Developing an understanding of cross-protection by *Citrus tristeza virus*

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**INTRODUCTION**

*Citrus tristeza virus* (CTV) causes two citrus diseases that have caused devastating losses in global citrus production. The first disease is quick decline of trees propagated on the sour orange rootstock. The second disease is stem pitting, which severely affects a number of economically important citrus varieties regardless of the rootstock used and results in reduced tree growth and vigor as well as in reduced fruit size and quality. Both diseases continue to invade new areas. While quick decline could be effectively managed by the use of resistant and/or tolerant rootstocks, the only means to protect commercial citrus against endemic stem pitting isolates of CTV has been cross-protection with mild isolates of the virus. In some citrus areas cross-protection has been successful and allowed production of certain citrus cultivars despite the presence of severe stem-pitting isolates in those regions. However, many other attempts to find isolates that would provide sustained protection against aggressive isolates of the virus had failed. In general, there has been no understanding why some mild isolates were effective and others failed to protect. We have been working on the mechanism of cross-protection by CTV. Recent considerable progress has significantly advanced our understanding of how cross-protection may work in the citrus/CTV pathosystem. As we demonstrated, only isolates that belong to the same strain of the virus cross protect against each other, while isolates from different strains do not. We believe that the results of our research could now make finding protecting isolates relatively straightforward. This review discusses some of the history of CTV cross-protection along with the recent findings and our “recipe” for selection of protecting isolates.

*Keywords: cross-protection, superinfection exclusion, Citrus tristeza virus, citrus, homologous interference*

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*Citrus tristeza virus* (CTV) is the largest and most complex member of the family Closteroviridae, which contains viruses that cause severe economic losses in crops including vegetables, grains, grapes, and fruit trees (Bar-Joseph et al., 1979; Dolja et al., 1994, 2006; Agranovsky, 1996; Kataeva, 2000). The natural host range of CTV is restricted to citrus and citrus relatives. Among viruses that infect citrus plants, CTV has been the most destructive. Following the large dissemination from its origin, which is thought to be South East Asia, into new regions at the end of nineteenth century due to active movement of different citrus varieties between continents, the virus caused severe disease epidemics in citrus and nearly destroyed whole citrus industries in various countries around the globe (reviewed by Moreno et al., 2008). Furthermore, in many citrus growing regions severe isolates of the virus continue to limit citrus production.

As the only option to suppress some of the aggressive virus isolates after they become endemic, cross-protection with mild isolates has been extensively explored in different production areas (reviewed by da Graça and van Vuuren, 2010; Roistacher et al., 2010). Earlier attempts to use this approach had erratic results. When successful, the mild protecting isolates have enabled the commercial production of certain citrus varieties in some citrus areas. However, protecting isolates have not been found in other regions or for other varieties. In many cases mild CTV isolates failed to protect or provided only short-term protection against severe disease.

Elucidation of the mechanism of CTV cross-protection has been an important component of the research program in our laboratory for a number of years. In this review I discuss some of the history of CTV cross-protection that goes back more than half of a century along with the recent findings of our research.

**THE COMPLEX OF CTV DISEASES**

Depending on the virus isolate and a citrus host scion/rootstock combination, CTV causes two major diseases, which have had a major impact on global citrus production. The first disease is quick decline of trees on the sour orange (*Citrus aurantium*) rootstock, which results from a virus-induced graft incompatibility between the scion and rootstock. During the last century severe epidemics of CTV-caused quick decline that developed in citrus growing regions destroyed almost 100 million trees (reviewed by Moreno et al., 2008). These losses prevented further usage of this popular rootstock for propagation of trees in citrus areas where decline-causing isolates of CTV were endemic. Alternative rootstocks, which create scion/rootstock combinations that do not respond with the decline syndrome to such virus isolates, were put in use. Although this allowed effective management of CTV-induced quick decline, those rootstocks often did not perform as well as the well-adapted sour orange rootstock.
Another disease that is caused by some of the CTV isolates is stem pitting. The disease severely affects grapefruit (C. paradisi Macfadyen), sweet orange (C. sinensis L. Osbeck), and lime (C. aurantiifolia (Christm.) Swingle) trees regardless of the rootstock used. Stem pitting results from disrupted differentiation of the cambium as the stem of an infected tree grows, which leads to the development of pits in areas of virus multiplication (Belansky et al., 2002; Tatineni and Dawson, 2012) resulting in reduced tree growth and vigor as well as in reduced fruit size and quality, which are highly important economic concerns (Roistacher and Moreno, 1991; Garnsey et al., 2005; Moreno et al., 2008). The CTV-associated stem pitting has caused significant economic damage for citrus industries in many different countries, including Brazil and other countries in South and Central Americas, South Africa, Australia, and a number of countries in Asia. In most of these regions stem pitting remains to be a major factor limiting citrus productivity.

