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SHORT COMMUNICATION

Effects of RNA silencing during antagonism between citrus exocortis viroid and citrus bark cracking viroid in Etrog citron (*Citrus medica*)

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Abstract

Citrus exocortis viroid (CEVd) and citrus bark cracking viroid (CBCVd) are two important viroids that infect citrus plants and frequently occur as mixed infections in orchards. However, the mechanism of antagonism between the two viroids in mixed infections remains unclear. The CEVd/CBCVd–citron system and small RNA sequencing (sRNA-seq) were used to study the antagonism. When CBCVd was inoculated before CEVd, the CEVd titre was significantly reduced and the symptoms were attenuated. Viroid-derived sRNAs (vd-sRNAs) from CEVd and CBCVd were predominantly 21-nucleotide (nt) and 22-nt in length and had similar 5′ base biases. Homologous sequences of the two viroids in the terminal right (TR) region are rich in vd-sRNAs, and the high frequency vd-sRNAs selected from the CBCVd TR region can be used to degrade the transcripts of CEVd in vivo directly. These results suggest that RNA silencing may play an important role in the antagonism of the two viroids, thus deepening our understanding of the molecular interaction of long noncoding RNAs in woody plants.

KEYWORDS
antagonism, citrus bark cracking viroid (CBCVd), citrus exocortis viroid (CEVd), RNA silencing, small RNA

Yuanjian Qiu and Yafei Wang contributed equally to this work.

Viroids, the simplest known pathogens infecting plants, are one kind of long noncoding RNA molecule of 246–434 nucleotides (nt), with a single-stranded, covalently closed circular genome (Wang, 2021). Viroids replicate and move in hosts, eliciting disease in some plants, such as vegetables, flowers, and fruit trees (Navarro et al., 2021). Citrus is a collection of perennial woody *Rutaceae* plants infected by abundant viruses and viroids, including eight known viroids, such as citrus exocortis viroid (CEVd) and citrus bark cracking viroid (CBCVd, or citrus viroid-IV, CVd-IV; Chambers et al., 2018; Duran-Vila et al., 1986; Ito et al., 2001; Serra et al., 2008; Wang et al., 2018). Citrus viroids are widespread, and they are often mixed in orchards around the world. CEVd is the causative agent of exocortis disease, which is described as a bark-scaling disorder on trifoliate orange (*Poncirus trifoliata*) and its hybrids (Duran-Vila et al., 1988; Semancik & Weathers, 1972). An interesting phenomenon was observed in field investigations that citrus trees manifesting mild bark-scaling and exocortis-scaling symptoms were infected by both CEVd and CBCVd (Vernière et al., 2006), thus suggesting that co-infection may moderate the virulence of CEVd. However, the mechanism of interaction between the two viroids remains to be elucidated.

There are two opposite types of interactions between two viruses or two strains of the same virus co-infecting the same plants, termed antagonism and synergism (Chavez-Calvillo et al., 2016; Crespo et al., 2019; Syller, 2012). Similar situations also occur in plants co-infected with two viroids (Pallas & Flores, 1989; Serra et al., 2008). Antagonism, also known as cross-protection in virology, is a common ecological relationship between viruses and viroids in the same niche (Duran-Vila & Semancik, 1990; Folimonova et al., 2010), which can be interpreted as the ability of a pre-infected mild viroid to hinder symptom expression and replication of a severe viroid strain (Niblett et al., 1978; Power, 1996). Although cross-protection measures have been applied in glasshouses and fields (Fulton, 1986; Hanssen et al., 2010), the underlying mechanism is still not well elucidated (Ziebell & Carr, 2010). One possible explanation is that RNA silencing affects the challenging viral RNA by sequence-specific degradation (Fagoaga et al., 2006; Ratcliff et al., 1999).

