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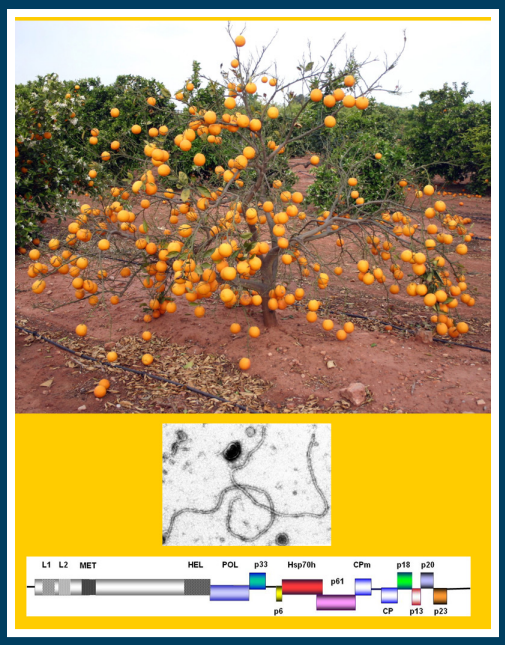

CLOSTEROVIRIDAE

Topic Editors
Ricardo Flores, Pedro Moreno, Bryce Falk,
Giovanni P. Martelli and William O. Dawson

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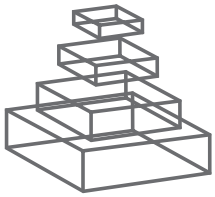

frontiers

ISSN 1664-8714
ISBN 978-2-88919-229-8
DOI 10.3389/978-2-88919-229-8

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CLOSTEROVIRIDAE

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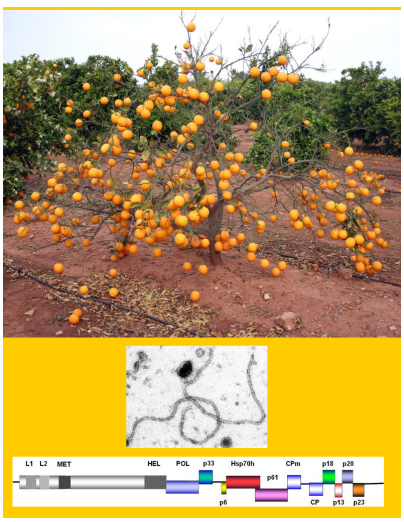

Upper panel: Sweet orange on sour orange tree dying from infection with citrus tristeza virus.
Middle panel: Citrus tristeza virus particles.
Upper two panels: "photo courtesy of Dr. Pedro Moreno"

Lower panel: Genomic organization of CTV. Schematic representation of the genomic CTV RNA with boxes denoting open reading frames (ORFs) flanked by untranslated regions (UTRs). ORFs 1a and 1b contain several domains: PRO, papain-like protease; MT, methyltransferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase. The functional role of some of the protein products is indicated. HSP70, CPm, and CP refer to a homolog of the plant heat shock protein 70 and to the minor and major coat proteins, respectively.
Taken from: Flores R, Ruiz-Ruiz S, Soler N, Sánchez-Navarro J, Fagoaga C, López C, Navarro L, Moreno P and Peña L (2013) Citrus tristeza virus p23: a unique protein mediating key virus–host interactions. *Front. Microbiol.* 4:98. doi: 10.3389/fmicb.2013.00098

Plant viruses grouped within this family have remarkable properties, prominent among which is their genomic size: Citrus tristeza virus (CTV) has the largest (19.3 kb) genome reported for a plant monopartite single-stranded RNA (+) virus. Virions are filamentous and typically flexuous particles, approximately 12 nm in diameter and 650 to 2000 nm in length, with a unique bipolar ("rattlesnake") morphology: the major coat protein (CP) encapsidates most of the genomic RNA, with a minor CP (CPm) coating a small 5'-terminal fragment (virion tail) and other viral-encoded proteins being also incorporated to this tail. The genome is monopartite (genus *Closterovirus*, type member *Beet yellows virus*, and genus *Ampelovirus*, type member *Grapevine leafroll-associated virus 3*) or bipartite (genus *Crinivirus*, type member *Lettuce infectious yellows virus*, with at least one example of tripartite genome). The genomic RNA (or RNA1 in criniviruses) directs translation of the two 5'-proximal ORFs (via a peculiar ribosomal frameshift mechanism and proteolytic processing) that encode replication-related components, with the 3'-proximal ORFs encoding proteins expressed from 3'-coterminal subgenomic RNAs. A genomic signature of members of the family *Closteroviridae* is the presence of a five-gene block of proteins involved in virion assembly and movement that, in addition to the CP and CPm, includes a small transmembrane protein, a homologue of the HSP70 class of heat-shock proteins and a diverged CP. Members of this family encode suppressors of RNA silencing differing in number (up to three in CTV), and in mode of action: intracellular, intercellular, or both. In this same context Sweet potato chlorotic stunt virus codes for a singular suppressor: an RNase III that catalyzes cleavage of the small interfering RNAs mediating RNA silencing. Host range is usually narrow and, in order to expand it, some member(s) of the family, illustrated by the case of CTV, have evolved by acquiring multiple non-conserved genes. Virion accumulation is restricted to the phloem, with aphids, mealybugs and whiteflies (depending on the genus) operating as natural vectors. Disease symptoms may be expressed in leaves, fruits and trunk of the woody hosts. Natural hosts include plants like beet, lettuce, tomato, citrus and grapevine, and damages can be economically very relevant: CTV has changed the course of the citrus industry. Altogether these properties make the family *Closteroviridae* particularly attractive from both a basic and applied point of view.

