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RESEARCH ARTICLE

High-Level Accumulation of Exogenous Small RNAs Not Affecting Endogenous Small RNA Biogenesis and Function in Plants

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Abstract

RNA silencing is a fundamental plant defence and gene control mechanism in plants that are directed by 20-24 nucleotide (nt) small interfering RNA (siRNA) and microRNA (miRNA). Infection of plants with viral pathogens or transformation of plants with RNA interference (RNAi) constructs is usually associated with high levels of exogenous siRNAs, but it is unclear if these siRNAs interfere with endogenous small RNA pathways and hence affect plant development. Here we provide evidence that viral satellite RNA (satRNA) infection does not affect siRNA and miRNA biogenesis or plant growth despite the extremely high level of satRNA-derived siRNAs. We generated transgenic *Nicotiana benthamiana* plants that no longer develop the specific yellowing symptoms generally associated with infection by *Cucumber mosaic virus* (CMV) Y-satellite RNA (Y-Sat). We then used these plants to show that CMV Y-Sat infection did not cause any visible phenotypic changes in comparison to uninfected plants, despite the presence of high-level Y-Sat siRNAs. Furthermore, we showed that the accumulation of hairpin RNA (hpRNA)-derived siRNAs or miRNAs, and the level of siRNA-directed transgene silencing, are not significantly affected by CMV Y-Sat infection. Taken together, our results suggest that the high levels of exogenous siRNAs associated with viral infection or RNAi-inducing transgenes do not saturate the endogenous RNA silencing machineries and have no significant impact on normal plant development.

Key words: *Nicotiana benthamiana*, transformation, satellite RNA, RNA silencing, small RNA

INTRODUCTION

RNA silencing is a conserved gene controling mechanism in eukaryotic organisms that is induced by double-stranded RNA (dsRNA) (Baulcombe 2004). In plants, dsRNA is processed by four different Dicer-like (DCL) proteins into small RNAs (sRNAs), including 21 (DCL4), 22 (DCL2) and 24 (DCL3) nucleotide (nt) small interfering RNAs (siRNAs) and 20-24 nt microRNAs (miRNAs) (DCL1) (Bouche *et al.* 2006). These sRNAs are incorporated into an Argonaute (AGO) protein to form an RNA-induced silencing complex (RISC), and guide the RISC to homologous target mRNA or DNA to direct mRNA cleavage or DNA methylation (Eamens *et al.* 2008; Wang *et al.* 2012). RNA silencing plays an essential role in plant defence and development (Baulcombe

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2004; Moissiard and Voinnet 2004). The 21-22 nt siRNAs are important in defence against viruses and in regulating endogenous gene expression, whereas the 24 nt siRNAs guide the *de novo* cytosine methylation process called RNA-directed DNA methylation, and play an essential role in silencing transposons and maintaining genome stability (Eamens *et al.* 2008). miRNAs are essential for plant development as they control the expression of many regulatory genes such as transcription factor genes, and are involved in developmental processes such as cell division, leaf formation and flower development (Bartel 2004; Chen 2005). Disruption of RNA silencing pathways, especially the miRNA pathway, in plants can result in developmental defects. For instance, *Arabidopsis* plants with complete loss-of-function mutation in miRNA pathway factors such as DCL1 and AGO1 are unviable (Bohmert *et al.* 1998; Kurihara and Watanabe 2004).

Plants are often exposed to exogenous siRNAs. For instance, infection with viruses and subviral RNAs are generally associated with the accumulation of large quantities of siRNAs corresponding to the entire viral genome (Wang *et al*. 2012). Transformation of plants with RNA interference (RNAi) constructs, such as long hairpin RNA (hpRNA) constructs, results in the production of additional siRNAs (Fusaro *et al.* 2006). Both viral siRNAs and hpRNA-derived siRNAs are processed by the same endogenous siRNA machineries such as DCL4, DCL2 and DCL3 (Fusaro *et al.* 2006). This raises the question whether these exogenous siRNAs would compete with endogenous sRNAs for the RNA silencing machineries and interfere with sRNA-mediated gene regulation and plant development.