The RNA silencing machinery of plants can be induced by double-stranded RNAs (dsRNAs) and involves three basic components, namely, Dicer or Dicer-like (DCL), RNA-dependent RNA polymerase (RDR), and Argonaute (AGO) family proteins (Baulcombe, 2004; Ding, 2023; Guo et al., 2016). Previous studies have shown that viroidal RNAs are activators and targets of RNA silencing in the host (Itaya et al., 2001; Wang et al., 2019; Zheng et al., 2017). Conversely, viroid-derived siRNAs (vd-siRNAs) produced thereafter by the host also play a key role in the pathogenesis of viroids by targeting RNA transcripts of host genes (Bao et al., 2019; Delgado et al., 2019). Because of the common sequence and structural features between the CEVd and CBCVd genomes (Semancik & Vidalakis, 2005), the antagonism of CBCVd on CEVd in citrus plants may be related to RNA silencing and vd-siRNAs.

To systematically study the antagonistic mechanism of the two viroids in citron, CEVd and CBCVd sources were separately isolated and analysed to construct infectious clones, as described previously (Wang et al., 2019; Figure S1a, Table S1 and File S1). The pGEM-T Easy vector, into which was ligated the viroid dimeric sequence, was linearized by the restriction enzyme SpeI and transcribed in vitro using T7 RNA polymerase (Promega). Citron plants infected with only CEVd or CBCVd were obtained by slash inoculation with buffer containing 150 ng of viroid RNA, and used as the initial inoculum sources. Twenty healthy citron plants and 20 CBCVd-infected citron plants obtained by bark grafting were prepared. After 2 months of growth in the greenhouse, 10 healthy and 10 CBCVd-infected citron plants were inoculated with CEVd by bark grafting. All citron materials were grown in the greenhouse, and the symptoms were observed 3 months after CEVd inoculation.

As shown in Figure 1a, healthy citron plants grew well without any symptoms, whereas viroid-inoculated citron plants exhibited the typical symptoms of leaf epinasty, dwarfing and necrosis. CEVd single-infected (CEVd-SI) citron plants showed severe dwarfing and new leaves on the top were severely curled with necrosis of the midrib and petiole. CBCVd single-infected (CBCVd-SI) citron plants showed intermediate symptoms, and the top new leaves were curled but without the petiole necrosis symptom. The combined infection of the two viroids caused relatively mild dwarfing symptoms compared to the CEVd-SI citron plants and no petiole necrosis symptom occurred. From the perspective of symptoms, CBCVd had an antagonistic effect on CEVd in the early stages of co-infection.

Northern blotting and reverse transcription (RT)-PCR experiments were performed to verify the infection of CBCVd and CEVd in citron plants (Figures 1b,d and S1b,c). The results indicated that the two viroids successfully infected citron plants, but their RNA accumulations under the single and co-infection conditions were significantly changed in the opposite direction (Figure 1b–e). Replication of CEVd (Figure 1b,c) decreased, while that of CBCVd increased (Figure 1d,e) in the co-infected citrons compared to the single infections. These trends in co-infection were also supported by RT-PCR detection and dot-blot hybridization of viroidal RNAs (Figure S1b–e). Moreover, another experiment was conducted using CBCVd single-inoculated, CEVd single-inoculated and CBCVd/CEVd simultaneous-inoculated to verify the antagonistic effects observed in mixed infection of CEVd-CBCVd in *Citrus medica*. The results of symptoms and dot-blot hybridization assays indicated that CBCVd and CEVd inoculated simultaneously also caused milder symptoms and lower viroid titres compared to CEVd inoculated singly (Figure S2). Taken together, it is thought that CBCVd has an antagonistic effect on CEVd replication during the early infection stage, which is consistent with the simultaneous suppression of CEVd-associated symptoms.

