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Superinfection exclusion by *Citrus tristeza virus* does not correlate with the production of viral small RNAs

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ABSTRACT

Superinfection exclusion (SIE), a phenomenon in which a preexisting viral infection prevents a secondary infection with the same or closely related virus, has been described for different viruses, including important pathogens of humans, animals, and plants. Several mechanisms acting at various stages of the viral life cycle have been proposed to explain SIE. Most cases of SIE in plant virus systems were attributed to induction of RNA silencing, a host defense mechanism that is mediated by small RNAs. Here we show that SIE by *Citrus tristeza virus* (CTV) does not correlate with the production of viral small interfering RNAs (siRNAs). CTV variants, which differed in the SIE ability, had similar siRNAs profiles. Along with our previous observations that the exclusion phenomenon requires a specific viral protein, p33, the new data suggest that SIE by CTV is highly complex and appears to use different mechanisms than those proposed for other viruses.

Introduction

Superinfection exclusion (SIE), also referred to as homologous interference, is a phenomenon in which a preexisting viral infection prevents a secondary infection with the same or closely related virus. First observed between strains of *Tobacco mosaic virus* (McKinney, 1926, 1929), SIE was found to be common for viruses in different systems, including important pathogens of humans, animals, and plants (Salaman, 1933; Bennett, 1951; Steck and Rubin, 1966a, 1966b; Bratt and Rubin, 1968; Hull and Plaskitt, 1970; Johnston et al., 1974; Whitaker-Dowling et al., 1983; Adams and Brown, 1985; Fulton, 1978; Delwart and Panganiban, 1989; Lecoq et al., 1991; Wen et al., 1991; Strauss and Strauss, 1994; Karpf et al., 1997; Singh et al., 1997; Kong et al., 2000; Hull, 2002; Geib et al., 2003; Gal-On and Shiboleth, 2005; Lee et al., 2005; Wildum et al., 2006). The phenomenon plays an important role in the pathogenesis and evolution of viral populations, and, therefore, has clear implication in treating viral infections. With plant viruses, for instance, SIE has been used as a tool to reduce infection and crop losses due to severe virus isolates by purposely pre-infecting plants with mild isolates of the virus, a procedure that has been referred to as 'cross-protection' (reviewed in Hull, 2002; Gal-On and Shiboleth, 2005). With viral diseases of animals and humans, the phenomenon was thought to decrease evolution of drug and vaccine resistance by limiting virus recombination and, consequently, variability, thus aiding the development of antiviral treatments in the medical and veterinary fields (Webster et al., 2013). On the other hand, in some situations SIE showed negative effect by interfering with repeated applications of virus-based vaccines to individuals with persistent infections (Strauss and Strauss, 1994; Ehrengruher and Goldin, 2007).

Several mechanisms acting at various stages of the viral life cycle have been proposed to explain SIE. For animal and human viruses, those included prevention of the incoming virus entry into cells (Steck and Rubin, 1966a, 1966b; Lee et al., 2005), inhibition of translation or interference with replication (Adams and Brown, 1985; Karpf et al., 1997; Lee et al., 2005; Schaller et al., 2007). For plant viruses, initial explanations included competition between primary and challenging viruses for host factors or intracellular replication sites and interference with disassembly of the secondary virus resulting from the expression of the coat protein by the primary virus (Sherwood and Fulton, 1982; Abel et al., 1986; Lu et al., 1998; Beachy, 1999; Bendahmane and Beachy, 1999; Hull, 2002; Gal-On and Shiboleth, 2005; Ziebell and Carr, 2010). However, most cases of homologous interference in plant virus systems have been attributed to induction of RNA silencing, a host surveillance mechanism that is mediated by small RNAs and plays important roles in various regulatory processes, including the

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defense against viruses (Baulcombe, 2004; Ding and Voinnet, 2007). RNA silencing relies on a set of conserved reactions that are triggered by double-stranded RNA (dsRNA) and lead to a homology-dependent degradation of RNA molecules (Voinnet, 2005; Ding and Voinnet, 2007). According to this model, dsRNAs of the primary virus, such as structured regions in the genome or replication intermediates, are recognized by the RNA silencing machinery and cleaved into small interfering RNAs (siRNAs) of 21–24 nt in length by an RNase III-type enzyme Dicer. These siRNAs, which represent a hallmark feature of RNA silencing in all organisms, are incorporated into the multisubunit RNA-induced silencing complex and guide degradation of RNA sequences that share perfect or near perfect homology with siRNAs, such as those of the incoming challenge virus (Ratcliff et al., 1997, 1999; reviewed in Hull, 2002). Primary silencing-based antiviral response is further strengthen by the function of host RNA-dependent RNA polymerases, which are thought to use viral templates to produce dsRNA substrates for secondary siRNA synthesis (Mourrain et al., 2000; Yu et al., 2003). siRNAs also appear to function as a mobile signal that spreads to more distant tissues ahead of the invading virus, thus generating the defense response against the same or sequence-related virus at the systemic level (Hamilton et al., 2002; Dunoyer et al., 2010).