In this paper, we investigated this question by using *Cucumber mosaic virus* (CMV) Y-satellite RNA (Y-Sat) as the source of exogenous siRNAs. Satellite RNAs (satRNAs) are one of the smallest plant pathogens and are regarded as parasites of plant viruses as they do not encode functional protein and depend entirely on the associated virus (helper virus) for replication and spread (Roossinck *et al.* 1992; Simon *et al*. 2004). SatRNAs usually replicate at high efficiency and form stable secondary structures, and accumulate at high levels relative to their helper viruses. CMV Y-Sat is one of the best-studied satRNAs (Takanami 1981; Masuta and Takanami 1989), and our previous studies have shown that *Nicotiana* plants infected with CMV Y-Sat contained extremely high levels of Y-Sat-specific siRNAs. For instance, Northern blot hybridization showed that the amount of Y-Sat siRNAs is much higher than that of siRNAs expressed from a hpRNA transgene in *Nicotiana tabacum* (Wang *et al.* 2004). Also, 5′ end-labelling of total sRNA from CMV Y-Sat-infected *N. tabacum* suggested that Y-Sat-specific siRNAs are more abundant than the total endogenous sRNAs (Ebhardt *et al.* 2005). In addition, the CMV helper virus strain is a type-II CMV strain which encodes a weak 2b suppressor of RNA silencing (Ye *et al.* 2009) that would have minimum effect on host sRNA accumulation. Therefore, CMV Y-Sat infection provides a good model system for examining the possible effect of exogenous sRNAs on endogenous RNA silencing pathways and plant development.

RESULTS AND DISCUSSION

CMV Y-Sat infection does not affect plant growth despite high levels of Y-Sat siRNAs

In this study, we used *Nicotiana benthamiana* as the host for CMV Y-Sat, as this plant system is highly susceptible to CMV Y-Sat infection, shows no visible symptoms when infected with the mild CMV strain (Q-CMV) used in this study, and is widely used for studying RNA silencing *via Agrobacterium*-infiltration (Baulcombe 1999). We observed that *N. benthamiana* was more susceptible to Y-Sat infection and contained a higher level of Y-Sat RNA than *N. tabacum* (Smith *et al.* 2011). As CMV Y-Sat-infected *N. tabacum* accumulates extremely high levels of Y-Sat siRNAs, we expected that *N. benthamiana* would accumulate similar or even higher amounts of Y-Sat siRNAs. However, CMV Y-Sat infection of *N. benthamiana* plants is associated with strong yellowing symptoms (Smith *et al*. 2011). These yellowing symptoms have been shown to be caused by Y-Sat siRNA-directed silencing of the chlorophyll biosynthetic gene *CHLI* and can be prevented by introducing a sat siRNA-

resistant version of the *CHLI* gene (*mCHLI*) into the plant (Smith *et al.* 2011).

Since the yellowing symptoms affect plant growth and would interfere with the visualization of other abnormal phenotypes associated with developmental defects, we transformed *N. benthamiana* plants with the Y-Sat siRNA-resistant *mCHLI* construct (Smith *et al.* 2011). Thirty putative T_0 transgenic plants were transferred to soil, of which 19 were confirmed by PCR to contain the *mCHLI* transgene (Fig. 1). To further verify the transgenic plants, 10 of the PCR-positive *mCHLI* transgenic lines plus 10 wild-type (wt) *N. benthamiana* plants were infected with CMV Y-Sat. The Y-Sat-induced yellowing symptoms were completely abolished in the transgenic *mCHLI* plants, while all the CMV Y-Sat-infected wt *N. benthamiana* plants showed yellowing symptoms (Fig. 2-A). This indicated that the *mCHLI* mRNA was expressed in the transgenic plants and delivered resistance to Y-Sat siRNA-directed silencing, allowing normal production of chlorophyll in CMV Y-Sat-infected plants. This result is consistent with that observed for transgenic *mCHLI N. tabacum* plants (Smith *et al.* 2011).