One possible explanation for antagonism is that RNA silencing exerts an effect on challenging viral RNA by sequence-specific degradation (Fagoaga et al., 2006; Ratcliff et al., 1999). To examine whether RNA silencing plays a role in the antagonism of the two citrus viroids, the expression levels of RNA-silencing components with CBCVd and/or CEVd infection were measured. In contrast to CEVd single infection on citron in previous studies, CBCVd single infection and two viroids co-infection only induced the upregulation of the

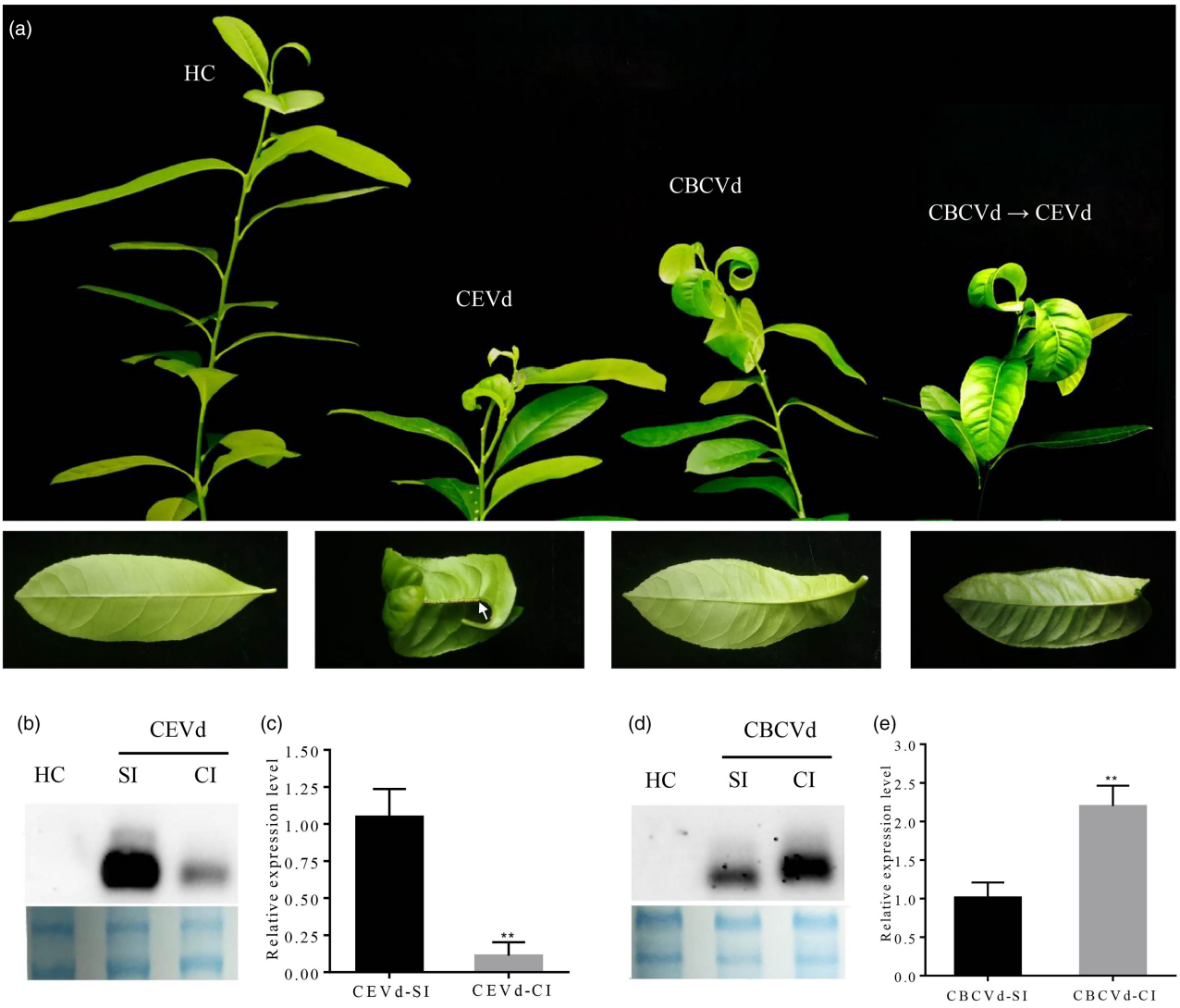

FIGURE 1 Symptoms of citron plants from different treatments and accumulation levels of viroid RNA at 3 months post-inoculation (mpi). (a) The Etrog citron plants exhibited dwarf and leaf-curl symptoms. Magnified symptoms on the leaves are shown aligned under the corresponding plants with a white arrowhead indicating necrotic lesions: healthy control (HC), single infections of CEVd (citrus exocortis viroid), CBCVd (citrus bark cracking viroid), and stepwise infection of CBCVd→CEVd. (b) and (d) Comparison of the titres of CBCVd/CEVd single-inoculation (SI) and the two viroid co-inoculation (CI) by northern blotting. Total RNAs (4 μg) were fractionated by electrophoresis in 2% (wt/vol) agarose gels and hybridization with digoxigenin (DIG)-labelled viroid complementary RNA (cRNA) probe. Ribosomal RNAs (rRNAs) stained with methylene blue are used as loading controls. (c) and (e) Relative levels of CEVd and CBCVd RNA as determined by reverse transcription-quantitative PCR (RT-qPCR). Experiments were performed in eight citron plants and the data are expressed as the mean ± *SD*. Error bars indicate the *SD* of eight biological replicates of RT-qPCR analysis. $^{**}p < 0.01$ using Student's *t* test.

