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Brief Report

The past and present status of *Citrus tristeza virus* in Florida.

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Abstract

The *Citrus tristeza virus* (CTV) strains present in an area can determine the type and severity of disease produced. Using real time RT-qPCR, we screened a series of representative samples collected throughout Florida from 1964 to 2002 for CTV strain presence. We found that contrary to previous reports, the historical samples were infected with both the T30 and T36 strains, the latter often at low titer. The VT strain was rare, with a scattered distribution. We compared this to samples collected in 2014 and 2015, and found that T30 and T36 are prevalent in nearly all commercial groves; VT strain incidence has increased markedly. With changing cultural practices, such as an increase in sour orange rootstock use, tristeza disease continues to be a significant threat to the Florida citrus industry.

Keywords: *Citrus tristeza virus*, strain, Florida

Introduction

Few viral pathogens have changed the character of their respective industry to the extent that *Citrus tristeza virus* (CTV) has to citrus. The CTV decline epidemics that swept through South America in the 1930s through to Spain in the 1980s led to a wide-scale abandonment of the sour orange rootstock, while in many parts of the world pressure from endemic CTV stem pitting limits the production of many sweet orange and grapefruit cultivars.

CTV have historically been referred to as "strains" in terms of biology, referring to T36 as a "decline" strain, or T30 as a "mild" strain. However, in the genomic era, these terms (strains) are archaic and misleading. The type and severity of disease induced is only relevant within a specific context, a context defined by the cultivar grown, what rootstock it is grown on, and the CTV isolates present. For example, the isolate T36 will indeed cause decline of sweet orange on sour orange rootstock, yet so will VT (Bar-Joseph et al. 1989) as well as some T30 isolates (Harper, unpublished). Similarly, some T30-like isolates cause pitting and stunting of grapefruit, while T36-like isolates do not (Harper, unpublished). Which is the "mild" strain?

There are, at time of writing, a total of 7 CTV genetic lineages. These lineages are defined as strains, grouped by their sequence homology and named after their respective type isolates (Harper 2013). It is important to note that the members within any given genotype, or strain, share few phenotypic characteristics; while host range and tissue tropism is common between members (Harper et al. 2010; Harper et al. 2014), vector transmission rates and the type and/or severity of disease produced are not. For example, not all VT isolates cause stem pitting, nor are all T30 isolates "mild" (Harper, unpublished). Complicating this further is the fact that nearly all natural infections of CTV are comprised of populations of 2 or more CTV strains (Scott et al. 2013). The members of these populations have the potential to interact (Harper et al. 2015a), another factor that can determine the extent and type of disease produced.

CTV has also had a significant impact on the direction of the Florida citrus industry. Periodic freezes from the 1890s onwards drove the industry further south, along the central ridge and into the sandy flatwoods where, in the early-to-mid 20th century, various selections of sour orange (*Citrus aurantium*) were the rootstock of choice for both orange and grapefruit production. Prior to the arrival of the brown citrus aphid (*Toxoptera citricida*) in late 1995 (Halbert et al. 2004), CTV disease was not a major issue; while sporadic outbreaks of tristeza decline occurred, the inefficient aphid vector species present (*Aphis gossypii*, *A. spiraecola*, and *T. aurantii*) reduced the spread and limited the damage caused by tristeza disease.

The arrival of the brown citrus aphid in 1995, and subsequent spread throughout the state during the following 2 years, triggered a series of severe CTV outbreaks. These severe decline epidemics accelerated a change in rootstock choice, away from sour orange, to Swingle citrumelo (*Poncirus trifoliata* x *C. paradisi*), and Carrizo citrange (*P. trifoliata* x *C. sinensis*); regrettably

neither rootstock provided the agronomic performance of sour orange, particularly in the flatwoods. It has been suggested that the brown citrus aphid transmitted "severe" strains already present in Florida more efficiently than the previously predominant vectors (Halbert et al. 2004). Yet, at the time, CTV strain classification was in its infancy; most strains were typed using antisera, or molecular probes based on conserved regions of the genome (Halbert et al. 2004) that did not directly correlate with genetic lineages or pathogenicity.

