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Research Article

Mapping sequences involved in induction of decline by *Citrus tristeza virus* T36 on the sour orange rootstock.

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Abstract

Historically, decline (or tristeza) has been a devastating disease of citrus caused by *Citrus tristeza virus* (CTV). Decline is a man-made disease based largely on propagation of sweet orange, grapefruit, and mandarins on the sour orange rootstock. In Florida, the major problem caused by CTV has been decline, since severe stem-pitting isolates are absent. Although this disease can be controlled by using alternative rootstocks, there are soils in which all other rootstock choices are less desirable in terms of fruit quality and yield. A major goal has been to develop measures that allow growers to use the sour orange rootstock in the presence of CTV. Florida has 2 predominant strains of CTV, a decline (T36) strain and a non-decline strain (T30). A first step was to map the viral determinant that induces decline. This was done by creating hybrids with T30 sequences substituted into T36 to identify sequences correlated with loss of decline symptoms. This project was delayed considerably because greenhouse assays to definitively assay decline symptoms did not work. In order to examine decline in field trees, a permit from the US Department of Agriculture Biotechnology Regulatory Service was obtained to test the recombinant-DNA-produced virus hybrids. This permit required that we test small trees inoculated with CTV at the time of planting. Under these conditions, those constructs that retained the T36 p23 and 3′ non-translated sequences induced greater amounts of stunting.

Keywords: *Citrus tristeza virus*, CTV, decline, tristeza, hybrids, stunting

Introduction

The tristeza disease, also referred to as decline or quick decline, has been one of the more devastating diseases of citrus, killing almost 100 million trees in South America, the United States, and Spain in the early 20th century (Moreno et al. 2008). The disease is man-made, primarily occurring when certain isolates of *Citrus tristeza virus* (CTV) infect sweet orange, grapefruit, mandarins, or other citrus trees grafted onto sour orange rootstocks. Although sour orange has been primarily associated with industry losses, other rootstocks susceptible to tristeza decline include grapefruit, lemon, shaddocks, and some tangelos and citranges. Interestingly, intact trees of these varieties on their own roots are not susceptible to tristeza when infected as mature trees. However, when graft inoculated with certain CTV isolates as young seedlings, they respond with seedling yellows symptoms (Wallace 1959; Dawson et al. 2015).

After the worldwide epidemic of decline, most citrus industries utilized alternative rootstocks that did not decline in response to CTV infection. However, this change came with considerable economic costs because the alternative rootstocks are less adapted to certain soil types. An example of the superiority of the sour orange rootstock is that growers in Florida are returning to the use of the sour orange rootstock, even with endemic CTV decline isolates present, because of the present huanglongbing (HLB) endemic. They are attempting to accelerate growth to allow productivity before trees are killed by HLB or CTV.

There is little information concerning how CTV induces decline, particularly what specific viral protein or RNA sequence is involved in triggering symptom development. This information would be useful for a number of different management approaches. In response to the economic losses due to CTV-induced decline along with stem pitting, another disease phenotype that causes severe disease losses in numerous industries, most citrus producing countries have developed regulatory systems to

manage these diseases, largely by excluding disease inducing CTV isolates. Consequently, importation of new citrus varieties requires that plant material is screened to prevent simultaneous incursion of disease inducing CTV isolates. To that end, an ELISA (Permar et al. 1990) was developed in Florida that enables discrimination between decline inducing T36 and non-decline inducing T30 isolates (Bar-Joseph et al. 1989). Nursery trees infected with CTV isolates that fail to react with the MCA-13 monoclonal antibody, indicating that the isolate is non-decline inducing, are permitted to grow in Florida. However, this antibody has often failed in other countries; isolates of CTV that were MCA-13 negative were found to induce decline. The MCA-13 antibody recognizes an epitope encompassing amino acid 124 (Tyr) in the coat protein (Pappu et al. 1993); this epitope does not correlate with the induction of tristeza (Satyanarayana et al. 2005). Identification of the precise nucleotides of CTV that induce decline could allow development of detection protocols that could be much more effective in other citrus industries.

Another potential tool for managing CTV disease would be to identify or create non-decline isolates to cross protect trees against decline isolates of the virus. Because superinfection exclusion only occurs between isolates of the same strain of CTV, one approach would be to obtain a non-decline isolate from the endemic strain whose members cause decline (Folimonova et al. 2010). Thus, knowing the viral element that causes decline would enable the selection or engineering of a non-decline isolate that would cross protect against endemic decline isolates.