Both diseases continue to spread into new areas, mainly via movement of infected plants or vegetative propagation of infected budwood followed by further local spread by several aphid species (Hilf et al., 2007; Moreno et al., 2008; Matos et al., 2013). There have been multiple examples of inadvertent introduction of severe CTV into many citrus-producing countries due to the international movement of citrus varieties despite established quarantine practices (Moreno et al., 2008). The discovery of new exotic CTV isolates in commercial citrus plantings in California (M. Polek and R. Yokomi, personal communication) and in Florida, USA (Sieburth and Nolan, 2005; Hilf et al., 2007) represent some of the recent examples. Once introduced, new isolates can be readily dispersed within a region via natural transmission of the virus by its aphid vector. The potential for future crop losses from CTV is much greater than what has been seen to date. Therefore, the development of means to protect citrus plantings against aggressive isolates is critical for virus suppression.

MANAGING CTV DISEASES VIA CROSS-PROTECTION

Cross-protection, a phenomenon in which a pre-existing viral infection presents a secondary infection with the same or closely related virus, was first demonstrated by McKinney (1926, 1929) in tobacco mosaic virus (TMV) infection prevents a secondary infection with the same or closely related virus, was first demonstrated by McKinney (1926, 1929) in tobacco mosaic virus (TMV) between two genotypes of Tobacco mosaic virus (TMV) related virus, was first demonstrated by McKinney (1926, 1929) in tobacco mosaic virus (TMV). Cross-protection was initially used as a test of virus relatedness to define whether two virus isolates were “strains” of the same virus or represented different viruses (McKinney, 1929; Salaman, 1933; reviewed by Hull, 2002; Gal-On and Shibolet, 2006). With plant viruses, cross-protection was initially used as a test of virus relatedness to define whether two virus isolates were “strains” of the same virus or represented different viruses (McKinney, 1929; Salaman, 1933; reviewed by Hull, 2002; Gal-On and Shibolet, 2006). Subsequently purposeful infection with a mild isolate was implemented as a protective measure against endemic isolates of the virus that caused severe disease, which in some cases was called “pre-immunization” (reviewed by Hull, 2002; Gal-On and Shibolet, 2006). The practical aspect of the cross-protection phenomenon is reflected in the more focused definition of the phenomenon used by Gonzalez and Garnsey (1989) as well as a number of other researchers, who described cross-protection as “the use of a mild virus isolate to protect plants against economic damage caused by infection with a severe challenge strain(s) of the same virus.” The ability of mild isolates to protect against challenge with other isolates of the same virus has been demonstrated for a large number of plant viruses (reviewed by Ziebell and Carr, 2010). However, practical measures for virus suppression in the field were developed for only a few of them. In addition to CTV, some of the examples of viruses for which such applications were shown to be successful include Zucchini yellow mosaic virus in squash, melon, and watermelon (Cho et al., 1992; Yarden et al., 2000), Cacao swollen shoot virus in cocoa (Hughes and Olliffen, 1994), Tomato mosaic virus in tomato and pepper (Tien and Zhang, 1985), and Papaya ring spot virus in papaya (Teh et al., 1988). In most cases, however, the use of cross-protection was eventually abandoned due to the breakdown of protection or development of alternative control means, such as generation of resistant plants. Remarkably, one of the first examples of the commercial exploitation for prevention of severe viral infections was cross-protection against severe CTV stem pitting with mild virus isolates (Grant and Costa, 1951). Cross-protection has continually played a major role in maintaining profitability of citrus production in several industries around the world (reviewed by Moreno et al., 2008).