The transgenic *mCHLI* plants showed no visible

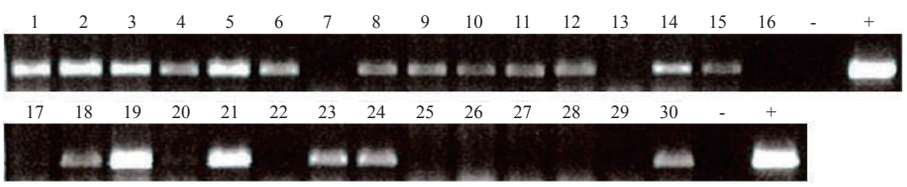

Fig. 1 PCR detection of a 519-bp *mCHLI* sequence in 30 putative transgenic *N. benthamiana* plants (lanes 1-30). -, the negative control; +, the positive plasmid control.

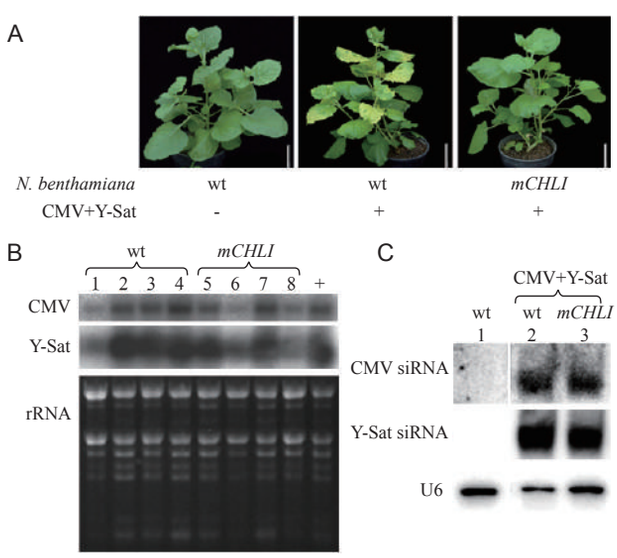

Fig. 2 Transgenic *mCHLI* plants show no abnormal phenotype despite the presence of high-abundance CMV and Y-Sat siRNAs. A, phenotypes of an uninfected wild-type *N. benthamiana* plant (left), and CMV Y-Sat-infected wild-type (middle) or transgenic *mCHLI* (right) *N. benthamiana* plant. Photographs were taken at 12 d post inoculation. Scale bar=5 cm. B, Northern blot hybridization of wt and transgenic *mCHLI* plants infected with CMV Y-Sat at 22 d post inoculation. The top panel shows the level of CMV RNA; the middle panel shows the level of Y-Sat RNA; and the bottom panel is ethidium bromide-stained rRNA for use as loading control. C, Northern blot hybridization of CMV and Y-Sat siRNAs in wt and transgenic *mCHLI* plants infected with CMV Y-Sat at 35 d post inoculation. U6 RNA was hybridized for use as loading control.

phenotypic difference to the wild-type, untransformed *N. benthamiana* plants (data not shown), which allowed us to examine if CMV Y-Sat infection would cause any abnormal growth phenotypes. As shown by the example in Fig. 2-A, CMV Y-Sat-infected transgenic *mCHLI* plants showed no visible phenotypic differences to uninfected wt *N. benthamiana*, consistent with previous observations with transgenic *mCHLI N. tabacum* (Smith *et al.* 2011). Thus, CMV Y-Sat infection did not cause any abnormal phenotypes to the *mCHLI* transgenic plants. Northern blot hybridization showed that CMV Y-Sat-infected *mCHLI* plants contained similar levels of Y-Sat genomic RNA (Fig. 2-B) and Y-Sat siRNAs (Fig. 2-C) to the wt plants. These results indicate that a high level of Y-Sat siRNA accumulation did not affect normal plant development in *N. benthamiana*.