Historically, differentiating CTV isolates from one another relied on biological indexing on select indicator species and, later, discrimination using strain-specific antibodies such as MCA13 (Permar et al. 1990). But with the advent of sequencing and strain-specific molecular assays, we can describe and quantify CTV isolates at the population level (Harper et al. 2015b). Given the long history of CTV in Florida, we wished to determine what CTV strains were present prior to the introduction of the brown citrus aphid in 1995, and whether this, and whether subsequent tree removal from both CTV and greening, had changed endemic CTV strain incidence. We found that strains T30 and T36 are just as prevalent now as they were 50 years ago, while the incidence of strain VT has increased markedly. Furthermore, these strains almost always occur in mixture, complicating both the prediction and control of tristeza disease.

Materials and Methods

To examine CTV strain presence and diversity in Florida, 2 sets of samples were examined. We began by screening sub-propagations of plant samples collected as part of the USDA-ARS CAPS survey (Courtesy S Garnsey) maintained at the University of Florida Citrus Research and Education Center. The original samples had been collected between 1964 and 2002, and sequentially propagated on various sweet orange (*C. sinensis*), rough lemon (*C. limon*), or Alemow (*C. macrophylla*) cultivars (Table 1) over the following decades. To see whether there had been any change in virus presence subsequent to the introduction of the brown citrus aphid, a second set of samples was collected from commercial and research groves throughout Florida in the fall and summer seasons of 2014 and 2015. Tristeza-associated symptoms present on these plants were noted when present.

Young flush bark and leaf tissue was collected from 3 randomly selected sites on each plant, pooled, and the total RNA extracted from a 100 mg sample using TRIzol (Life Technologies, Carlsbad, CA) as per the manufacturer's instructions. Extracts were then diluted 1:10 in water to reduce the effect of inhibitory substances present. Field samples were first screened for CTV presence using a real time RT-qPCR assay targeting the coat protein (p25) gene between bases 16649-16761; older USDA samples were not screened as they were known to be CTV positive. For amplification, the SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies) was used with 400 nM of sense (5'-ACCGGAGCTGGCTTGACTGAT-3') and antisense (5'-CCAAGCTGCCTGACATTAGTAA-3') primers, and 100 nM of 6-FAM/BHQ-1 labelled TaqMan probe (5'-AGAGTGTGCTGTGTACATACAAGCTAAAGA-3'), and 2 µl of diluted RNA template in a reaction volume of 10 µl. Cycling conditions were as follows: 50 °C for 5 minutes, 94 °C for 2 minutes, then 40 cycles of 94 °C for 10 seconds and 60 °C for 40 seconds.

All samples were screened for the presence of CTV strains T36, T30, and VT, using strain-specific real time RT-qPCR as described in Harper et al. (2015a). Samples were tested in technical replicates of 3, and positive samples called against a cutoff determined by a weakly positive (Ct 36 cycles) control sample. CTV strain titer was determined in select samples by relative quantification of strain-specific Ct values against weakly CTV positive control samples using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and normalized using titer of the citrus ACTB and GAPDH genes, as per Harper et al. (2014).

Results

Screening a series of samples collected by the USDA from 1964 to 1996 (Table 1) showed that, prior to the arrival of the brown citrus aphid in 1995, the T36 and T30 strains predominated. The dogma was that most plants were solely infected with T30, the so-called "mild" isolate, while T36, the "decline" isolate, incidence was sporadic. This was due to use of the MCA13 antibody, which does not detect T30-like isolates while detecting isolates of other strains, such as T36 or VT (Permar et al. 1990). Plants that were CTV positive but MCA13 negative were assumed to be infected with only T30 (Irey et al. 1988), and were legally permitted to be propagated in nurseries. We found that most samples that contained T30-like isolates also contained T36 (Table 1). Quantification showed that in many cases T36 was at low titer, as demonstrated by samples T4 and T55 in Fig. 1, and hence are effectively latent. This is likely why T36 was not detected by MCA13 in previous studies, and potentially, why it did not cause disease.

Prior to 1995, VT-like isolates were rare, and associated only with Meyer lemon cultivars (Table 1). While the plants we tested in this study are in most cases several sub-propagations removed from the original, the strains present in the trees we tested are likely the same as in the parent given the relative fidelity of graft transmission (Harper et al. 2015b). The apparent increase in VT-like isolates present in plants collected in 2002 is interesting for it is the first appearance of VT-like isolates in hosts other than Meyer lemon in Florida (Sieburth and Nolan 2005). Given that these isolates were retained precisely because they contain VT, it remains unknown whether this was representative of the frequency of VT in citrus in Florida in 2002.

Table 1
Historical samples examined for CTV strains T36, T30, and VT, collected before the arrival of the brown citrus aphid (1964 to 1996) and after the arrival of the aphid (1997 to 2002).