Additionally, knowing the decline-inducing element would solve another problem. We have CTV-based expression vectors (Folimonov et al. 2007; Hajeri et al. 2014) that are being considered as a tool to fight HLB by expressing anti-bacterial or anti-psyllid sequences. However, this vector is built from CTV-T36, a decline isolate. If this vector were used on trees on the sour orange rootstock, it would induce decline. Mapping the determinant of T36 decline could allow creation of a T36 mutant or T36/T30 hybrid vector that would not induce decline of trees on sour orange rootstocks.

One method to identify viral components involved in symptom induction is to examine hybrids between virus isolates that do and do not induce the disease phenotype. The determinant of T36 that induces seedling yellows was mapped using this approach; by examining hybrids between T36, which induces seedling yellows, and T30, which does not induce seeding yellows. T36 hybrids containing the *p23* gene plus 3′ NTR from T30 failed to induce the symptom, demonstrating that this region of T36 was the determinant of seedling yellows (Albiach-Martí et al. 2010).

The objective of this project was to identify the determinants of the decline isolate T36 by similarly examining hybrids between T36 (decline) and isolate T30 (non-decline). Difficulties in observing definitive decline symptoms in greenhouse assays (Garnsey et al. 2005) led to the need for assays of larger trees in the field. Since the hybrids assayed in the field were created by recombinant DNA methods, the experiments had to be conducted according to the conditions of a US Department of Agriculture (USDA) Biotechnology Permit. Here we show that T36/T30 hybrids containing the *p23* gene and 3′ NTR from T30 failed to stunt trees in the field assay, correlating these sequences to the T36 decline phenotype.

Materials and methods

Virus constructs and assay plants

CTV isolates T30 (the type isolate of the T30 strain) and T36 (the type isolate of the T36 strain) were used as viral RNA sources for the generation of the T36/T30 hybrid constructs. The T30 isolate was originally obtained from a naturally infected sweet orange tree (Albiach-Martí et al. 2000) and was maintained in Madam Vinous sweet orange and *Citrus macrophylla* plants. The T36 infectious clone pCTV9 (Satyanarayana et al. 1999, 2001, 2003) and the hybrid T36/T30 constructs were maintained in *C. macrophylla* plants. The hybrids were built and described previously (Albiach-Martí et al. 2010).

Sour orange rootstock liners were purchased from a commercial nursery and moved to the greenhouse onto which Valencia sweet orange buds were grafted. The plants were graft inoculated 3 to 4 months later with the different CTV constructs, using bark pieces from infected *C. macrophylla* plants. Each plant was tested by ELISA to ensure that it was infected. After another 4 months in the greenhouse, the trees were planted 1 June 2010.

A requirement of the USDA permit was that the plants be maximally treated with both systemic and contact insecticides to prevent transmission of the constructs by aphids from the inoculated trees. In addition, the trees were inspected weekly for aphids, followed by immediate insecticide application if aphids were detected. Healthy trees were planted as sentinels adjacent to the test trees, and were assayed periodically by ELISA, followed by sequencing, for movement of the hybrids by aphids. If any sentinel trees became infected with any of the constructs, the experiment would be terminated and the trees burned. The trees were fertilized to promote rapid tree growth and fruit production, before losing the trees to HLB, as per current procedures.

The growth and symptoms of the 3-year-old trees were rated by Garnsey (08-02-2013), Bar-Joseph (08-02-2013), and Robertson and Dawson (11-01-2013, 25-02-2013, and 02-07-2013). The experiment was terminated and the trees were pulled and burned when the permit expired (02-07-2013 and 03-07-2013).

Results

Greenhouse assays

Since T36 was our only infectious cDNA clone of CTV, we could only make hybrids in one direction; substituting 3′ genes of T30 that are not required for replication into the T36 backbone. The association of the

removal of T36 sequences with loss of induction of the decline phenotype should identify the T36 determinant. Toward this goal, we utilized a series of T36/T30 hybrids previously used to map the seedling yellows determinant (Albiach-Martí et al. 2010). Because the 2 coat proteins must precisely interact with HSP70h, p61, and the 5′ NTR to form virions (Gowda et al. 2003; Satyanarayana et al. 2004), a few of the hybrids, although capable of replicating in protoplasts, failed to produce viable virions, and could not be further passaged in protoplasts in order to produce sufficient virions to inoculate citrus trees. The surviving hybrids used to inoculate citrus are shown in Fig. 1.

