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Brief Communication

With a little help from my friends: Complementation as a survival strategy for viruses in a long-lived host system

S.J. Harper *, S.J. Cowell, W.O. Dawson

Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA

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ABSTRACT

In selective host species, the extent of *Citrus tristeza virus* (CTV) infection is limited through the prevention of long-distance movement. As CTV infections often contain a population of multiple strains, we investigated whether the members of a population were capable of interaction, and what effect this would have on the infection process. We found that the tissue-tropism limitations of strain T36 in selective hosts could be overcome through interaction with a second strain, VT, increasing titer of, and number of cells infected by, T36. This interaction was strain-specific: other strains, T30 and T68, did not complement T36, indicating a requirement for compatibility between gene-products of the strains involved. This interaction was also host-specific, suggesting a second requirement of compatibility between the provided gene-product and host. These findings provide insight into the 'rules' that govern interaction between strains, and suggest an important mechanism by which viruses survive in a changing environment.

Introduction

The study of viruses primarily focuses on pure cultures, yet in nature hosts generally contain viral populations, the dynamics of which will dictate the persistence, virulence, and pathogenicity of the infection (García-Arenal et al., 2001; Mideo, 2009; Syller, 2012; Alizon et al., 2013). This is particularly true of hosts that permit persistent infection, such as perennial plants, which over their lifespan have the potential for repeated superinfection by different viral species, strains, or variants (Moreno et al., 2008).

The interactions between different components of a population result in an array of biological and phenotypic effects not seen during infection by single, pure isolates (Syller, 2012), and may be synergistic or complementary, allowing a more extensive or virulent infection (Scheets, 1998; Untiveros et al., 2007), or permitting the persistence of less fit components that would otherwise be lost (Čičin-Šain et al., 2005). Conversely, the interaction may be antagonistic or competitive, with displacement or exclusion of one or more components of the population (Miralles et al., 2001; Capote et al., 2006; Predajňa et al., 2012; Syller and Grupa, 2014). Alternatively, components may show no direct interaction and produce a stable coinfection (Barker and Harrison, 1978; Capote et al., 2006; Bellecave et al., 2009). However, despite decades of study (García-Arenal and Fraile, 2011; Syller, 2012) the 'rules' governing viral population dynamics, of any species, remain largely unknown. For example, what determines whether interaction between viruses in a population will be positive as opposed to antagonistic or competitive? Does the former require co-infection of the same cell, and, by extension, does superinfection-exclusion and segregation of viruses prevent synergism? Are these interactions constant, or are they dependent on the host and/or environmental factors? What determines the structure of the population? Do the components reach an equilibrium? Indeed, are there 'rules' at all, or is each interaction due to a unique set of circumstances?

One virus species particularly amenable to the study of such questions is *Citrus tristeza virus* (CTV), a positive-sense ssRNA virus, with a genome of 19.2 kb that is limited to phloem-associated cells of *Citrus* spp. and citrus relatives in the family Rutaceae (Moreno et al., 2008). CTV has, at time of writing, at least seven characterized strains that are clearly delineated from one another by sequence, and not by pathogenicity (Harper, 2013). Unlike several species for which interactions have been examined (Barker and Harrison, 1978; Lee et al., 2005; Capote et al., 2006), CTV strains readily co-infect the same host (Folimonova et al., 2010; Harper et al., 2010; Scott et al., 2013).

One aspect of CTV biology that is poorly understood is that of movement and cell entry via the phloem, which is a crucial step in the infection process. This process generally requires the precise interactions of viral and host proteins (Marsh and Helenius, 2006). For those viruses that utilize direct cell-to-cell connections, such

* Correspondence to: Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA Tel.: +1 863 9568719.
E-mail address: sjharper@ufl.edu (S.J. Harper).

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as plasmodesmata, this involves the interaction of viral movement proteins interacting with or hijacking the host's macromolecular transport pathway (Carrington et al., 1996; Marsh and Helenius, 2006). But what of viruses that move through tissues that are unable to support viral replication, such as plasma-borne *Poliovirus* in humans (Hogle, 2002), or plant viruses in phloem sieve elements? In the latter, the sieve elements form a continuous pathway for movement of carbohydrates throughout the plant, a pathway that, for the virus, theoretically permits access to nearly every tissue in the host. Yet, once the virus arrives at a target cell, it still has to transit the cell wall. How plant viruses achieve this remains largely unknown (Carrington et al., 1996; Hipper et al., 2013).

For CTV in *Citrus* the process of systemic movement is exceedingly inefficient, as only a small proportion of phloem-associated cells become infected (Folimonova et al., 2008). This proportion decreases further in more resistant hosts, and reaches an extreme wherein the virus is almost undetectable in trifoliate orange (*Poncirus trifoliata*) (Harper et al., 2014). This would suggest, given that CTV readily replicates in the protoplasts of these species (Albiach-Marti et al., 2004), that the ability to move and enter cells varies between hosts. In addition, not only are there differences in the number of cells infected, differential infection of root and not shoot tissue also occurs (Harper et al., 2014). This phenomenon, a root-specific tropism, was found to be strain-specific; some strains were capable of systemic infection of a given host, whilst others were limited to the roots, suggesting host-specific adaptation of CTV strains (Harper et al., 2014).