Indeed, plant viruses have been shown to be strong inducers as well as targets of RNA silencing (reviewed in Voinnet, 2001, 2005; Ding and Voinnet, 2007). For many different viruses, the accumulation of viral siRNAs was reported at the sites of the initial virus invasion and in systemic tissues of infected plants and was correlated with lowering virus titer (Hamilton and Baulcombe, 1999; Szittya et al., 2002, 2010; Molnar et al., 2005; Pantaleo et al., 2007; Donaire et al., 2008, 2009; Qu, 2010). Furthermore, the recovery phenotype, a long-time known characteristic feature of the infection course of a number of plant viruses, which is manifested as attenuation or elimination of the symptoms in newly developed leaves after the initial symptomatic infection coupled with reduction of virus accumulation and sequence-specific resistance to further virus infection, was linked to RNA silencing (Covey et al., 1997; Ratcliff et al., 1997, 1999). Additionally, it was found that many instances of pathogen-derived resistance to viruses appear to be explained based on RNA silencing (reviewed in Goldbach et al., 2003; Sudarshana et al., 2007; Prins et al., 2008; Simon-Mateo and Garcia, 2011). Transgene or transient expression of virus sequences, in some cases shorter than 100 nucleotide residues, was shown to confer resistance against homologous viruses in experimental and natural hosts (Wesley et al., 2001). Best results were achieved using constructs that encoded self-complementary RNA sequences derived from the genomes of target viruses. These constructs appeared to be highly potent initiators of RNA silencing apparently due to the dsRNAs generated upon their transcription being fed directly into the silencing pathway, thus, leading to nearly 100% efficiency against homologous viruses (Smith et al., 2000; Helliwell and Waterhouse, 2003; Wesley et al., 2001). In such studies, the level of virus resistance was positively correlated with generation of siRNAs from different parts of the transgene (Kalantidis et al., 2002; Chen et al., 2004; Bucher et al., 2006; Leibman et al., 2011). Along with these observations, it was demonstrated that incorporation of cognate sequences into genomes of replicating heterologous viruses could trigger degradation of RNA molecules containing these sequences. To this end, the pioneering work of Ratcliff et al. (1999) showed that primary infection of *Tobacco rattle virus* carrying the green fluorescent protein (GFP) gene exhibited cross protection against challenge inoculation of *Potato virus X* encoding a fragment of the GFP ORF. The results obtained in that study have been later reproduced with other combinations of viruses in which the primary and challenging viruses shared a common genomic fragment (Tamura et al., 2013). Protection against the challenge virus was correlated with the amplification of siRNAs corresponding to the shared common sequence upon infection with the primary virus, indicating that the primary virus triggered silencing to the target region. Altogether, these findings supported the hypothesis attributing homologous interference of viruses to a small RNAs-mediated mechanism.

We are examining SIE by *Citrus tristeza virus* (CTV). CTV is a member of the family *Closteroviridae*, which contains viruses with mono-, bi-, and tripartite genomes (Bar-Joseph et al., 1979; Dolja et al., 1994, 2006; Agranovsky, 1996; Karasev, 2000). CTV has long flexuous virions (2000 nm × 10–12 nm) encapsidated by two coat proteins and a single-stranded positive-sense RNA genome of approximately 19.3 kb. The major coat protein (CP) covers about 97% of the genomic RNA, and the minor coat protein (CPm) encapsidates the rest of the genome at its 5′ end (Febres et al., 1996; Satyanarayana et al., 2004). The RNA genome of CTV encodes twelve open reading frames (ORFs) (Pappu et al., 1994; Karasev et al., 1995) (Fig. 1). ORFs 1a and 1b are expressed from the genomic RNA and encode polyproteins required for virus replication. Ten 3′ end ORFs are expressed by 3′ co-terminal

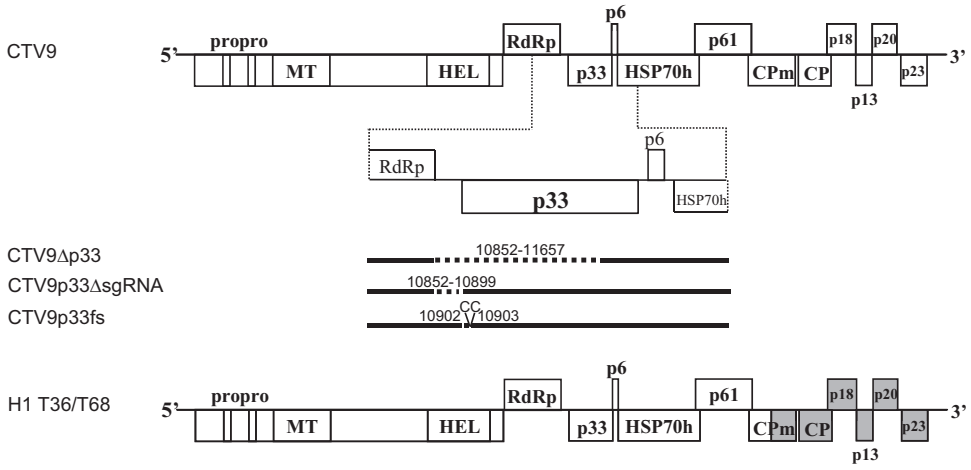

Fig. 1. Schematic diagram of the genome organization of wild type CTV T36 (CTV9) and its derivatives. The open boxes represent ORFs and their translation products. PRO, papain-like protease domain; MT, methyltransferase; HEL, helicase; RdRp, an RNA-dependent RNA polymerase; HSP70h, HSP70 homolog; CPm, minor coat protein; CP, major coat protein. The enlarged view of the region containing the p33 ORF and schematic diagrams of CTV mutants are shown below. The sequences deleted in mutants are indicated by dotted lines with corresponding nucleotide numbers. Solid lines represent sequences present in the genomes of mutants. "CC" indicates two extra cytidylates inserted in CTV9p33fs construct. Sequences substituted from the genomes of T68-1 isolate are shown in gray.

subgenomic RNAs (sgRNAs) (Hilf et al., 1995, Karasev et al., 1997) and encode CP, CPm, p65 (HSP70 homolog), and p61 that are involved in assembly of virions (Satyanarayana et al., 2000), a hydrophobic p6 protein with a proposed role in virus movement (Dolja et al., 2006; Tatineni et al., 2008), p20 and p23, which along with CP are suppressors of RNA silencing (Lu et al., 2004); and p33,