Among the two diseases caused by CTV, stem pitting is the most difficult to control. The disease affects both scion and rootstock, so changing to tolerant rootstocks is not effective. At present, the only means to protect commercial citrus varieties from severe CTV-associated stem pitting is cross-protection with appropriate mild CTV isolates. This approach has been most extensively used in Brazil where more than 80 million Pera sweet orange trees are protected. It also has been used in Australia for protection of Marsh grapefruit against severe stem pitting isolates widely distributed in the country as well as for protection of Star Ruby grapefruit in South Africa, Navel orange and lime in Peru, red grapefruit in Argentina, and C. hassaku trees in Japan where it allowed commercial production of those citrus varieties despite the presence of aggressive stem pitting isolates in those regions (reviewed by da Graça and van Vuuren, 2010; Roistacher et al., 2010).

With all the successes in the use of cross-protection described above, an enormous difficulty of making cross-protection work needs to be understood. The reality is that without knowing rules of CTV cross-protection it was very hard and in most cases impossible to find protecting isolates. In Brazil, for instance, it took over a decade and half for the establishment of commercial orchards of cross-protected Pera sweet orange (Costa and Müller, 1980). Prior to finding a satisfactory mild isolate, many sweet orange, lime, and grapefruit plantations were surveyed in order to identify trees that were doing well in groves severely affected by the stem pitting disease. Forty five selections were used for further field tests that involved almost 2,300 trees. Among those 45 mild isolates, only six were satisfactory, which included three for Pera...
Although an acceptable degree of Marsh grapefruit protection was achieved, difficulties have been experienced in pre-immunizing red grapefruits and no mild isolates that could confer protection against stem pitting of sweet orange were found (Broadbent et al., 1991). Complete lack of success in developing cross-protection-based means to control CTV was reported in California. There it proved highly difficult to find local mild isolates which will protect against severe stem pitting isolates. Evaluation of over 100 mild isolates collected from throughout California yielded no protection (Roistacher and Dodds, 1993). In addition to the efforts to develop effective protection against stem pitting, extensive experimentation has been done in order to achieve protection against quick decline. As discussed above, in contrast to the stem pitting disease, quick decline could be effectively managed by the use of resistant and/or tolerant rootstocks in combination with pathogen-free germplasm. This, however, does not negate an importance of finding mild virus isolates that could provide sustained protection against this disease. Due to the high adaptability of sour orange rootstock to a variety of soil types and its tolerance to the oomycetes-associated root rot diseases as well as the ability to support scions that produce high yields of fruit, it would be desirable in many situations to preferentially use this rootstock. The development of an effective cross-protection strategy against quick decline would bring it back into play. A number of experiments were conducted in this attempt worldwide, however, all were unsuccessful, and no effective protective CTV isolate has been found (reviewed by da Graça and van Vuuren, 2010; Roistacher et al., 2010).

Overall, finding protecting isolates has been empirical and rarely successful. The general approach for selecting protecting isolates was to find infected plants showing little or no symptoms in areas where severe isolates have caused serious disease and test them for the ability to protect against severe isolates in different varieties, which required years of evaluation. Researchers have spent their whole careers trying to develop a cross-protection-based approach to control CTV. Often mild CTV isolates failed to protect or provided only limited short-term protection against severe disease. Best results were obtained when mild isolates derived from certain citrus varieties were used for pre-immunization of the same varieties; the same isolates usually performed poorly when used with other citrus varieties. In general, there has been no understanding why some mild isolates were effective and others failed to protect.

