Brief Communication

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

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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 (Cèn-Sain 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; Predajia 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
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 trifoliate) (Harper et al., 2014). This would suggest, given that CTV readily replicates in the protoplasts of these species (Albiach-Martí 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 (Albiach-Martí et al., 2004), that the ability to move and enter cells was 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>
<thead>
<tr>
<th>Host species</th>
<th>T36</th>
<th>T36 + VT</th>
<th>T36 + T30</th>
<th>T36 + T68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sour orange</td>
<td>5/5</td>
<td>4/5</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Sun Chu Sha mandarin</td>
<td>4/5</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Swingle citrange</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Carrizo citrange</td>
<td>3/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Trifoliate orange</td>
<td>6/5</td>
<td>3/5</td>
<td>0/5</td>
<td>3/5</td>
</tr>
</tbody>
</table>

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 co-infected 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 citrange 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 $28.2 \pm 11.5$, in sour orange sections infected with T36-GFP alone. In contrast, there was a marked increase to an average of $145.4 \pm 27.7$ (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.
Fig. 1. Comparison of the normalized titer of T36 (white) versus T36 complemented with VT (dark grey), T30 (light grey), and T68 (black) strains in (a) Sour orange, (b) Sun Chu Sha mandarin, (c) Carrizo citrange, and (d) Swingle citrumelo as determined by relative quantification real time RT-qPCR. Significant changes (P < 0.05) are indicated with an asterisk (*).

Table 2
Average frequency of fluorescent cells in 10 × 5 mm sections of (A) sour orange and (B) Sun Chu Sha mandarin plants at 12–16 weeks post-inoculation. A total of five sections were taken from shoot tissue of each plant; fluorescent cells were determined visually under UV-fluorescence microscopy.

<table>
<thead>
<tr>
<th>Strain Combination</th>
<th>Average fluorescent cells per section</th>
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<tr>
<td>T36</td>
<td>28.44 ± 0.20</td>
</tr>
<tr>
<td>T36+VT</td>
<td>27.72 ± 17.18</td>
</tr>
<tr>
<td>T36+T30</td>
<td>145.37 ± 17.18</td>
</tr>
<tr>
<td>T36+T68</td>
<td>7.58 ± 0.00</td>
</tr>
<tr>
<td>SE</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Discussion

We have previously demonstrated in the CTV–Citrus spp. host system that susceptibility is a matter of degree, and differs between species (Folimonova et al., 2008), between tissues and between extant CTV strains (Harper et al., 2014). This, in selective species, was observed to lead to a restriction of specific CTV strains to the root tissues alone (Harper et al., 2014). In this study we examined whether the barriers to movement and cell entry in differential hosts could be overcome in CTV populations comprised of strains with varying tropisms and levels of infection. We found that the components of CTV populations are capable of positive interaction, in the form of complementation, during the co-infection of selective host species. As a result, the tropism-limited T36 strain was able to more readily infect phloem cells in shoots of two differential hosts that it, alone, infects only at trace levels. While changes in host range or tropism have been reported between different virus strains (Mansky et al., 1995) and more frequently, species (Carr and Kim, 1983; Hacker and Fowler, 2000; Ryang et al., 2004), this, to the best of our knowledge, is the first instance where co-infection between strains of the same virus species has specifically affected long-distance movement.

Strain- and host-specific interactions

Although the limited ability of T36 to infect shoot tissue of differential hosts was overcome by addition of a second CTV strain, this interaction was found to be strain-specific, for complementation was only observed between representatives of the T36 and VT strains; neither T30 nor T68 significantly increased the T36 titer in any of the four selective or resistant hosts examined. This was not due to the inability of T30 or T68 to infect cells, as both accumulated to a titer comparable to that of VT in all hosts. Nor was T36 negatively affected