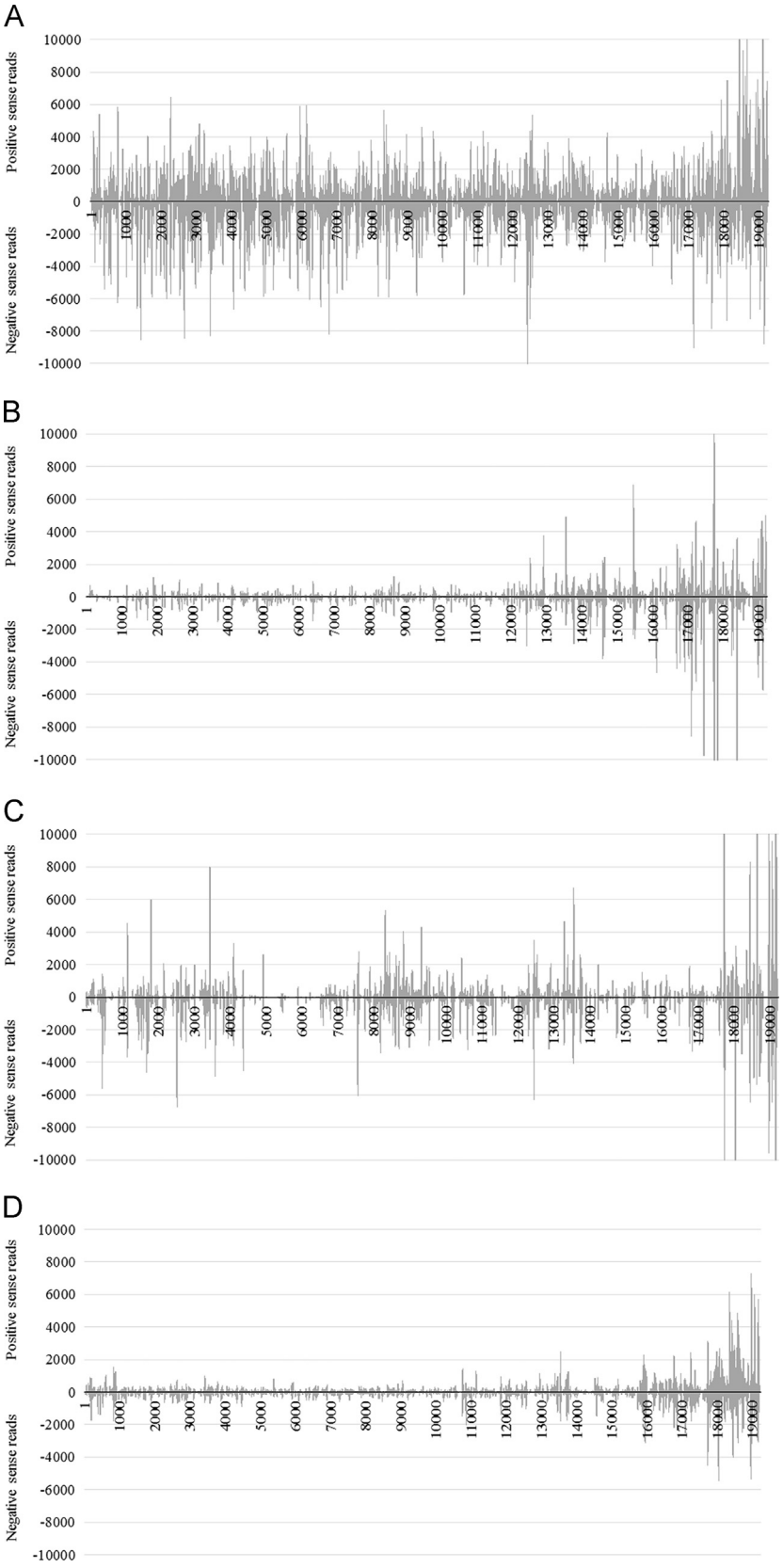

Fig. 2. Profiles of siRNAs produced by isolates (A) T36, (B) T30-1, (C) FL202 isolate of the VT strain, and (D) T68-1 upon their infection of *C. macrophylla* plants. The small RNA reads were mapped against the reference genome sequences of their respective strains.

p13, and p18. The latter three proteins function in extending the virus host range (Tatineni et al., 2011), however, most citrus varieties can be infected with mutants in which their genes were deleted (Tatineni et al., 2008).

Upon infection of citrus plants, CTV induces production of a large amount of siRNAs, which represent nearly 50% of the total small RNAs in most susceptible citrus varieties (Ruiz-Ruiz et al., 2011). CTV siRNAs are produced from all genomic regions, however the amount of siRNAs generated from the 5′ half and central areas of the genome is reduced, compared to large quantities that map preferentially at the 3′-terminal region (Ruiz-Ruiz et al., 2011). Recently, it was also demonstrated that CTV could silence an endogenous host gene: expression of citrus phytoene desaturase (PDS) gene sequences from CTV resulted in photo-bleaching phenotype, accompanied by synthesis of PDS-specific siRNAs and down-regulation of PDS mRNA in infected leaves (Hajeri et al., 2014). Therefore, the virus has a potential to induce silencing of RNA targets containing homologous sequences. With that, however, in spite of widely accepted hypothesis of the involvement of RNA silencing in homologous interference of plant viruses, our previous observations appeared not to support the idea that SIE by CTV is operated by an RNA silencing-based mechanism. The results of the study presented here further strengthen this conclusion.

Recently we demonstrated that SIE by CTV occurs only between isolates of the same strain, but not between isolates of different strains of the virus (Folimonova et al., 2010). This powerful mechanism completely prevents superinfection of a host plant by a homologous virus from the same virus strain. Remarkably, exclusion of a challenging isolate was absolute even though the primary virus infected only a small proportion of susceptible cells in a host. Not only the cells that contained the primary virus were protected, the cells that were not pre-infected became "immune" to the challenging virus (Folimonova et al., 2010), suggesting that the exclusion phenomenon appears to be systemic and must be able to spread beyond the infected cells.