Collection Period	Isolate Name	Year Collected	Original Host	Location	MCA13	T36	T30	VT
Prior to *T. citricida* (1964 to 1996)	FS43	1964	*C. limon* cv. 'Meyer'	Polk	+	+	-	+
	T4	1969	*C. medica*	Polk	-	+	+	-
	FS217	1971	*C. sinensis* cv. 'Hamlin'	Orange	+	+	+	-
	FS252	1973	*C. sinensis* cv. 'Valencia'	St Lucie	+	+	+	-
	T26	1983	*C. sinensis* cv. 'Valencia'	Polk	-	+	+	-
	T30	1983	*C. aurantifolia*	Polk	-	+	+	-
	T55	1983	*C. sinensis* cv. 'Hamlin'	Orange	-	+	+	-
	T66	1983	*C. paradisi* cv. 'Marsh'	St Lucie	+	+	+	-
	FS319	1983	*C. sinensis* cv. 'Hamlin'	Orange	+	+	+	-
	FS505	1987	*C. sinensis* cv. 'Hamlin'	Hendry	-	+	+	-
	FS523	1987	*C. sinensis* cv. 'Hamlin'	Hendry		+	+	-
	FL77	1995	*C. sinensis* cv. 'Pineapple'	Martin	+	+	+	-
	FL128	1995	*C. sinensis* cv. 'Valencia'	Glades	-	+	+	-
	FL134	1995	*C. reticulata* cv. 'Murcott'	Hendry	+	+	+	-
	FL141	1995	*C. sinensis* cv. 'Valencia'	Hendry	+	+	+	-
	FL145	1995	*C. sinensis* cv. 'Hamlin'	Manatee	+	+	+	+
	FL149	1995	*C. reticulata*	Orange	-	+	+	-
	FL139	1995	*C. paradisi*	Lee	+	+	+	-
	FL186	1996	*C. limon* cv. 'Meyer'	Hardee	-	+	+	-
	FL188	1996	*C. limon* cv. 'Meyer'	Polk	+	+	+	-
	FL207	1996	*C. limon* cv. 'Meyer'	Pasco	+	+	+	+
	FL278	1996	*C. reticulata* cv. 'Temple'	Lee	-	+	+	-
	FS627	1996	*C. sinensis* cv. 'Hamlin'	Polk	+	+	+	+
Post *T. citricida* (1997 to 2002)	FS669	2002	*C. sinensis* cv. 'Valencia'	Polk	+	+	+	+
	FS672	2002	*C. sinensis* cv. 'Roble'	Hillsborough	+	+	+	+
	FS674	2002	*C. sinensis* cv. 'Khalily'	Polk	+	+	+	+
	FS685	2002	*C. sinensis*	Polk	+	+	+	+
	FS692	2002	*C. sinensis*	Polk	+	+	+	+
	FS701	2002	*C. reticulata*	Polk	+	+	+	+
	FS703	2002	*C. reticulata*	Polk	+	+	+	+

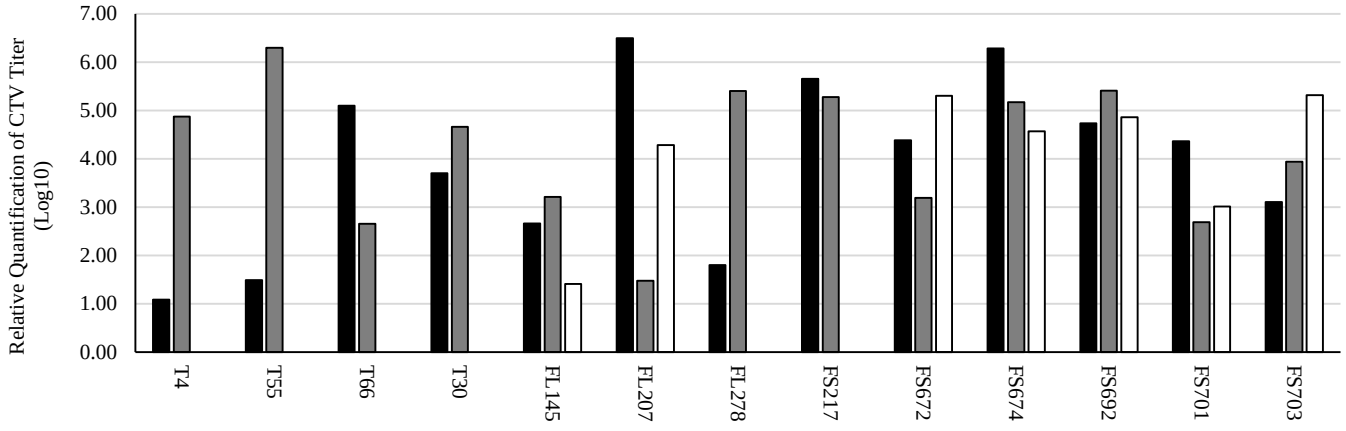

Fig. 1. Comparison of the relative titer of CTV strains T36 (black), T30 (grey), and VT (white) in selected samples from the historical collection, as determined by real-time RT-qPCR.