As per Harper et al. (2014), no fluorescent cells were observed in sections of Sun Chu Sha mandarin infected with only T36-GFP (Table 2), a result expected given the low titer of this strain in this host. However, when T36-GFP was co-inoculated with VT, we located an average of 29.2 ± 6.9 fluorescent clusters per section (Fig. 2), suggesting that complementation overcomes the tropism limitation of T36 in this host, allowing the infection of cells in the shoot. This also, albeit indirectly, confirms the titer increase observed by RT-qPCR. No consistent effect was observed after co-infection of T36-GFP with either T30 or T68-1 (Table 2); clusters of 1–4 fluorescent cells were observed in one plant infected with T30. However, the remainder of the T36-GFP and T30 co-infected plants were negative, suggesting that T30 has no significant or consistent effect on the titer of T36 in Sun Chu Sha mandarin. What we have observed here then, is that complementation with specific strains not only increases the titer of the complemented strain, as observed by RT-qPCR, but achieves this by increasing the frequency at which cells in the shoot are infected, and in selective hosts, such as Sun Chu Sha mandarin, overcomes tropism limitations.
by the presence of a second strain, as has been observed in studies of potyvirus populations (Capote et al., 2006; Syller and Grupa, 2014); no reduction of T36 titer was observed. Instead, strain-specificity suggests that for complementation to occur, the gene-products provided by the donor virus must be compatible with those of the recipient. This complementary effect was found not only to be strain-specific, but was also host-specific. The T36-VT strain-pairing did not provide a uniform increase in all hosts tested, with a smaller increase observed in sour orange versus Sun Chu Sha mandarin and Carrizo citrange, and none in other hosts. This suggests that complementation is dependent on the degree to which the donor is adapted to each host species. Indeed, one could propose that in Sun Chu Sha mandarin, T68-1 would be a more optimal partner than VT, as it has a titer ~70 times greater. Finally, in Swingle citrumelo the addition of VT had no significant effect on the titer of T36, indicating that, quite simply, what works in one host may not work in another (González-Jara et al., 2004; Wintermantel et al., 2008).

**Maladapted proteins**

It is apparent that the total number of phloem companion cells infected by T36 in sour orange or Sun Chu Sha mandarin shoots, for example, is far fewer than those that may be potentially infected, as we have seen here when a second, complementary strain is added. One could attribute this to low levels of gene expression, for it has been shown that ectopic expression of CTV p23, a suppressor of silencing, will also increase the number of cells infected by T36 (Fagoaga et al., 2011). However, our findings differ from those of Fagoaga et al. (2011) in that ectopic expression of p23 increased cell-to-cell movement to neighboring cells, whereas complementation caused an increase in scattered primary infection of companion cells, suggestive of a change in long-distance movement or cell entry. From this we propose that the CTV T36 strain possesses one or more maladapted gene-products or proteins necessary for interaction with the cells of these hosts, whereas complementation caused an increase in scattered primary infection of companion cells, suggestive of a change in long-distance movement or cell entry. From this we propose that the CTV T36 strain possesses one or more maladapted gene-products or proteins necessary for interaction with the cells of these hosts, reducing entry and infection. Those of VT, and presumably T30 and T68, on the other hand are adapted and hence, functional. Which components are involved can only be speculated upon, for a number of proteins from CTV and other closteroviruses have been implicated in long-distance movement and infection, including the leader-proteases (Peng et al., 2003; Liu et al., 2009), heat-shock protein homologues (Prokhnevsky et al., 2002; Napuli et al., 2003; Alzhanova et al., 2007), and proteins of unknown function (Tatineni et al., 2008).

![Fig. 2. The distribution of the GFP-expressing infectious clone of strain T36 in single infection, and co-inoculated with strain VT in C. aurantium sour orange (A, B) and in C. reticulata cv. Sun Chu Sha mandarin (C, D) at 12 weeks post-inoculation.](image-url)
'Rules' of population interaction

What, then, is the significance of strain- and host-specific complementation between CTV strains that increases the number of cells infected? It is apparent that the gene products of each CTV strain required for systemic movement and/or cell entry are adapted to a subset of their potential host range, and that in resistant hosts these produce a root-limited tropism fatal to the survival of the virus, in that they would be prevented from further spread (Harper et al., 2014). Here we have seen that such limitations may be overcome through complementation with other strains, from which it may be suggested that existence as a population is a possible survival strategy for CTV. Yet, for complementation to occur, the strains, or rather their gene products, must be compatible with both one another, and with their host, forming what may be described as ‘complementation groups’ (Mansky et al., 1995). Whether these ‘rules’ are products of circumstance remains to be seen, but what we have described here is a first look into the interactions that occur within CTV populations, and their effect on the biology of the virus.