Further data demonstrated that the phenomenon requires production of a specific viral protein, p33 (Folimonova, 2012). SIE was conferred by the protein rather than the RNA sequence: the mutants that retained the entire sequence of the p33 ORF, yet, had a deletion of the subgenomic mRNA controller element for the p33 sgRNA or a frameshift mutation within the p33 ORF failed to exclude the wild type virus as well as to exclude a secondary infection by the same mutant virus (Folimonova, 2012). In this study, we show that SIE by CTV does not correlate with the production of viral siRNAs or, in other words, production of specific siRNAs does not determine the ability of a virus variant to exclude another virus containing sequences homologous to those siRNAs. CTV variants, which expressed differences in the SIE ability, had similar siRNAs profiles. This argues that SIE by CTV is highly complex and appears to use different mechanisms than those proposed for other viruses.

Results and discussion

Isolates of heterologous strains of CTV share certain siRNAs

CTV has numerous isolates with distinctive biological and genetic characteristics, which can be classified into six major genotype groups or strains: T3, T30, T36, VT, T68, and RB (Harper, 2013). In our previous work we demonstrated that SIE by CTV works only between isolates of the same strain group and not between isolates of different strains. While isolates within the same strain show minor sequence divergence, generally less than 5% throughout the entire genome, isolates of different strains share nucleotide identity that ranges from 80.5% to 92.4% across the length of the genome (reviewed in Harper, 2013). Diversity between the genomes of the latter isolates is greater in the 5′ half region, with levels of sequence identity being as low as 72.3–90.3%. At the same time, sequences in the 3′ halves are more similar, reaching about 90–95% nucleotide identity towards the areas closer to the 3′ end. Importantly, this region of the genome has been shown to generate production of most siRNAs upon CTV infection that would be expected to act as a potent trigger of RNA silencing machinery against virus variants containing homologous sequences (Ruiz-Ruiz et al., 2011).

Considering high sequence similarity found between isolates of distinct strains in the 3′-terminal area, we analyzed siRNA profiles of isolates T36, T30, FL202, and T68-1 representing four heterologous strains of CTV – T36, T30, VT, and T68, respectively – to assess whether they generate certain homologous siRNAs upon plant infection. The total number of small RNA reads obtained for each sample ranged between 13.52 and 16.79 million reads, which was reduced to between 2.58 and 5.09 million reads per sample after depletion of host and non-target sequences. Aligning the remaining reads against the appropriate reference genome sequences produced totals of between 2.03 and 3.01 million reads mapped per isolate, with the exception of the T68-1 isolate, for which the number of reads was lower. All isolates, however, showed a similar asymmetrical profile of siRNA accumulation, which was in the agreement with the findings reported previously (Ruiz-Ruiz et al., 2011; Fig. 2). siRNAs of (+) and (−) polarity were generated from all the regions throughout the virus genome, with the highest level of those been produced from the 3′-terminal 2500 nt. An increased level of the siRNAs in the 3′-terminal portion of the virus genome was most likely related to the production of ten 3′-co-terminal sgRNAs that form dsRNA molecules in infected tissues (Moreno et al., 1990), and thus could be suitable substrates for Dicer-mediated degradation.

To reveal common siRNAs, the small RNA reads obtained upon sequencing samples from citrus plants infected by an isolate of the T36 strain were mapped against the reference genome sequences of the T36, T68, VT, and T30 strains (Fig. 3). A similar procedure was done by mapping siRNAs generated in plants each of which was infected with an isolate of one of the four strains against the T36 reference genome sequence. The analysis identified a number of homologous siRNAs generated by different isolates (Fig. 3). These siRNAs were mapped to the region of approximately 6000 nt in the 3′-terminal area of the virus genome, with most of the identical siRNAs found in close proximity to the 3′ end. The distribution of the homologous siRNAs appeared to reflect the levels of sequence homology between individual virus isolates. Thus, the isolates of the T36 and T30 strains showed a significant number of common siRNAs produced within a region between nt 12,500 and 17,000, which corresponded to the higher level of sequence similarity between the genomes of these two isolates that share about 93.0% identity in the 3′ half region, compared to 90.1% identity found between those of the T36 and T68 or VT strains (Harper, 2013; Fig. 3D). The T36 and T68-1 isolates, however, do share higher sequence homology to each other in the terminal ~2 kb (92.9–91.6% nucleotide identity), which apparently explains the large number of T36 generated reads mapped to the T68-1 genome in this region as shown in Fig. 3B.

Exclusion of a secondary virus infection is not triggered by high-level expression of homologous small RNAs

Homologous siRNAs produced by isolates of different strains of CTV upon their infection of citrus plants were detected in a relatively low amount (Fig. 3). Therefore, one could suggest that the limiting concentration of the guide siRNAs may not be enough