expression of genes *CmDCL2* and *CmSDE3*, without the upregulation of the expression of other RNA silencing genes (*CmRDR1*, *CmAGO2*, *CmAGO7*, *CmDCL1* and *CmDCL4*; Figure 2). Given that DCL2 participates in generating 22-nt siRNAs, four small RNA libraries were prepared for deep sequencing to assess the expression and characteristics of vd-siRNAs in citron plants. Despite different inoculations with CBCVd and/or CEVd, and whether they were of sense strand or antisense strand origin, 22-nt vd-siRNAs associated with DCL2 were highly expressed as the predominant vd-siRNAs, as expected (Figure S3). The proportion of CEVd vd-siRNAs in total sRNAs was significantly decreased in co-infection (CEVd-CI, 43,522, 0.19%) compared to single infection (CEVd-SI, 1,485,910, 4.99%), probably affected by low viroid titre, while little change was found for CBCVd vd-siRNAs (CBCVd-SI, 257,350, 0.95%; CBCVd-CI, 217,685, 0.98%) (Figure 3 and Table S2). vd-siRNAs with a 5′-end nt preference toward U and C corresponding to AGO1 or AGO5, respectively, are the major vd-siRNAs in count (Minoia et al., 2014; Figure S3). It was predicted that similar RNA-silencing pathways were activated in citrons on different viroidal infections.

The distribution of vd-siRNAs in the viroidal sense or antisense strand was visualized by mapping vd-siRNAs in the corresponding genomes (Figure 3), and the frequency of identical siRNAs was counted for ranking. The results showed one common hotspot of vd-siRNAs in the terminal right (TR) region of the genome (Figure 3,