**UNDERSTANDING CROSS-PROTECTION BY CTV**

**EXAMINATION OF THE ABILITY OF DIFFERENT ISOLATES OF CTV TO PREVENT SUPERINFECTION BY ANOTHER ISOLATE OF THE VIRUS**

CTV has long flexuous virions (2000 nm × 10–12 nm) that are encapsidated by two coat proteins. A single-stranded RNA genome of CTV, which is ∼19.3 kb, encodes twelve open reading frames (ORFs; Pappu et al., 1994; Karasev et al., 1995) (Figure 1). ORFs 1a and 1b are expressed from the genomic RNA and encode polyproteins required for virus replication. ORF 1a encodes a 349 kDa polyprotein that has two papain-like protease domains plus methyltransferase-like and helicase-like domains. Translation of the polyprotein is thought to occasionally continue through the polymerase-like domain (ORF 1b) by a +1 frameshift. Ten 3′ end ORFs are expressed by 3′ co-terminal subgenomic RNAs (sgRNAs; Hilf et al., 1995; Karasev et al., 1997). Those ORFs encode the following proteins: major (CP) and minor (CPm) coat proteins, p65 (heat shock protein 70 (HSP70) homolog), and p61 that are involved in assembly of virions (Satyanarayana et al., 2000); a hydrophobic p6 protein with a proposed role in virus movement (Dvola et al., 2006; Tatineni et al., 2008); p20 and p23, which along with CP are suppressors of RNA silencing (Lu et al., 2004); and p53, p12, and p18, which play a role in extending the virus host range (Tatineni et al., 2011). Yet, trees of most citrus varieties can be infected with mutants that have the genes for the latter three proteins deleted (Tatineni et al., 2008).

CTV has numerous isolates with distinctive biological and genetic characteristics. The isolates can be classified into six major CTV genotype groups or strains: T3, T3h, T3e, VT, T68, and resistance breaking (RB), with some isolates being unclassified (Folimonova et al., 2010; Harper, this series). Strains are defined as phylogenetically distinct lineages of CTV based upon analysis of nucleotide sequences of the 1a ORF (Hilf et al., 2005; Folimonova et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series). This region of the genome displays high genetic diversity between CTV variants, with levels of sequence identity ranging between 72.3 and 90.3% (Mawassi et al., 1996; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Hilf et al., 2010; Harper, this series).
of different genotypes and recombinants between these geno-
types (Grant and Higgins, 1957; López et al., 1998; Kong et al.,
2000; Rubio et al., 2001; Vives et al., 2005; Weng et al., 2007; Martin
et al., 2009).

Earlier we developed a green fluorescent protein (GFP)-
expressing CTV vector based on an infectious cDNA clone of
CTV T36, the type isolate of the T36 strain (Folimonov et al.,
2007; GFP-T36 CTV herein). This virus contains an extra ORF
that of GFP inserted into the viral genome between the CPm and
2007; GFP-T36 CTV herein). This virus contains an extra ORF,
CTV T36, the type isolate of the T36 strain (Folimonov et al.,
expressing CTV vector based on an infectious cDNA clone of
phloem-associated cells of those hosts (Folimonova et al., 2008).
observation of which allowed visualization of virus distribution in
T36 CTV in different citrus varieties produced GFP fluorescence,
in infected plants (Folimonov et al., 2007). Multiplication of GFP-
developing systemic infections and produced similar symptoms
the wild type T36. Both viruses showed similar time intervals for
expression of symptoms of a severe virus iso-
late by pre-inoculation of a host with a mild isolate. We define
cross-protection as superinfection exclusion or, in other words,
as the ability of a primary virus infection to completely exclude
secondary infection with the same or closely related virus.

After examination of many different CTV isolates, it was
found that superinfection exclusion occurs between isolates of
the same strain, but not between isolates of different CTV strains
(Folimonova et al., 2010). When citrus trees pre-infected with an
isolate of one of the five genotypes (strains) of CTV (T36, T3, T68,
VT, or T36) were sequentially challenged with GFP-marked T36
CTV, all of them with the exception of the plants that were initially
infected with isolates of the latter T36 genotype displayed GFP
fluorescence similar to that observed in control plants that had
no primary infection and were inoculated only with the challenge
virus (Figure 2). The isolates of heterologous strains had no inter-
ference with the secondary infection by the T36-based virus. In
contrast, no GFP fluorescence was detected in plants first infected
with isolates of the T36 strain. The T36 isolates completely pre-
vented superinfection by the GFP-tagged virus of the same T36
strain. The results were “black and white.” The isolates from het-
rogenous strains conferred no protection. The isolates from the
same strain protected totally. Additional experiments in which
interactions of several different combinations of primary and chal-
lenging virus isolates were evaluated using reverse transcription
polymerase chain reaction (PCR)– or serology-based differenti-
ation between genotypes of the virus demonstrated that CTV
isolates that have established a systemic infection in citrus trees
prevent superinfection by an isolate of the same strain, but not by
isolates from different strains (Folimonova et al., 2010). Remark-
ably, similar results were obtained using two different citrus hosts
for CTV: highly susceptible C. macrophylla and less susceptible
sweet orange in which fewer cells become infected with the virus
compared with the former host. In both hosts exclusion among
isolates of the same strain of CTV was absolute, while isolates from
different strains demonstrated complete lack of exclusion. Fur-
thermore, with the GFP-marked virus used as a challenge virus,
we saw no difference in the proportion of cells infected or in the
intensity of GFP fluorescence per infected cell in trees infected ini-
itially with isolates of heterologous strains compared to inoculation
of trees with no primary infection. The isolates of heterologous
strains that were established initially appeared to have no effect on
infection, movement, and replication of the challenge virus. Addi-
tionally, when trees were initially infected and later challenged
with isolates belonging to the same strain, there was no evidence