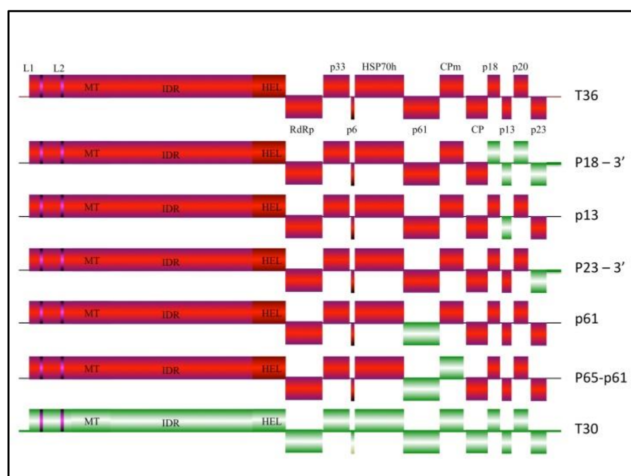

Fig. 1. CTV hybrids. Red are T36 sequences and green are T30 sequences.

Assays of decline of sweet orange on sour orange rootstocks in the greenhouse are known to be problematic (Garnesy et al. 2005). Yet, we initially attempted to assay decline in the greenhouse by inoculating the wild type parents and each hybrid individually on small trees of sweet orange grafted onto sour orange rootstocks. However, when maintained under these conditions, trees infected with decline isolates of CTV failed to develop a visible decline or stunting phenotype (data not shown). Sweet orange on sour orange trees non-infected, infected with non-decline isolates, and infected with decline isolates all grew similarly. A similar result was observed with other decline isolates of CTV that are available to us (T68, T3, and several field isolates containing mixtures of T30, T36, and VT).

Pina et al. (2005) developed a unique greenhouse decline assay by grafting sour orange buds into sweet orange trees already systemically infected with CTV. We attempted similar assays using the parental isolates and the T36/T30 hybrids. However, our assays were complicated by the fact that our decline isolates of CTV also induce seedling yellows symptoms on sour orange. The wild type T36 and several of the hybrids induced yellowing and reduced growth of the sour orange shoots, but we could not be sure that this was a decline reaction and not a seedling yellows reaction.

Field assays

Since the decline phenotype could not be measured accurately in the greenhouse, a field assay was required. A permit was obtained from the USDA to test the hybrids in the field over 4 years. Trees were ready for planting at the end of the first year, resulting in a 3-year time period in which to observe growth and symptom development. The field test limited us to examining small trees that were inoculated in the greenhouse with CTV before transplanting them in the field. Under these conditions, sweet orange trees on sour orange rootstocks generally do not decline, rather they become severely stunted (Cohen and Burnett 1961).

Trees were inoculated with the hybrids (Fig. 1), the T36 wild type, or VT674, which is a decline-inducing population containing VT, T36, and T30. We monitored the plants over the 3-year period. Some of the plants were severely stunted after the first year in the field, and other plants became obviously stunted later. Representative stunted and normal trees are shown in Fig. 2. Near the end of the third year, the trees were evaluated independently by Garnsey, Bar-Joseph, and Dawson and Robertson, with almost identical results (Table 1). The total numbers of trees in the different treatments were the same initially, but some trees died early due to other causes. Inoculation with the decline isolates (T36 and VT674) and constructs p13, p61, and p65-p61 resulted in 10 to 13 stunted trees out of 15 to 19 total trees, whereas constructs mid-p18 to 3′ end, and p23 to 3′ end resulted in only 6 and 4 stunted trees out of 18 total. There were also 2 of 15 non-inoculated trees that were stunted. Thus, both constructs having the *p23* gene and the 3′ NTR resulted in substantially less stunting, suggesting that the determinant of T36-induced decline occurred within these sequences.

Discussion

Instead of the dramatic quick decline and eventual death of sweet orange trees on sour orange rootstocks infected by CTV that occurs in large grove trees, we observed different degrees of stunting in the small trees. This phenotype was initially reported for small trees in Florida (Cohen and Burnett 1961), and was an epidemic in the 1980s when short nursery tree supply resulted in T36-infected budwood being used (Garnsey and Jackson 1975; Brlansky et al. 1986; SM Garnsey, personal communication). We did not see completely uniform results, but the trend of stunting was associated with T36 constructs retaining the p23 to 3′ end sequences. A few of the non-inoculated trees and those inoculated with constructs containing the p23 to 3′ end sequences from T30 were also stunted. This could have resulted from any number of maladies in the field. Also, a few of the trees containing the p23 to 3′ end sequences from T36 failed to show stunting. It should be noted that all of the trees were grown under the maximal fertilization regime currently used in Florida, which pushes trees to grow rapidly and become productive before HLB damage can occur. The lack of quick decline of the small trees is better