These data may be compared with the status of CTV strain incidence in 2014 and 2015 (Table 2). As with earlier samples, plants infected with both T36 and T30 strains were prevalent throughout the state and, again, T36 was latent in most samples (data not shown). Some trees, including nearly half of the samples in St Lucie County, and to a lesser extent in Lake and Collier counties, possessed only T30. As with the post-brown citrus aphid samples from 2002, VT incidence is high, though never found in isolation; VT was regularly detected in mixture with T36 and T30, or more rarely, with T36 or T30 alone (Table 2). VT incidence remains largely restricted to Polk and Hillsborough counties in central Florida, with single groves in Marion and Collier counties, in center-north and south Florida respectively, also showing some VT infection; this pattern was also observed by Sieburth and Nolan (2005). There was no correlation between strains present and scion (X_2=0.005, d.f.=25, p>0.05) or rootstock (X_2=0.0035, d.f.=10, p>0.05) variety; this is to be expected as all commonly used commercial citrus varieties used in Florida are susceptible to CTV infection.

CTV strains continue to spread in commercial citrus, particularly within groves. We screened CTV presence in replants within a single older grove that consisted of 20-year-old, CTV-positive, Hamlin on sour orange. These replants were initially virus free, having been obtained from certified nursery stock, and were under a heavy spray regime for vector control. When tested at 6 months post-planting, we found that 9 of 45 (20%) replants had become infected with CTV. All 9 had strain T30, and 1 had T36 as well.

Finally, due to the extensive use of non-sour orange rootstock, few tristeza-like symptoms were observed during this survey. Scattered decline symptoms were observed within older groves on sour orange rootstock, although not all trees in any individual grove were affected, and the severity differed markedly. We therefore compared CTV in decline-symptomatic versus asymptomatic trees in 3 commercial groves in central Florida to see whether there were differences between these trees that correlated with symptom expression. Quantification of the CTV strains present (Fig. 2) revealed that while all trees, both asymptomatic and symptomatic, possessed T36 and T30, those expressing decline symptoms had a T36 titer approximately 1000 times higher than asymptomatic trees. In contrast, the titer of T30 was similar between both groups. This would suggest that T36 titer, and not simply presence, affects disease expression.

Discussion

We began this survey with the intent of determining whether the arrival of the brown citrus aphid, and subsequent loss of trees through decline, as well as more recent losses due to greening, had changed the type or frequency of CTV strains present in Florida. We found that T30 and T36 strains were prevalent in trees sampled in both 1970 and 2015. There was a marked increase in VT strain incidence, and in hosts other than Meyer lemon; interestingly this appears to be restricted to select groves and/or counties rather than a statewide phenomenon. The 3 strains, T36, T30, and VT, are in all major commercial scion/rootstock species tested. There is remarkable uniformity in CTV strain presence, particularly in older groves where tree age has given ample opportunity for admixture of strains via tree-to-tree vector spread or root grafts. A few groves however, possessed more disparate CTV populations, where only some trees had T36 or VT in addition to T30, which may reflect differences in management practices or more effective vector control.

Just as there is little evidence that changes in cultural practices, in rootstock and scion selections, and removal of trees have caused significant changes in the distribution of T36 and T30 strains, there is no reason to suppose that virulent CTV isolates are no longer present. As we observed in this study, decline-inducing CTV isolates continue to exist and spread in Florida, and continue to cause disease under the right circumstances; the absence of decline in groves not using sour orange is due to rootstock choice, not an absence of CTV. Indeed, we observed that trees on Swingle citrumelo maintain a very high titer of T36 and T30 strains (data not shown), making them a potent source of inoculum for neighboring trees and groves.

Table 2
Incidence of CTV strains T36, T30, and VT in Florida citrus in 2014 & 2015, divided by county.