![Figure 1](Image 1) Schematic diagram of the genome organization of wild type
CTV and its derivative GFP-T36 CTV encoding green fluorescent protein
(GFP). The open boxes represent ORFs and their translation products. PRO,
papain-like protease domain; MT, methyltransferase; HEL, helicase; RdRp,
rna-dependent RNA polymerase; HSP70h, HSP70 homolog; Cpm, minor coat protein; CP, major coat protein. Bent arrows indicate
positions of beet yellows virus (BYV) or CTV CP sgRNA controller
elements (CE).
FIGURE 2 | Observation of GFP fluorescence in phloem-associated cells of C. macrophylla trees upon challenge with GFP-T36 CTV. Left panel represents a non-inoculated healthy tree. Other panels represent trees with no primary infection (second panel) or pre-infected with isolates belonging to five CTV strains, which were sequentially challenged with GFP-T36 CTV. Observations were done on the internal surface of bark at 2 months after challenge inoculation using a dissecting fluorescence microscope. Scale bar = 0.4 mm. Figure 2 as it appears in this review is similar to that published in the original manuscript (see Figure 3 in Folimonova et al., 2010).

As discussed above, isolates of CTV are generally classified into phylogenetically distinct lineages or strains based on sequence analysis of the more diverged 5’ half of the genome (Harper, this series). This grouping reflects the pattern of exclusion, suggesting the sequence divergence in this region of the genome may affect inter-virus interactions resulting in the complete lack of superinfection exclusion between isolates of different CTV strains. This contradicts with the premise of one of the original uses for superinfection exclusion as a measure of virus relatedness, in which non-excluded viruses were identified as different viruses (Matthews, 1991). Apparently, that is not the case with CTV. Superinfection exclusion defines excluding CTV isolates as members of the same strain, not different strains.

EXCLUSION OF SUPERINFECTION BY ISOLATES OF CTV IN THE FIELD

The findings from our basic research discussed above correlate well with other observations that we have made while analyzing the dynamics of CTV populations in the Dominican Republic. Our data demonstrated a dramatic change in CTV populations that occurred in this region over a period of 10 years, which was characterized by tremendous increase in the incidence of the VT genotype and the introduction of two new virus genotypes, T36 and RB (Matos et al., 2013). Remarkably, the VT isolates of CTV were able to move in and spread in commercial citrus despite the fact that prior to their introduction into the country most citrus trees have been already infected with mild T30 isolates of the virus. The pre-existing isolates of the T30 genotype apparently did not provide protection against the isolates of the VT genotype. The same was true for the newly found T36 and RB genotypes. These viruses appeared to be able to superinfect trees that appeared to be infected with other genotypes of the virus prior to their invasion. Multiple infections of trees resulted in formation of complex virus populations composed of various combinations of different genotypes. Since a systemic infection with a CTV isolate in citrus trees prevents superinfection by an isolate of the same genotype, but not by isolates from other genotype groups of the virus, the widely spread isolates of the T30 genotype could not prevent dissemination of the isolates of the VT and T3 genotypes that were introduced in the Dominican Republic later. Further, the pre-existing infection with isolates of all these genotypes could not exclude invasion of isolates of the two other genotypes, the T36 and RB.