We previously demonstrated that CTV strains show differences in ability to systemically infect specific citrus species that we refer to as differential (Harper et al., 2014). In this study, we used these differences to examine how CTV behaves whilst infecting as a population of multiple strains, rather than as a single strain. To do this, we compared the infection of the type isolate of strain T36, an isolate previously shown to poorly infect some host species (Folimonova et al., 2008) and be restricted to the roots of others (Harper et al., 2014), alone and in combination with other, more infectious CTV strains (Harper et al., 2014) in differential and resistant host species.

We found that complementation with a second strain could overcome the tropism limitations of T36, increasing the viral titer and number of cells infected in select differential and resistant host species. This suggests that the second strain provides adapted components that interact more efficiently with these hosts than those of T36. However, this effect was found to be strain-specific: complementation was only observed when T36 was paired with VT, but not T30 or T68. We also found that complementation could occur in a host-specific manner, for the T36-VT pairing did not provide an increase in titer in all species tested. These findings are a first step into understanding the 'rules' that govern interaction of CTV strains with one another, and with their host. They also provide insight into the infection process of this complex, phloem limited virus.

Results

Infectivity of CTV mixtures

To confirm that CTV populations were infectious and transferred intact irrespective of host species, we compared the frequency of infection by strain T36 alone versus T36 plus members of the T30, VT, or T68 strains. We found that T36 was detectable alone in flush tissue of all hosts but trifoliate orange by RT-qPCR (Table 1), and that there were no significant differences (Fisher's exact test $P=0.371$) in infectivity of T36 in these species alone versus in mixture. However, T36 was only found in trifoliate orange flush tissue when in company with either VT or T68, suggesting that complementation between movement capable and deficient strains can overcome the tropism limitations of T36 in resistant species.

Table 1
Frequency of T36 strain presence in mixture in permissive and selective citrus host species at 12–18 weeks post inoculation.

Host species	T36	T36+VT	T36+T30	T36+T68
Sour orange	5/5	4/5	5/5	3/5
Sun Chu Sha mandarin	4/5	3/5	3/5	4/5
Swingle citrumelo	5/5	5/5	4/5	3/5
Carrizo citrange	3/5	5/5	5/5	5/5
Trifoliate orange	0/5	3/5	0/5	3/5

Accumulation in flush tissue

We previously observed that the exclusion of T36 from flush tissue of selective or resistant species is not absolute: this strain can be detected by a sensitive technique such as RT-qPCR, whilst remaining below the level of detection of both ELISA and fluorescence microscopy (Harper et al., 2014). In this study we found that the titer of the T36 strain can be increased in several of these selective species by co-infection with a second strain. In sour orange (Fig. 1a), a host that T36 can infect, albeit weakly (Folimonova et al., 2008), quantification revealed a significant, ten-fold increase (Tukey HSD $P>0.05$) in T36 titer when complemented with a representative of the VT strain ($F(1,9)=17.00$, $MS=2.18$, $p=0.003$) over infection by T36 alone. Minor increases were also observed in this host during co-infection with representatives of strains T30 and T68, although the differences were not significant.

A significant increase in T36 titer was also observed in Sun Chu Sha mandarin ($F(1,19)=8.03$, $MS=11.39$, $p=0.01$) and Carrizo citrange ($F(1,14)=8.95$, $MS=8.22$, $p=0.01$) when co-infected with VT (Fig. 1b and c). In these two hosts, the effect of complementation with VT was more pronounced than in sour orange, with an increase of approximately forty to fifty times more T36 present. Again, no significant changes in T36 titer were observed through complementation with T30 or T68, although a minor decrease in T36 titer was observed in Carrizo citrange when T36 was co-infected with T30. In Swingle citrumelo however, there were no significant changes in the titer of T36 when co-infected with VT, T30, or T68 (Fig. 1d).

Effect of complementation on movement and cell entry

Having observed that complementation causes an increase in T36 titer, we next examined whether this was caused by an increase in infectivity, resulting in more infected cells. Therefore, a visual comparison of infection by GFP-expressing T36 alone to infection complemented by other isolates was made at the time of the second post-inoculation flush. We observed few, scattered fluorescent cells, an average of 28.2 ± 11.5, in sour orange sections infected with T36-GFP alone. In contrast, there was a marked increase to an average of 145.4 ± 27.7 (Table 2) fluorescent cells per section in plants co-infected with VT (Fig. 2). The addition of VT did not, however, change the size of fluorescent clusters; infection remained limited to single fluorescent cells, albeit at greater frequency (Fig. 2). Co-infection with T30 showed no major difference from T36 alone (not shown), whilst no fluorescence was observed in plants co-infected with T68-1, although no change in T36 titer was observed. These results correlate with the increase in titer observed by RT-qPCR.