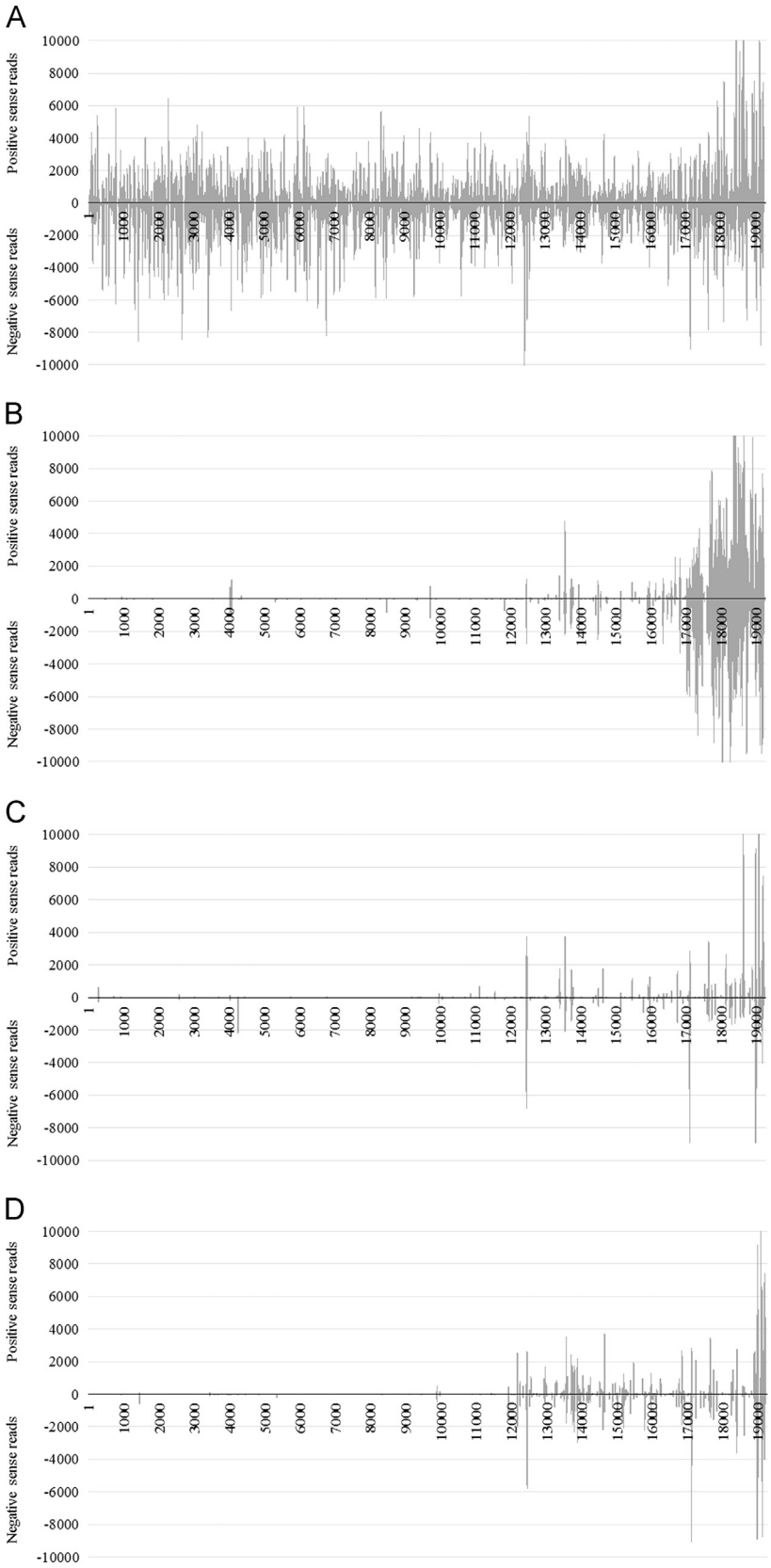

Fig. 3. Strain-specific siRNA reads generated from isolate T36, mapped against the reference genome sequences of strains (A) T36; (B) T68; (C) VT; and (D) T30.

to effectively target genomes of heterologous virus isolates. Thus, as the next step, we assessed production of siRNAs by a hybrid virus, which genome contained sequences of isolates from two heterologous strains, and compared the profiles with siRNA profiles of the donor isolates. In our earlier work we engineered several hybrids that contained substitutions of the sequences in the 3′ half of the T36 genome with the cognate sequences from the genome of the T68-1 isolate (Folimonova et al., 2010). For the

analysis here, we chose the H1 T36/T68 hybrid described in the aforementioned study, because it contained substitution of the largest fragment of 3, 697 nt in size, compared to the other created hybrids. This substitution comprised half of the CPm ORF, ORFs of the CP, p18, p13, p20, and p23 proteins as well as the 3′ untranslated region (Fig. 1; Folimonova et al., 2010). Comparison of siRNA profiles generated upon infection of plants by this hybrid and the T68-1 isolate revealed that the hybrid virus produced large amounts of T68-specific siRNAs from the respective region of approximately 3.7 kb, with the overall level of those been similar or even higher than that of the siRNAs produced by the T68-1 isolate (note the levels of T68-specific siRNAs generated from the H1 hybrid compared to those from T68-1 in Fig. 4).

As we reported in the earlier study, wild type isolates exclude secondary infection by the same isolate or by an isolate of the same strain (Folimonova et al., 2010). Therefore, the T68 isolate, apparently, is capable of excluding its own secondary infection. H1 hybrid, as we show above, triggers production of a large amount of T68-specific siRNAs from an extended region. The size of this region (3697 nt) is significantly greater than those of inserts successfully used for targeting of the cognate RNA molecules in various experiments (Ratcliff et al., 1999; Thomas et al., 2001; Voinnet, 2001; Tamura et al., 2013), including the size of a PDS gene fragment (391 nt) that induced silencing of the respective endogenous gene in citrus plants infected with CTV carrying that gene fragment (Hajeri et al., 2014). In spite of generating T68-specific siRNAs, the H1 hybrid was not capable of excluding the secondary infection by the T68 isolate (Folimonova et al., 2010). The result presented in the latter manuscript was confirmed in this study: challenge-inoculation of H1 hybrid-preinfected plants with the T68 isolate in an experiment set up that duplicated that described previously (Folimonova et al., 2010; see Materials and methods section) showed that T68 successfully established infection in those plants (data are not shown). In addition to the H1 hybrid, the earlier study described several other T36/T68 hybrids that showed a similar "non-excluding" phenotype (Folimonova et al., 2010). Yet, such hybrids containing substitutions of various genes from the T68 isolate would be expected to generate abundant T68-specific guide RNAs, similarly to what we reported here for the H1 hybrid. An observation that the expression of T68-specific siRNAs did not result in the exclusion of the respective isolate describes the situation that would not be expected if the operating mechanism was based on RNA silencing.