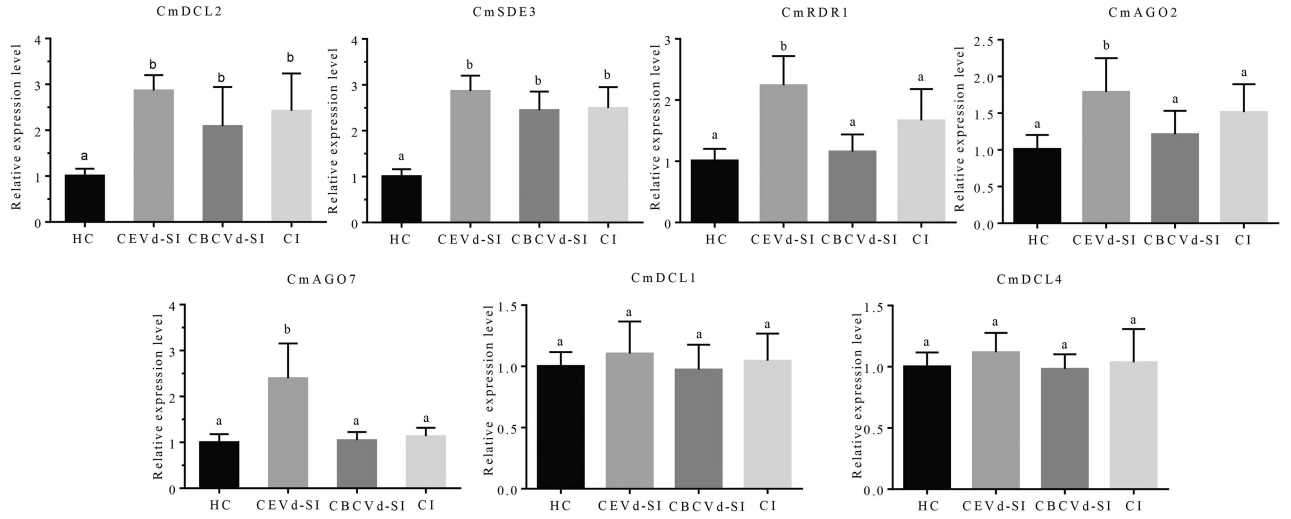

FIGURE 2 Relative expression of RNA silencing pathway genes with reverse transcription-quantitative PCR in Etrog citron (*Citrus medica*). HC, healthy control; CEVd/CBCVd-SI, CBCVd/CEVd single-inoculation; CI, the two viroids co-inoculated (CI) to citron plants. Experiments were performed in eight citron plants and the data are presented as the mean ± *SD*. The expression levels of citron *actin* were used as internal reference. Bars with different letters above them differ significantly at $p < 0.05$ using Student's *t* test.

