencing at a step after RDR6.

DCLs in Arabidopsis act in combination to counteract RNA virus and DNA virus invasion, with RNA viruses mainly
affected by DCL4 and DNA viruses targeted by all four DCLs (Blevins et al., 2006; Deleris et al., 2006). CaMV is reported to silence host gene expression through the coordinated action of four DCLs in Arabidopsis (Moissiard & Voinnet, 2006). The accumulation of viral RNAs is significantly increased in DCL mutants, especially double or triple mutants (Deleris et al., 2006). With DCL4 suppressed by TCV CP, TCV was targeted by DCL2 and produced 22 nt siRNAs in Col-0 (Deleris et al., 2006).

Taking our findings together, we postulate that HCRSV CP interferes with PTGS and ta-siRNA biogenesis at the RNA recognition step downstream of RDR6, which is common to the ta-siRNA pathway and amplicon-induced silencing pathway. The phenotypes observed in CP6 may be a sum effect of the interplay by miRNAs, ta-siRNAs or unknown endogenous siRNA pathways and other transcriptional or translational regulation mechanisms.

ACKNOWLEDGEMENTS

We thank Dr. Peter Palukaitis of the Scottish Crop Research Institute, UK, and Dr. Hao Yu of the National University of Singapore for helpful discussions; the Arabidopsis Biological Resource Center for providing seeds of the rdr6-11, sg3-11 and zip-1 mutants, Dr. Herve Baucheret for the L1 line, Dr. David Baulcombe for the amplicon lines, Dr. James Carrington (Oregon State University, USA) for mutant line dcl4-2 and Dr. Milton Zaitlin from Cornell University, USA, for editing the manuscript. The work was supported by National University of Singapore research grants R-154-000-252-112 and R-154-000-341-112.

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