understood considering the seminal anatomical work done by Schneider (1954). The decline of sweet orange on sour orange rootstocks is associated with necrosis of phloem cells (Schneider 1954). However, it does not result directly from a massive death of phloem immediately below the bud union. The earliest abnormality that Schneider (1954) observed was the necrosis of some sieve tubes and companion cells of sour orange at the bud union, along with hypertrophy of adjacent parenchyma cells. This was followed by necrosis of sieve tubes and companion cells in the sweet orange cells above the bud union.

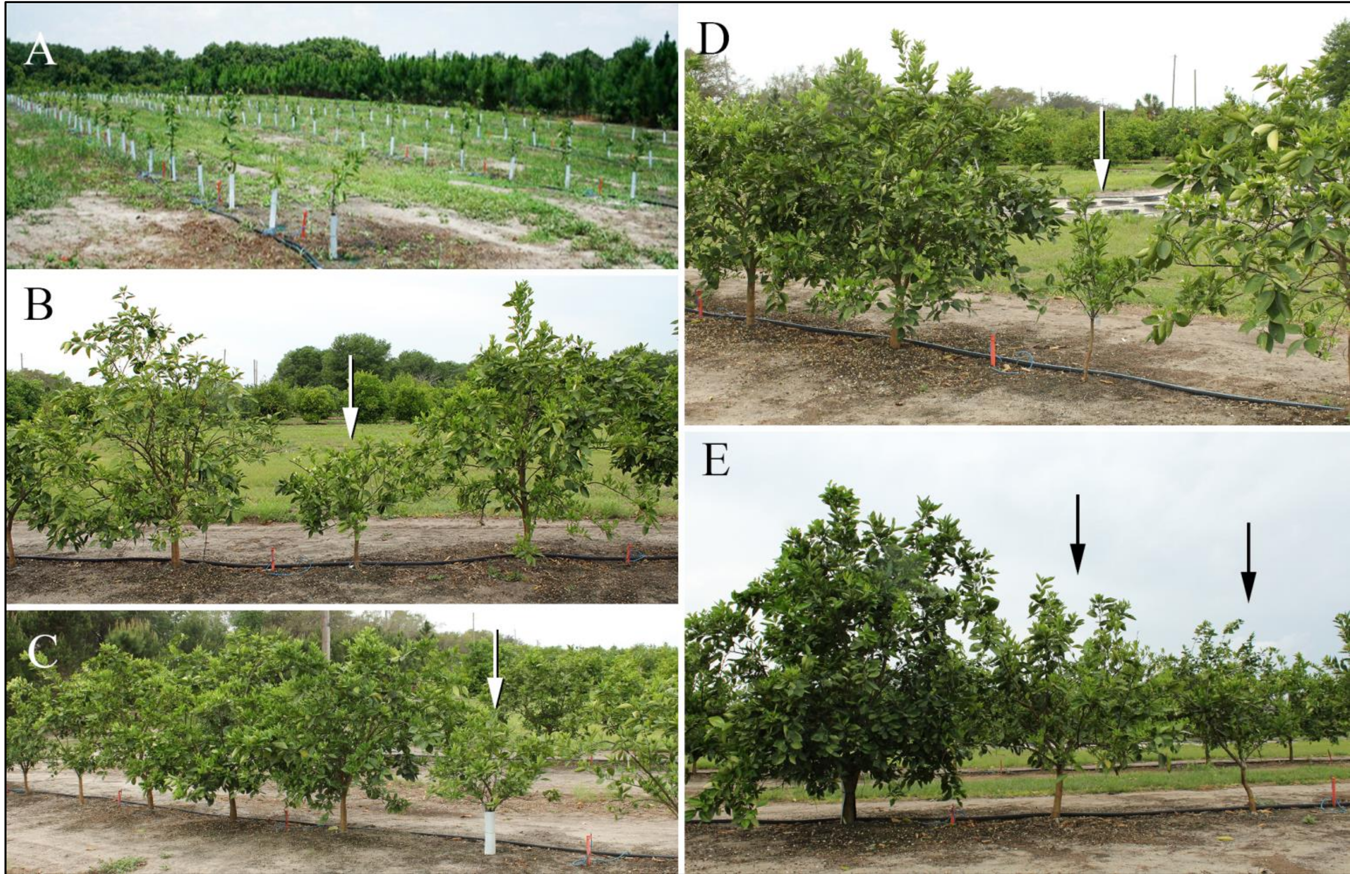

Fig. 2. A) shows the field after planting; B-E) shows examples of stunted trees marked by arrows.