SIE is not determined by production of small RNAs

As described above, the H1 hybrid contained substitutions of the 3′-terminal regions, and, therefore, T68-specific siRNAs were generated from the corresponding regions in that area of the virus genome. On the other hand, since the hybrid was built based on a cDNA clone of the T36 isolate, the largest portion of the hybrid's genome contained sequences of the latter isolate, and, as we showed above, this part of H1 induced production of T36-specific siRNAs (Fig. 4). The hybrid retained the ability to exclude the T36 isolate, while was not capable of excluding the T68-1 isolate, the donor of the 3′-terminal sequences (Folimonova et al., 2010). Based on these observations, there was a possibility that production of siRNAs from regions other than regions in the 3′ area could be important for the exclusion phenomenon. To assess this possibility, we analyzed the profiles of siRNA that are produced by several "non-excluding" virus variants and compared them to those CTV variants that display the SIE ability. Three virus variants – a mutant virus containing a deletion within the p33 ORF (CTV9Δp33; Tatineni et al., 2008), a mutant with the deletion of the subgenomic mRNA controller element for the p33 sgRNA (CTV9p33ΔsgRNA; Folimonova, 2012), and a virus that contained a frameshift mutation within the p33 ORF leading to a shift of the normal reading frame and generation of a new stop codon (CTV9p33fs; Folimonova, 2012; Fig. 1) – were used for this analysis. As we reported earlier, all these viruses lost the ability to exclude the parental wild-type virus due to their inability to produce a functional p33 protein, one of the determinants of SIE by CTV (Folimonova, 2012). Here we were interested to compare siRNA profiles generated in plants infected by these virus variants with those of the wild type T36 and the H1 hybrid, both of which were capable of excluding the T36 virus. Remarkably, mapping of reads obtained upon sequencing of small RNAs from plants infected with the above viruses revealed that the profiles of siRNAs were very similar between those viruses as well as were similar to the siRNAs profiles of H1 and the wild type CTV (Figs. 5 and 4). The total amount of siRNAs produced by three "non-excluding" viruses as well as the number of siRNAs generated from different genomic regions of those viruses were similar to that of the wild type T36, with the exception of siRNAs corresponding to the sequences deleted in CTV9Δp33 and in CTV9p33ΔsgRNA that were apparently missing in samples from the latter mutant viruses (Fig. 5). The samples obtained from plants infected with CTV9Δp33, CTV9p33ΔsgRNA or CTVp33fs ranged between 2.80 and 3.01 million reads per sample mapped to the T36 genome, respectively, compared to nearly 2.70 million reads generated by the T36 isolate, suggesting similar levels of accumulation and/or infection to the wild-type. The siRNA profile of CTVp33fs, a virus variant that differed from the wild type by only two nucleotides that were inserted in the p33 ORF of the mutant, was highly comparable to that of the wild type virus (Fig. 5). High similarity was also found upon comparison of siRNAs from the three CTV mutants with T36-specific siRNAs detected from the H1 hybrid, the virus that was capable of excluding the parental wild-type T36. All the five viruses similarly showed high abundance of virus siRNAs from different regions throughout the genome in the infected plants, which indicated that all of them effectively engaged the host surveillance system that triggered degradation of viral RNAs. Remarkably, though, accumulation of such siRNAs did not correlate with the exclusion ability of the viruses: three of the five viruses – CTV9Δp33, CTV9p33ΔsgRNA, and CTVp33fs – were unable to exclude the T36 virus, while H1 and the wild type virus did as we demonstrated in our previous work (Folimonova et al., 2010; Folimonova, 2012) and confirmed here by repeating our earlier SIE experiment using plants that were analyzed in this study. Similarly to what we observed previously, challenge-inoculation of CTV9Δp33-, CTV9p33ΔsgRNA-, CTVp33fs-, H1- or T36-preinfected plants with the T36 virus isolate showed that the secondary virus T36 was able to establish infection only in plants that had primary infection with a virus variant containing mutation within p33, while was excluded from plants preinfected with the wild type T36 or the H1 hybrid (data not shown).

Conclusions

SIE of viruses has been related to a number of different mechanisms acting at various stages of the viral life cycle, including prevention of the incoming virus entry into cells, competition between primary and challenging viruses for host factors and intracellular replication sites, interference with disassembly, translation or replication of the secondary virus (Steck and Rubin, 1966a, 1966b; Sherwood and Fulton, 1982; Adams and Brown, 1985; Abel et al., 1986; Karpf et al., 1997; Lu et al., 1998; Beachy, 1999; Lee et al., 2005), and induction of RNA silencing by the protector virus that leads to sequence-specific degradation of the challenge virus RNA (Ratcliff et al., 1997, 1999; reviewed in Hull, 2002). Most of the proposed mechanisms, with the exception of the latter one, apparently act at the level of cells that were preinfected with the primary virus, leaving uninfected cells