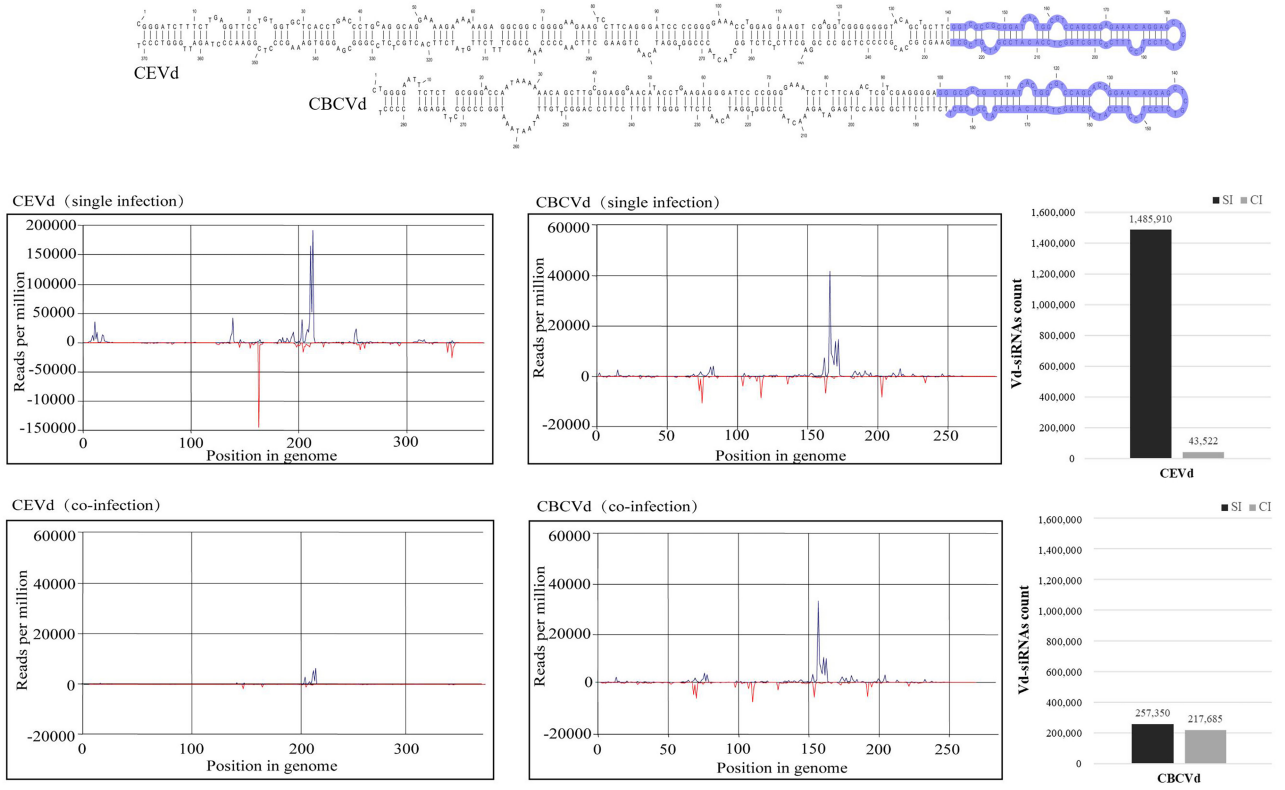

FIGURE 3 Analysis of hot spot regions on viroid-derived small RNAs. The histograms compare the total counts of unique vd-sRNAs from CEVd/CBCVd single-infected and co-infected Etrog citron.

highlighted in blue). Comparing the genomic sequences of CEVd and CBCVd, the TR regions were also highly homologous, with 89.4% sequence identity (nine nucleotides difference) in aligned sequences of 85 nt in length (red box in Figure S1a). Moreover, the top three vd-siRNAs by frequency were all derived from TR regions.

To further study whether vd-siRNAs from TR regions can directly silence CEVd transcripts, a transient reporter gene system was used. The whole complementary genome sequence of CEVd was transcriptionally fused to the green fluorescent protein (GFP) coding sequence in the pCAMBIA1302-35S-mGFP vector. Two viroid-specific vd-sRNAs with the highest frequency from CBCVd, CB-sRNA1 and CB-sRNA2, and one with the highest frequency from CEVd, CE-sRNA1, were selected to construct a plant expression vector with the pCAMBIA-35S-GN-NOS-OsamiR528 vector as backbone. We

conducted co-infiltration of the pCAMBIA1302-35S-mGFP:CEVd vector with the transient sRNA expression vector or empty vector control into cv. Etrog citron or *Nicotiana benthamiana* at the four-leaf stage. As shown in Figure 4a,b, co-infiltration of CB-sRNA1, CB-sRNA2 or CE-sRNA1 vector with GFP:CEVd vector showed less GFP fluorescence than GFP:CEVd alone or co-infiltrated with the empty vector. The results of confocal laser scanning microscopy and RT-quantitative PCR (RT-qPCR) on *N. benthamiana* confirmed that fused GFP:CEVd transcripts were degraded when co-infiltrated with sRNA vector expressing CB-sRNA1, CB-sRNA2 and CE-sRNA1 (Figure 4c,d). In addition, the result of 5′ RACE PCR showed that the CEVd transcripts were degraded by sRNA and the cleavage site was between positions 10 and 11 of CB-sRNA1 (Figures 4e,f and S4a). We also performed relevant experiments on citrus, and the GFP signal was weaker in CBCVd-infected plants when the CEVd-GFP reporter was used (Figure S4b,c). These results indicate that the sRNAs from TR regions of CBCVd or CEVd can cleave CEVd transcripts and hinder the replication of CEVd in citron plants.

Antagonism is common in fungi, bacteria and viruses (Moreno & Lopez-Moya, 2020; Syller & Grupa, 2016). A similar phenomenon has been observed in citrus orchards co-infected with CEVd and CBCVd (Vernière et al., 2006). Due to the lack of an infectious clone and an efficient research system, studies on the interaction between CEVd and CBCVd are limited. Here, we built a research system by constructing their infectious clones using a one-step fast RT-PCR method and Etrog citron sensitive to them as indicator plants for symptom observation.