Table 1
The effect of CTV infection on the stunting of Valencia sweet orange trees on sour orange rootstock.

Construct	Decline/Total
Non-inoculated	2/15
mid p18 to 3′ end (*T30 sequences*)	6/18
p13 *(T30 sequences)*	13/18
p23 to 3′ end (*T30 sequences*)	4/18
p61 (*T30 sequences*)	12/19
p65-p61 (*T30 sequences*)	10/18
T36	10/15
VT674	13/15
Summary of p23-3′ replacement	**28%**
Summary of other infections	**64%**

Over time, as early symptoms developed, the bands of functional phloem both above and below the bud union were reduced. However, except during certain times of the year, some functional phloem remained, even as the trees declined. As sieve elements became necrotic, new sieve elements were produced, but they were smaller than normal. In this respect, young trees differed from mature trees. In mature trees, the wide band of phloem was only partially replaced by small sieve elements, whereas young trees sometimes had almost complete replacement of sieve elements. Occasionally, trees did not die; new growth was still produced on otherwise defoliated trees. These trees had greatly reduced functional phloem width both above and below the bud union.

It is possible that the T36 present in Florida induces less severe decline than the T36 present in other countries, thus the stunting phenotype that was observed instead of decline in small trees in Florida. In terms of seedling yellows, the phenotypes of different CTV isolates range from severe chlorosis resulting in almost white leaves

with almost no new growth and rare recovery, to only a transient yellowing and uniform recovery. Most isolates of the T36 strain exhibit the latter phenotype; the chlorosis is marginal and transient. It is possible that different CTV isolates differ in severity or rapidity of decline, just as they do in seedling yellows, and that T36 is a milder decline isolate.

The field test had to be planned and approved before beginning the experiment, with no deviations from the protocol proposed for the permit. It may have been better to inoculate older field trees, but this was not possible under the permit conditions. In addition, large trees free from natural CTV and HLB infections would have been difficult, perhaps impossible, to find. The permit allowed 3 years of growth to be monitored. The results of CTV stunting would have been more distinct, with more trees likely being stunted, had we continued the experiment longer, but in the last year the test plot was in the early stages of HLB infection. Had we extended the field experiment we would have had difficulty distinguishing symptoms induced by CTV from symptoms of HLB.

The stunting of the trees mapped to the *p23* gene plus the 3′ non-translated region of isolate T36. All constructs that had these sequences from T36 induced a greater amount of stunting. Most of the differences between T36 and T30 in this region reside in the *p23* gene. There are 8 nt differences in the 3′ non-translated region and 57 within the *p23* gene. The phenotype of citrus has already been shown to be affected by the *p23* gene of T36; the induction of the seedling yellows phenotype had been mapped by substituting the *p23* gene plus the 3′ non-translated region of T36 with the homologous region of T30, which abolished the capacity of the hybrid virus to induce seedling yellows (Albiach-Martí et al. 2010).

The *p23* gene also induced abnormalities when expressed in transgenic citrus. Sweet and sour orange trees transformed with the *p23* gene exhibited severe symptoms, including vein clearing, epinasty, stunting, and leaf distortion (Fagoaga et al. 2005; Ghorbel et al. 2001). Since p23 is one of the suppressors of RNA silencing (Lu et al. 2004), it is possible that alteration of this process either by reduced activity of the T30 p23 protein or reduced activity in cooperation with the other suppressor proteins causes the stunting and decline. The mapping of decline and seedling yellows to the same region of the CTV genome is interesting, given that it has long been recognized that most isolates that cause decline also induce seedling yellows. It is possible, however, that other isolates of CTV may induce decline via other gene products or mechanisms.

The need to use sour orange root stock, coupled with the capacity of certain isolates of CTV to induce decline of trees on sour orange rootstocks, is a problem in almost all citrus industries. Understanding how CTV induces decline forms a foundation from which to develop new approaches for managing this disease. For example, it is now known which sequences in the T36-based CTV expression vector would need to be replaced, and that sequences from T30 can be substituted, to avoid the problem of decline if the vector is deployed as a tool to fight HLB in citrus trees on sour orange rootstock.

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