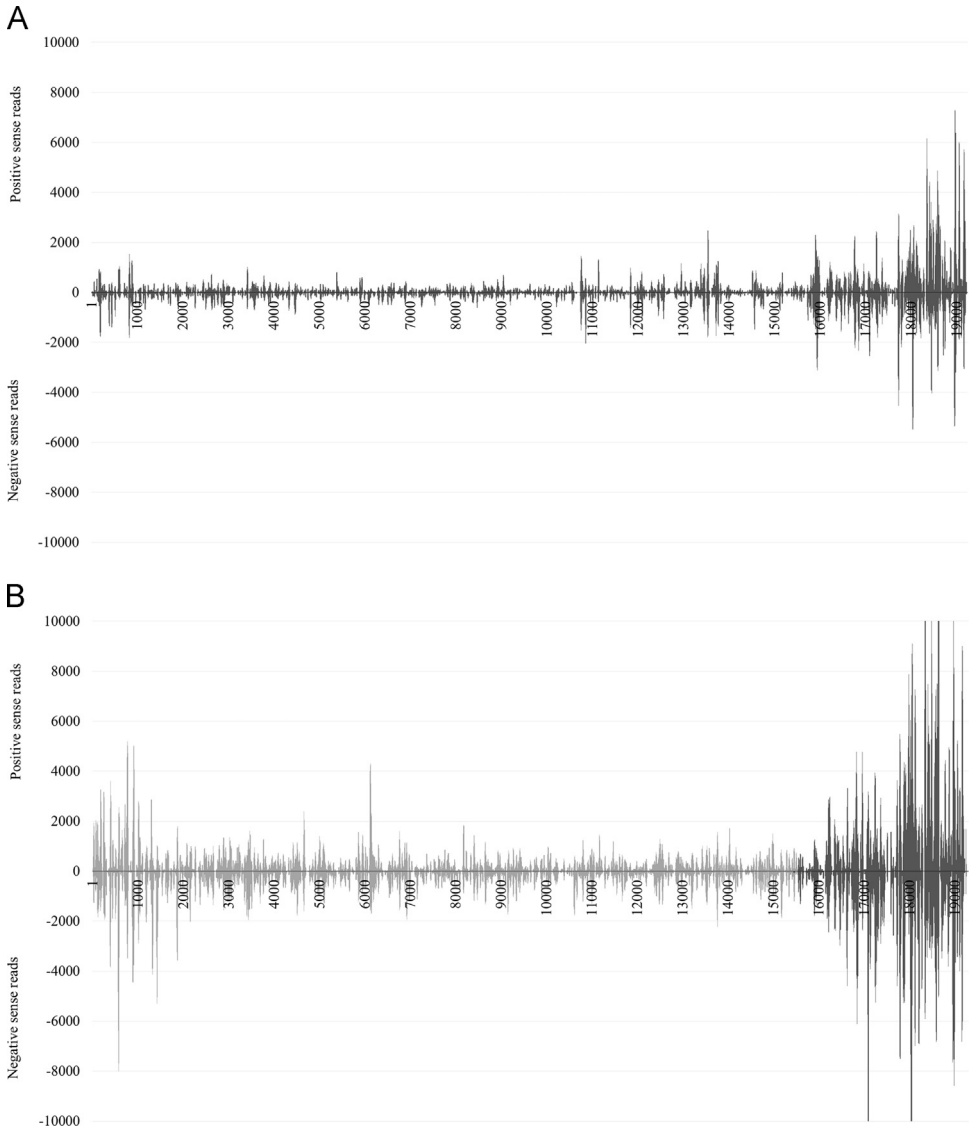

Fig. 4. Distribution of positive- and negative-sense siRNAs generated from (A) T68-1 and (B) T36/T68 hybrid H1 in *C. macrophylla*. T68-specific reads are shown in dark gray, T36-specific reads are in light gray.

susceptible to the secondary virus. On the other hand, RNA silencing, can function systemically: in cells that contained the primary virus and also in cells that were not pre-infected with the one. This feature makes RNA silencing a relevant mechanism that could potentially explain the "systemic" nature of SIE by CTV. To address a role of RNA silencing in CTV SIE, in our earlier work we attempted to trigger exclusion between heterologous CTV isolates by substituting extended regions in the genome of the protecting virus with the exact cognate sequences from the genome of the challenging virus (Folimonova et al., 2010). Nevertheless, despite the fact that such hybrid viruses drive the production of copious amounts of siRNAs homologous to the genomic sequences of the challenge virus as we demonstrated here, the hybrids were shown unable to exclude the latter isolate, thus indicating lack of positive correlation between the production of viral siRNAs and the SIE ability of the virus. Similarly, profiling of siRNAs generated upon infection of plants with the other three CTV variants that were not capable of excluding a secondary infection by either the parental wild type virus or by the exact same mutant variant revealed that they all produced large amounts of siRNAs at levels comparable to the wild type CTV, which was capable of exclusion. Thus, the observations obtained in this study indicate that production of specific siRNAs by a variant of CTV does not determine the ability of the virus to exclude another CTV variant containing sequences homologous to those siRNAs.

As we showed recently, SIE by CTV is due to a mechanism that requires production of the viral p33 protein. The exclusion ability of the virus could be removed by preventing production of p33. The mutants retain the ability to trigger an RNA-mediated host defense response targeted against the corresponding viral sequences that is evidenced by the abundant production if virus-derived siRNAs, yet are unable to exclude superinfection. These findings further argue for the intriguing complexity of CTV SIE phenomenon. More studies will be needed to determine whether SIE by CTV involves components of RNA silencing pathway or operates via another novel mechanism.

Materials and methods

Virus isolates and inoculation of citrus trees

The T36 isolate-based cloned virus CTV9 (56, 58), a set of mutant viruses: CTV9Δp33 (65), CTV9p33ΔsgRNA, CTV9p33fs