POTENTIAL MECHANISMS

Superinfection exclusion of viruses has been related to a number of different mechanisms acting at various stages of the viral life cycle, including prevention of the incoming virus entry into cells (Steck and Rubin, 1966a,b; Lee et al., 2005), competition between primary and challenging viruses for host factors and intracellular replication sites, interference with disassembly, translation or replication of the secondary virus (Steck and Rubin, 1966a,b; Sherwood and Fulton, 1982; Adams and Brown, 1985; Abel et al., 1986; Karpf et al., 1997; Lu et al., 1998; Beachy, 1999; Lee et al., 2003), and induction of RNA silencing by the protector virus that leads to sequence-specific degradation of the challenge virus RNA (Batcliff et al., 1997, 1999; reviewed in Hull, 2002). Most of the proposed mechanisms, with the exception of the latter one, could function only in cells that were infected with the primary virus, leaving uninfected cells susceptible to the secondary virus. Based on our data, such mechanisms would not be relevant for superinfection exclusion by CTV, since the phenomenon appears to be systemic and functions not only in cells infected with the primary virus, but also in cells that were not infected. Usually, in a host, CTV infects only a portion of the phloem associated cells: less than one-third of the cells even in the most susceptible varieties (Folimonova et al., 2008). However, even though the majority of cells were...
not infected by the primary isolate, exclusion of a challenging isolate of the same strain was absolute. Not only the one-third of the cells that contained the primary virus was protected, but the other two-thirds of the cells that were not infected became “immune” to the challenging virus (Folimonova et al., 2010). Thus, the exclusion phenomenon must be able to spread beyond the infected cells.

The “systemic” nature of superinfection exclusion by CTV parallels characteristics of RNA silencing that has been considered as the major antiviral defense mechanism in plants and invertebrates (Vance and Vaucheret, 2001; Voinnet, 2001, 2005; Baumbach, 2004; Li and Ding, 2005). RNA silencing can be triggered systematically: in cells that contain the primary virus and also in cells that were not pre-infected with the one. The mechanism elicits degradation of RNA molecules that have nearly identical sequences (Ratcliff et al., 1999; Jan et al., 2000; Thomas et al., 2001; Voinnet, 2001). Therefore, for a number of plant viruses RNA silencing was suggested as a mechanism that confers homologous interference of viruses (Ratcliff et al., 1997, 1999; Valkonen et al., 2002; reviewed by Hull, 2002; Gal-On and Shibolath, 2006). To examine the role of RNA silencing in CTV superinfection exclusion, we attempted to trigger exclusion between heterologous CTV isolates by substituting extended regions in the genome of the protecting virus with the exact cognate sequences from the genome of the challenging virus. The substituted regions contained 3’ end genes, which amplify large amounts of double-stranded RNAs (Moreno et al., 1990, 2008; Häf et al., 1993). This part of CTV genome directs production of most viral small RNAs upon CTV infection (Ruiz-Ruiz et al., 2011). Nevertheless, the hybrids in which these regions were substituted from the challenge isolate failed to exclude the latter isolate despite that they shared extended identical sequences (Folimonova et al., 2010). These results did not appear to support the RNA silencing-based model and further argued for the intriguing complexity of CTV superinfection exclusion phenomenon, posing a possibility of an existence of a novel mechanism for superinfection exclusion between virus variants.

Most recently, we demonstrated that superinfection exclusion by CTV is due to a mechanism that requires production of a specific viral protein, the p33 protein (Folimonova, 2012). The p33 is a non-conserved protein with no significant homology to other known proteins and is not essential for CTV infection in most citrus hosts (Tatímeni et al., 2008). Lack of the functional p33 completely abolished the exclusion ability of the virus. The virus mutants that failed to produce p33 failed to exclude superinfection by the parental wild-type virus. Superinfection exclusion was conferred by the protein rather than the RNA sequence: the mutants that retained the entire sequence of the p33 ORF yet, had a deletion of the subgenomic mRNA CE for the p33 protein. This mutant and sequentially challenged with the GFP-marked CTV showed GFP fluorescence, which distribution and intensity were comparable to that found upon inoculation of trees with no primary infection (Folimonova, 2012). More studies will be needed to determine whether superinfection exclusion by CTV involves components of RNA silencing pathway or operates via another novel mechanism. The p33 protein appears to function in a homology-dependent manner. The hybrid viruses with the p33 substitutions behaved, similarly, to the mutants that produced no p33. They were unable to interfere with the secondary infection by the wild type virus, indicating that a heterologous p33 could not confer the exclusion (Folimonova, 2012). These data suggest an existence of a precise interaction(s) of the p33 protein with some other viral factor(s) involved in superinfection exclusion.