Y-Sat-derived siRNAs do not interfere with the biogenesis of endogenous small RNA

The lack of abnormal phenotypes in CMV Y-Sat-infected *mCHLI* plants implied that CMV- and Y-Sat-derived siRNAs do not have significant impact on

host plant sRNA pathways. To investigate this, we first compared the level of hpRNA-derived siRNAs as well as target gene silencing between uninfected and CMV Y-Sat-infected *mCHLI* plants. Both infected and uninfected plants were *Agrobacterium*-infiltrated with a β-glucuronidase overexpression construct (*GUS*) and a hpRNA to induce GUS silencing (*hpGUS*), and the infiltrated tissues analysed for GUS expression and siRNA accumulation at 3 d post infiltration. As shown in Fig. 3-A, the level of *hpGUS*-induced silencing was similar between the uninfected and the CMV Y-Sat-infected *mCHLI N. benthamiana* plants. Consistent with the similar degree of GUS silencing, hpGUS-derived siRNAs accumulated at a similar level in uninfected and infected plants (Fig. 3-B). These results suggest that CMV Y-Sat infection did not affect the biogenesis of siRNAs or the downstream siRNA-directed degradation of mRNA despite the high-level accumulation of Y-Sat siRNAs.

The biogenesis of miRNAs requires DCL1, which is different to the biogenesis of hpRNA-derived siRNAs that depends primarily on DCL4 but also on DCL2 and DCL3 (Fusaro *et al.* 2006). We therefore compared the expression level of two miRNAs, miR159 and miR171, between uninfected and CMV Y-Sat-infected *mCHLI N. benthamiana* plants (Fig. 4). These two miRNAs are conserved among plant species and important in the control of plant development (Zhang *et al.* 2006, 2007). Both miR159 and miR171were accumulated at a similar level in uninfected, CMV or CMV plus Y-Sat-infected samples. This result suggests that, like siRNAs, the biogenesis of miRNAs is not affected by CMV Y-Sat infection despite the high levels of Y-Sat siRNAs. However, in this study, we did not test if miRNA-directed target gene silencing was affected by CMV Y-Sat infection. Given the lack of visible phenotypes in the CMV Y-Sat-infected plants, it is likely that CMV Y-Sat infection has little effect on target gene regulation by miRNAs.

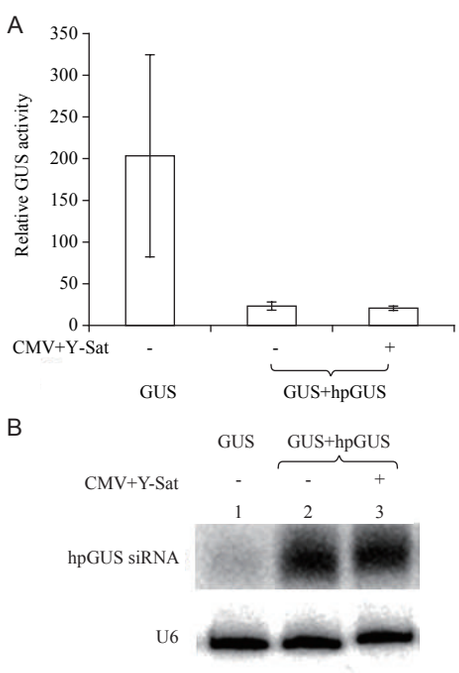

Fig. 3 The levels of *hpGUS*-induced GUS silencing (A) and *hpGUS*-derived siRNAs (B) are not affected by CMV Y-Sat infection. *Agrobacterium*-infiltration of transgenic *mCHLI* leaves were performed at 17 d post inoculation, and infiltrated leaf samples were collected and analysed at 3 d post infiltration. 5 μg of total protein was used in MUG assay to determine GUS activity (A), and 10 μg of total RNA was separated in denaturing polyacrylamide gel and hybridized with a RNA probe corresponding to the dsRNA stem region of the hpGUS sequence (B). U6 RNA was hybridized for use as loading control (B).