Although the phenomenon of antagonism between CBCVd and CEVd was discovered many years ago, knowing why and how CBCVd excludes CEVd is not completely understood. In this study, the mild leaf-curl symptom occurred on Etrog citron plants when inoculated with CBCVd dimeric RNAs alone. Similar symptoms caused

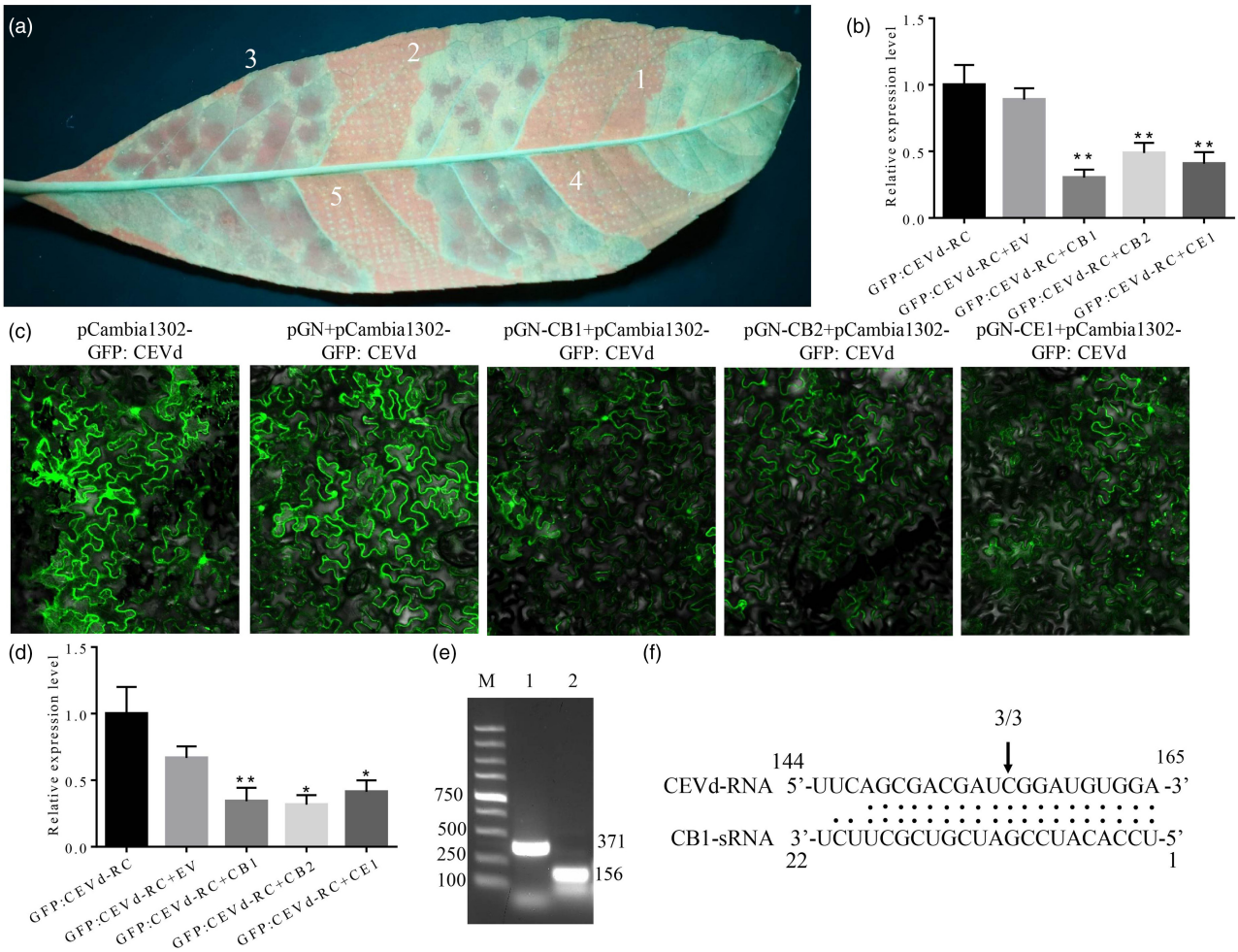

FIGURE 4 Predicted targeting of the CEVd transcripts by vd-sRNA. Etrog citron and *Nicotiana benthamiana* leaves were agroinfiltrated with (1) empty pCambia1302-GFP:CEVd vector (2) GFP:CEVd plus empty pGN vector, (3) GFP:CEVd plus pGN-CB1 sRNA, (4) GFP:CEVd plus empty pGN-CB2 sRNA vector, (5) GFP:CEVd plus pGN-CE1 sRNA vector. At 3 days post-infiltration (dpi), the Etrog citron leaves were photographed under UV illumination (a) and tested by reverse transcription-quantitative PCR (RT-qPCR) (b), while *N. benthamiana* leaves were examined for green fluorescence under a confocal laser scanning microscope (c) and RT-qPCR (d). (e) Nested PCR products obtained from 5′ RACE PCR and separated by 1.5% agarose gel electrophoresis. 1, empty pCambia1302-GFP:CEVd vector; 2, GFP:CEVd plus pGN-CB1 sRNA. (f) Predicted structure of the CEVd mRNA/vd-sRNA duplex formed by siRNAs derived from the CBCVd. Arrows indicate the 5′ termini of CEVd mRNA fragments isolated from the CB1-sRNA/CEVd co-infected plants, with the frequency of termini shown.

by CBCVd and CEVd on citron led us to suspect that their interactions were due to homologous sequences. The terminal right region was shown to be the homologous region with the most abundant vd-siRNAs. Previous studies have shown that the TR region can bind to a plant nuclear import protein (viroid RNA-binding protein 1, Virp1) to promote the endoduplication and movement of viroids (Gozmanova et al., 2003; Maniataki et al., 2003). Intriguingly, pre-inoculated CBCVd, which shares a homologous TR region with CEVd, resulted in attenuated symptoms and lower viroid titres than those of CEVd inoculated alone. In addition, the distribution patterns of vd-siRNAs of the two viroids were similar in TR regions, with 21-nt and 22-nt vd-siRNAs being predominant. As vd-siRNA is one core effector