County	Total No. Samples	CTV Strain / Combinations Present						
		T36	T30	VT	T36 T30	T36 VT	T30 VT	T36 T30 VT
Marion	19	0	0	0	9	0	0	10
Lake	14	0	3	0	11	0	0	0
Collier	6	0	2	0	2	2	0	0
Hardee	18	0	0	0	17	0	0	1
St Johns	7	0	0	0	7	0	0	0
St Lucie	30	0	14	0	16	0	0	0
Polk	13	0	0	0	0	0	0	13
Hillsborough	15	0	3	0	6	1	1	4

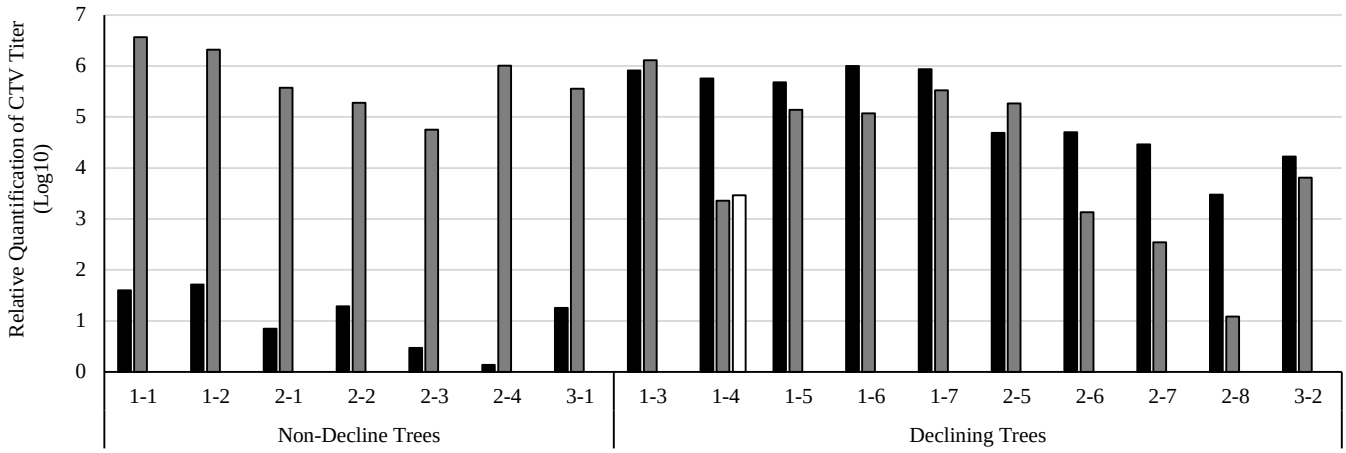

Fig. 2. Comparison of the relative titer of CTV strains T36 (black), T30 (grey), and VT (white), as determined by real-time RT-qPCR, in sweet orange trees on sour orange rootstock expressing decline symptoms against trees from the same groves that are asymptomatic.

The ineffectiveness of vector control was made clear by examination of clean replants in a single grove, nearly 20% of which had become CTV positive within 6 months of planting. The heavy spray regime used in this grove was insufficient to prevent CTV infection in an area of high inoculum pressure.

It has long been supposed, based on MCA13 ELISA assays, that Florida citrus was predominantly infected with only the so-called "mild" T30 strain (Powell et al. 2003). But, as we have seen in this study, nearly all commercial citrus also possess varying levels of T36, and have done so since the 1960s. Given the use of MCA13 in screening budwood for the industry, this is a cause for concern, as it is possible that high T30 can mask the detection of T36 using MCA13 (Powell et al. 2003). So T36, isolates of which can cause decline (Dawson et al. 2015), were present throughout Florida yet caused only localized outbreaks of disease. What effect then, did the brown citrus aphid have? Other researchers have speculated (Powell et al. 2003; Halbert et al. 2004) that this aphid transmitted "severe" strains of CTV, rather than the "mild" strains previously present, yet here we observed no significant change in strain incidence, with the exception of VT, whose spread remained localized to individual groves and/or counties. What happened? Are there more severe, phenotypically different variants of T36 present in Florida that were spread by the introduction of the brown citrus aphid? How do these differ from the T36 isolates prevalent in the 1960s through 1980s? This is an area that requires further research.

In summary, CTV remains an endemic problem for citrus production in Florida, and despite the loss of trees to decline, freezes, and eradication efforts for both citrus canker and greening, is widespread throughout the state. With the recent increase in use of sour orange as a rootstock for new plantings, up to 14% in 2014, the industry should be aware that the threat of a new decline epidemic is very real.

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