CONCLUSION

Our results suggest that a high level of exogenous siRNAs has no significant effect on host sRNA biogenesis and sRNA-directed target gene silencing. This implies that the key RNA silencing machineries, including DCLs and AGOs, are not saturable by exogenous siRNAs. Thus, transformation of plants with RNAi constructs such as the hpRNA constructs is unlikely to cause abnormal plant development due to interference with endogenous sRNA pathways.

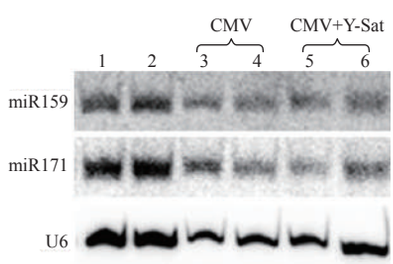

Fig. 4 CMV or CMV Y-Sat infection does not affect the accumulation of miR159 and miR171 in transgenic *mCHLI* plants. 10 μg of total RNA from uninfected (lanes 1-2), CMV-infected (lanes 3-4) or CMV Y-Sat-infected (lanes 5-6) plants were separated in denaturing polyacrylamide gel and hybridized with an antisense miR159 or miR171 oligonucleotide probe. The stronger bands in the uninfected samples (lanes 1-2) are due to over-loading of RNA samples as indicated by the stronger U6 RNA band.

However, our study focused on siRNAs derived from a hpRNA transgene and a viral satellite RNA, both of which are processed by DCL4/2/3, but not by DCL1 that is required for miRNA biogenesis. It would be interesting to examine if the expression of artificial miRNAs, which depend on miRNA machineries for processing and function, would affect the biogenesis of endogenous miRNAs and their function. However, there has been no report of abnormal phenotypes associated with the expression of artificial miRNA in transgenic plants. Thus, exogenous siRNAs, either from infecting viruses or from RNAi-inducing transgenes, are unlikely to have a direct effect on plant growth and development unless they have a target sequence in the plant genome.

MATERIALS AND METHODS

Plant growth, virus inoculation, *Agrobacterium* infiltration and MUG assay of GUS expression

Nicotiana benthamiana plants used for transformation were grown on MS medium in a 26°C growth room with 16 h/8 h light/dark cycle. Both wild type and transgenic *N. benthamiana* plants for viral inoculation were grown in a 25°C glasshouse with natural light. Infection of *N. benthamiana* with *Cucumber mosaic virus* (CMV) plus Y-Sat was performed as previously described (Smith *et al.* 2011). Basically, 3-4 wk old *N. benthamiana* plants were dusted with carborundum and rub-inoculated with leaf extracts of previously viral-infected leaves in 0.1 mol L^{-1} phosphate buffer (pH 7.2). *Agrobacterium* infiltration was performed as previously described (Liu and Lomonossoff 2002). Essentially, actively growing *Agrobacterium* cells containing plant expression constructs were suspended in infiltration buffer (10 mmol L^{-1} $MgCl_2$ and 150 μg mL^{-1} acetosyringone) and adjusted to a final optical density of 0.7 at 600 nm. The suspensions were incubated at room temperature for 3 h and infiltrated into expanded leaves with flat-pointed syringe. GUS expression was quantitatively measured using fluorometric MUG (4-methyl-umbelliferyl-β-D-gulcuronide) assay as described previously (Chen *et al.* 2005).

Agrobacterium-mediated transformation of *N. benthamiana*

Transformation of *N. benthamiana* was performed using the leaf disc method as described previously (Ellis *et al.* 1987). The Y-Sat siRNA-resistant *mCHLI* construct (Smith *et al.* 2011) was transferred into *Agrobacterium tumefaciens* strain LBA4404 *via* triparental mating (Ditta *et al.* 1980; Ellis *et al.* 1987). *Agrobacterium* cells were grown for 2 d at 28°C on LB medium with appropriate antibiotics, harvested by centrifugation and resuspended in MS broth. Actively growing *N. benthamiana* leaves were cut into small pieces (about 1 cm^2) and immersed in *Agrobacterium* cell suspension for 5 min. The leaf sections were then transferred to the MS0 plates and incubated for 48 h at 26°C. The leaf disks were washed in sterilized H_2O and transferred to MS9 shoot regeneration medium containing timentin (150 mg L^{-1}) and kanamycin (50 mg L^{-1}) at 26°C. Newly formed shoots (~1 cm long) were excised and transferred to MS4 rooting medium containing the same antibiotics (Ellis *et al.* 1987). Plants with established roots were transferred to soil and allowed to grow under normal growth conditions.