**RECIPE FOR CROSS-PROTECTION BY CTV**

As a result of our research efforts, now we know the basic rule of CTV cross-protection: sustained protection against a severe isolate of a particular CTV genotype (strain) can be achieved only by using mild isolates of the same genotype. We believe that this knowledge could make finding protecting isolates relatively straightforward. The first objective for development an effective cross-protection system is to identify the genotype of the severe isolate that needs to be controlled. Then a mild isolate of that same genotype needs to be found. If such an isolate does not occur naturally, it is possible through recombinant DNA methodologies to map the disease determinant(s) of the severe isolate and then remove it by substituting sequences from a mild isolate of a different strain. The resulting mild isolate should exclude the severe isolate. A similar approach was used for the decline isolate in Florida, USA (Aibuch-Martí et al., 2010).

To fulfill the first objective, or, in other words, to identify the “enemy,” an assessment of the pathogenic potential of CTV isolates in a given area needs to be conducted. This includes collection of CTV isolates from highly symptomatic trees in various locations and their biological characterization using standard indicator hosts (grapefruit, sweet orange, sour orange, and Mexican lime) and commercially important varieties. The following step is molecular characterization of those isolates in order to determine their genotype composition. At first, this can be done by amplifying genomic fragments with the oligonucleotide primers that specifically amplify sequences of particular CTV genotypes (strains) using nucleic acids extracted from collected plant material, followed by sequence analysis of the resulting products. We have used a similar strategy for characterization of CTV populations in the Dominican Republic (Matos et al., 2013). The approach has been also widely used by many other CTV researchers (Ribó et al., 2001; Häfl et al., 2005; Roy and Belansky, 2009; Scott et al., 2012). An alternative strategy, which recently became quite popular among different virologists, is the use of next-generation sequencing techniques for virus characterization (Wu et al., 2010; reviewed by Singh et al., 2012). Sequencing of full viral genomes could be done, for instance, via using viral small RNAs that are produced during infection. Those are purified and used for library construction, which is then subjected to a high-throughput sequencing that generates millions of short reads in a single sequencing run. The latter reads are further used for virus genome reconstruction via methods of computational analysis. This approach was recently used for analysis of CTV isolates from Spain and Florida, USA (Ruiz-Ruiz et al., 2011; Harper, this series).
Similarly, viral genome sequencing via next-generation sequencing techniques could be conducted using cDNA prepared from total or double-stranded RNA isolated from virus-infected plants as has been demonstrated in a number of recent publications (Adams et al., 2009; Coetze et al., 2010).

For the second objective, non-symptomatic trees in which CTV isolates frequently represent a mixture of different virus strains, for practical applications to control CTV diseases in the field, it would be valuable to develop a broad-spectrum cross-protection, for instance, by creating a virus for protection against multiple CTV strains. Our premise is that further research on the superinfection exclusion mechanism will define ways for more effective protection of citrus crop against CTV, including engineering transgenic resistance and developing methods to extend the effectiveness of cross-protection. Knowledge developed with CTV can be further transferred to other viruses that cause diseases in other economically important crops.

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FUTURE CONSIDERATIONS

Overall, our data demonstrate that superinfection exclusion by CTV is an active virus-controlled function. It is a powerful process that completely prevents a challenging infection by a closely related virus variant. At this point, its effectiveness is limited to isolates belonging to the same virus strain. However, because severe isolates of the virus frequently represent a mixture of different virus stains, for practical applications to control CTV diseases in the field, it would be valuable to develop a broad-spectrum cross-protection, for instance, by creating a virus for protection against multiple CTV strains. Our premise is that further research on the superinfection exclusion mechanism will define ways for more effective protection of citrus crop against CTV, including engineering transgenic resistance and developing methods to extend the effectiveness of cross-protection. Knowledge developed with CTV can be further transferred to other viruses that cause diseases in other economically important crops.

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