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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

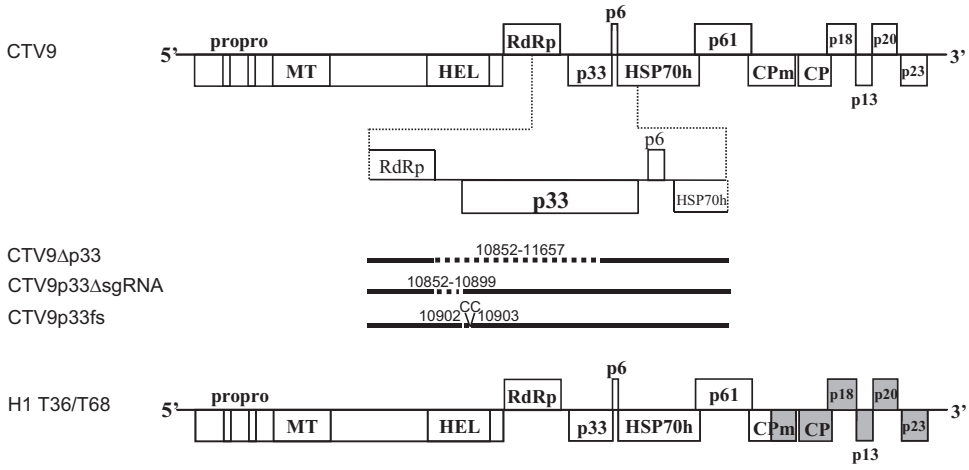

Fig. 1. Schematic diagram of the genome organization of wild type CTV T36 (CTV9) and its derivatives. The open boxes represent ORFs and their translation products. PRO, papain-like protease domain; MT, methyltransferase; HEL, helicase; RdRp, an RNA-dependent RNA polymerase; HSP70h, HSP70 homolog; CPm, 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.