of RNA silencing in plant defence mechanisms, the role of vd-siRNAs and the related RNA silencing genes in antagonizing citrus viroids are poorly understood. It has been reported that the expressions of RNA silencing genes *DCL2*, *RDR1*, *AGO2*, *AGO7* and *SDE3* were upregulated in CEVd single-infected citron plants (Wang et al., 2019). Genes whose expression was upregulated in CBCVd single infection and co-infection with CEVd also included *DCL2* and *SDE3*. Additionally, the upregulated expression of the *SDE3* gene, which encodes RNA helicase in *Arabidopsis*, has been implicated in producing secondary siRNAs for posttranscriptional gene silencing (Dalmay et al., 2001; Garcia et al., 2012). In another aspect, due to 22 nt vd-siRNAs that are probably generated by citrus DCL2 homologue being the most abundant, we randomly selected one (CB-sRNA1) from the CBCVd-TR region and demonstrated its ability to degrade CEVd transcripts. This may explain why the titre of CEVd in co-infected citron plants are also decreased. Thus, it is rational to propose that vd-siRNAs from the TR region of the pre-inoculated CBCVd disrupted the replication and movement of CEVd through RNA silencing.

These data suggest that inoculated CBCVd antagonizes CEVd in citron plants. As for the titres of CBCVd in two viroids co-infected plants, which increased 2.19-fold compared to that in single-inoculated plants, we speculate that CBCVd could be more likely to avoid AGO-guided silencing than CEVd due to the differences in secondary structure (Itaya et al., 2007). Some viruses exhibit antagonism or synergism depending on the order of infection in plants (Chavez-Calvillo et al., 2016). It would be interesting to confirm whether CBCVd and CEVd exhibit different interactions when the inoculation order changes. The vd-siRNAs from the CBCVd-TR region can affect the replication of CEVd due to high sequence similarity, but the function of the CEVd-TR region itself remains to be determined by site-mutation and chimeric viroids.

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DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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