Materials and methods

Virus isolates and inoculations

The eponymous type isolate of the strain T36 was selected for study, as it has a limited host range and has been shown to differentially infect Citrus reticulata cv. Sun Chu Sha mandarin and C. sinensis × P. trifoliate cv. Carrizo citrange (Harper et al., 2014). To examine the effect of the presence of additional strains on the infectivity of T36 in these and other species, we first constructed a series of source-combinations through the sequential inoculation of C. macrophylla first with T36, and then with one of three other strains extant in Florida: VT from an aphid-transmitted sub-isolate of isolate FL202 (Harper, 2013), T30 from the type isolate of the same name (Albiach-Martí et al., 2000), and T68 from the type isolate T68-1 (Harper, 2013). Population composition of these combinations was confirmed at three months post-inoculation using CTV strain-specific multiple-molecular markers (Matos et al., 2013).

Once confirmed, these sources were used to inoculate five plants each of C. aurantiuim cv. California Standard sour orange, C. reticulata cv. Sun Chu Sha mandarin, C. paradisi × P. trifoliate cv. Swingle citrulmo, C. sinensis × P. trifoliate cv. Carrizo citrange, and P. trifoliate cv. Rubidoux trifoliate orange, because previous research indicated that these species are selective and/or differential for T36 (Harper et al., 2014). Plants were cut back to force the production of new flush at three weeks post-inoculation, and maintained in greenhouse conditions, with an ambient temperature of 25–30 °C, throughout the course of the experiment. It should be noted here that the source isolates for the VT and T30 strains were found to contain trace amounts of a T36 variant that we have been unable to remove (Harper, unpublished), and so were not tested as individuals in differential hosts.

RT-qPCR and quantification

Samples of 100 mg of young green flush bark and leaf midrib tissue were processed and extracted using Trizol reagent (Life Technologies, Carlsbad CA), as per the manufacturer’s instructions, and eluted in 500 μl of H2O. The titer of each strain of CTV present was assessed by RT-qPCR and quantified against two reference genes, as previously described by Harper et al. (2014). Sequences of strain-specific primers and probes are given in Supplementary Table 1. Differences between treatments were examined by one-way analysis of variance, followed by Tukey’s post-hoc test.

Fluorescence microscopy

To visually examine the effect of complementation in selective species, we established a second series of source populations as above, but with the full length GFP-expressing cDNA clone of T36 in place of the wild-type T36 isolate. These sources were then used to graft-inoculate ten plants each of C. aurantiuim cv. California Standard sour orange and C. reticulata cv. Sun Chu Sha mandarin. Plants were maintained as described earlier, and tested for CTV presence by ELISA (Harper et al., 2014) at the second post-inoculation flush (approximately 12–16 weeks). CTV-positive plants were subsequently examined for fluorescence.

A total of five 10 × 5 mm sections of young green bark tissue were taken from the stem of each plant and examined for GFP fluorescence using a Zeiss Stemi SV 11 UV–fluorescence dissecting microscope (Carl Zeiss Jena GmbH, Jena, Germany). The total number of fluorescent clusters were counted for each section, and averaged for each plant and treatment for comparison. Next, these samples were set in Tissue Tek Cryo-OCt compound (Thermo-Fisher Scientific, Waltham, MA) and 30 μm sections were prepared on a WRC cryostat microtome (Harris Manufacturing Inc., Burlingame, CA). These sections were then placed on glass slides with 15 μl of VectaShield Hardset mounting medium (Vector Laboratories Inc., Burlingame, CA) and a coverslip. Prepared sections were then examined on an Olympus BX61 series compound microscope (Olympus America Inc, Melville, NY) using a TRITC–FITC filter cube, and photographed using an OMAX digital microscope camera (OMAX, Irvine, CA) with a 0.66 × conversion lens.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.041.

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