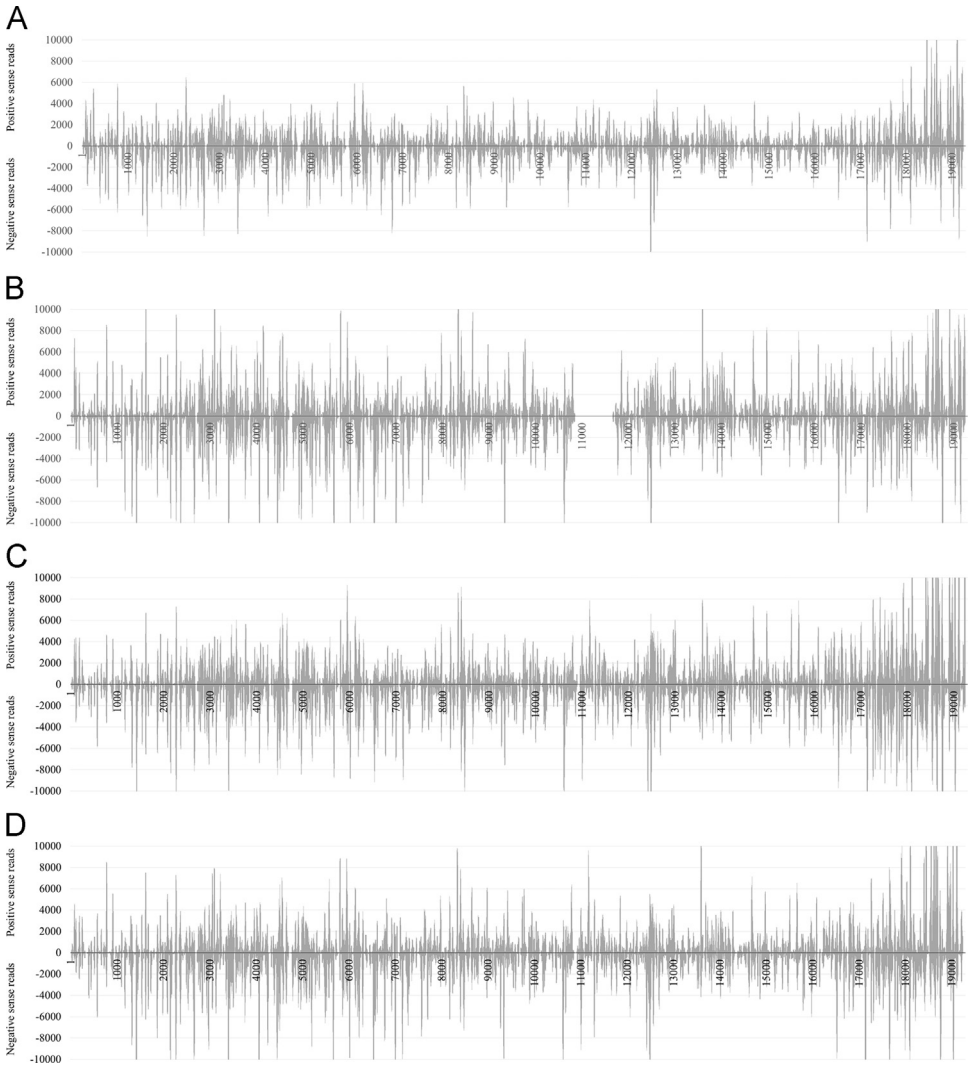

Fig. 5. Distribution of positive- and negative-sense siRNAs generated from (A) wild type T36 and its derivatives (B) CTV9Δp33, (C) CTV9p33ΔsgRNA, and (D) CTVp33fs in *C. macrophylla* mapped to the T36 genome sequence.

(Folimonova, 2012), hybrid virus T36/T68 H1 (Folimonova et al., 2010), CTV isolates T30-1 (3), T68-1 (16) and FL202 have been maintained in citrus plants under greenhouse conditions. These plants were used as sources of virus for subsequent graft-inoculations of young trees. For small RNA analysis and subsequent SIE tests, 9–12 months old trees of *Citrus macrophylla* Wester (usually 3 plants per treatment) were inoculated by grafting of virus-infected tissue from individual source plants. The plants in all treatments were inoculated at the same time. At eight weeks after inoculation, systemic tissue was assayed by ELISA using antibodies specific to CTV virions to confirm the establishment of infection as described (Folimonova et al., 2010). Young systemic flushes from the infected trees were then sampled for small RNA analysis as per Harper (2013). SIE tests were conducted as described (Folimonova et al., 2010; Folimonova, 2012). In brief, the plants pre-infected with the primary virus were subjected to secondary (challenge) inoculation by inserting a second graft of bark tissue infected with a challenge virus. When the graft healed, the upper flushes of leaves were trimmed to induce growth of a new flush, which was then evaluated for the ability of the challenge virus to establish systemic infection in plants that were previously infected with a primary virus. In the first SIE experiment, the ability of the challenge T68 isolate to superinfect trees pre-infected with the T36/T68 H1 hybrid was verified by reverse transcription PCR using T68-specific primer set and RNA extracted from the plants being tested as described earlier (Folimonova et al., 2010). The second SIE experiment, which tested the ability of different CTV mutants to exclude the T36 virus, was conducted using a green fluorescent protein-tagged CTV T36 as a challenge virus and following the experimental design described in details by Folimonova (2012).

Extraction, sequencing and analysis of siRNAs

The extraction and enrichment of viral and host small RNAs was performed as per Harper (2013). Small RNA libraries were constructed using the ABI SOLiD small RNA expression kit (Applied Biosystems Inc., Foster City, CA, USA) as per the manufacturer's protocol, bar-coded in groups of four, and sequenced using a SOLiD 5500xl platform, with single direction, 36 bp parameters, at the Interdisciplinary Center for Biotechnology Research, University of Florida. The resulting reads for each sample were trimmed to remove adapter sequences, and reads outside the desired size range of 19–25 nt were discarded. Each read library was then depleted of non CTV-specific reads that mapped to mirBase19 (Kozomara and Griffiths-Jones, 2011) and the plant snoRNA databases (Brown et al., 2003), the *Citrus sinensis* chloroplast sequence (Bausher et al., 2006), *C. sinensis* genome scaffolds and

the *Arabidopsis thaliana* mitochondrion sequence (Unseld et al., 1997) using CLC Genomics Workbench version 6.01 (CLC Bio, Aarhus, Denmark).

To examine the distribution of siRNAs generated from the CTV genome, the depleted read libraries for each sample were mapped against the reference CTV genomes for strains T36 (Karasev et al., 1995), T68-1 (Harper, 2013), T30 (Albiach-Marti et al., 2000), and VT (Mawassi et al., 1996) using SHRiMP v2.0 (David et al., 2010). The resulting maps were visualized using Tablet 1.13.07.31 (Milne et al., 2013).

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