Ball-bearing DNA extraction and PCR

DNA was isolated from *N. benthamiana* leaves using the ball-bearing methods as described previously (Colosi and Schaal 1993). Small leaf discs (~0.4 cm^2) were collected in PCR tubes containing 75 μL 0.5× CTAB and a ball bearing. Homogeniztion of tissues was carried out for 2 min at 30 s^{-1} frequency in a Tissuelyser (Qiagen, German). 50 μL chloroform was added to each tube and mixed vigorously. The tubes were then centrifuged at 3 800 r min^{-1} for 5 min. 0.5 μL supernatant was used as template in the 20 μL of PCR. For the detection of *mCHLI* in the transgenic plants, primers corresponding the mCHLI sequence (5′-CCGTACGTGACGCCGAATTACG-3′) and the OCS terminator (5′-GAGCTACACATGCTCAGG-3′) were used with the following cycling conditions: 1 cycle of 95°C, 3 min; 35 cycles of 95°C, 30 s, 54°C, 30 s, and 72°C, 1 min, and 1 cycle of 72°C, 5 min. PCR products were analyzed in 1% agarose gel.

RNA extraction and Northern blot hybridization

Total RNA was isolated from *N. benthamiana* leaves using Trizol reagent (Invitrogen, Australia) with overnight isopropanol precipitation to maximize recovery of the small RNA fraction. Northern blot hybridization was performed as previously described (Smith *et al.* 2010). For mRNA detection, total RNA was separated in formaldehyde-agarose gel and blotted onto a Hybond-N membrane (Amersham Life Science, USA), and then hybridized with [α-^{32}P] UTP-labelled CMV RNA3a or Y-Sat probes obtained by *in vitro* transcription (Smith *et al.* 2011). For small RNA Northern blot hybridization, approximately 10 μg of total RNA was separated in 17% urea-polyacrylamide gel, and blotted onto a Hybond-N^+ membrane (Amersham Life Science, USA). For small RNA hybridization, [α-^{32}P] UTP-labelled RNA

probe as in mRNA Northern hybridization was fragmented by treatment in carbonate buffer to reduce the probe to an average size of ~50 nucleotides (Wang *et al.* 2001); [γ-^{32}P] ATP-labelled locked nucleic acid probes were synthesized as described previously (Jones *et al.* 2006; Eamens *et al.* 2011) and used for detecting miR159 and miR171.

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检索结论	经检索，柑桔研究所申晚霞所提交的 1 篇论文被 SCI 收录，收录检索结果详细情况见附件 1 和附件 2。 检索人（签名）：李娇 职称：馆员 教育部科技查新工作站 2014 年 09 月 02 日
备注	本报告共 5 页（含封面）。

附件 1：SCI 收录

个人排名	题名	SCI 检索号	影响因子	分区(2012)		出版时间	语种	出版地
				学科	分区			
1	High-Level Accumulation of Exogenous Small RNAs Not Affecting Endogenous Small RNA Biogenesis and Function in Plants	000336107500011	IF_{2013}=0.625	AGRICULTURE, MULTIDISCIPLINARY	3 区	2014 年	English	国外

附件 2：
SCI 收录
标 题： High-Level Accumulation of Exogenous Small RNAs Not Affecting Endogenous Small RNA Biogenesis and Function in Plants
作者: Shen, WX (Shen Wan-xia); Smith, NA (Smith, Neil A.); Zhou, CY (Zhou Chang-yong); Wang, MB (Wang Ming-bo)
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