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frontiers in
MICROBIOLOGY

EDITORIAL
published: 27 December 2013
doi: 10.3389/fmicb.2013.00411

e-Book on *Closteroviridae*

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Keywords: closterovirus, ampelovirus, crinivirus, Citrus tristeza virus, citrus tristeza

This e-Book on the virus family *Closteroviridae* provides an overview on some representative members of this family. Most articles are reviews on different fundamental and applied aspects, but a few are original contributions focused on more specific issues. Even if biased toward closteroviruses, and more explicitly toward *Citrus tristeza virus* (CTV) due to its economic relevance, this compilation altogether results an attractive blend that we hope will attract the attention of the aficionados of this fascinating virus family.

First, Dawson et al. (2013) discuss CTV-host interactions, highlighting that in contrast to movement of viruses in herbaceous plants that largely occurs through adjacent cells, CTV infection relies mainly on long-distance movement. Moreover, infection of certain citrus species requires different combinations of three CTV genes, possibly acquired by the virus to expand its host range. Regarding pathogenesis, it is unknown why the virus incites severe disease in some citrus species and not in others, but p23, a CTV-specific protein that is a suppressor of RNA silencing and a regulator of viral RNA synthesis appears to be the cause of some tristeza syndromes, particularly seedling yellows (SY).

Flores et al. (2013) describe the properties of this protein (p23), with a putative zinc-finger domain and some basic motifs, which is unique to CTV. Besides the functions mentioned above, p23: (i) elicits CTV-like symptoms when expressed ectopically as a transgene in several citrus species, (ii) enhances systemic infection and virus accumulation in p23-transgenic sour orange, and (iii) releases the virus from the phloem in some p23-transgenic citrus species. Furthermore, p23 accumulates preferentially in the nucleolus—being the first closterovirus protein with such subcellular localization—as well as in plasmodesmata.

While one of the economically relevant CTV syndromes (decline) can be managed by using resistant/tolerant rootstocks, the other (stem pitting, SP) cannot. Folimonova (2013) reports on the recent progress achieved on elucidating how cross-protection may work in the citrus/CTV pathosystem. Only isolates that belong to the same strain or genotype group of the virus (there are six) cross-protect against each other, while isolates from different strains do not. Intriguingly, the mechanism of cross-protection (or superinfection exclusion) by CTV requires a specific viral protein, p33. These findings open the door for the selection of protecting isolates.

In this same context, Lee and Keremane (2013) elaborate on the history of CTV in Florida and on the methods developed to select mild isolates that could protect against strains inducing decline of trees grafted on sour orange, a rootstock much valued for the quality of the fruit produced and for its tolerance to citrus blight, a disease of unknown etiology, and to phytophthora root rot. The final aim was to identify mild isolates that when inoculated in the existing field trees could extend their productive life and facilitate a more graduate replanting with trees propagated on tolerant rootstocks.

Also on a historical framework, Wang et al. (2013) have examined the collection of Californian CTV isolates maintained in the Citrus Clonal Protection Program (CCPP) in Riverside since 1914. Analyses of isolates from this collection using multiple molecular markers have found genotypes T36, VT, and T30 at high frequencies, with T30 and T30 + VT being the most abundant. Phylogenetic reconstructions using the CTV coat protein gene have resulted in seven clades: five associated with standard genotypes (T36, VT, T30, T3, and B165/T68) and two unrelated. Reduced phylogenetic diversity and virulence was observed in isolates collected in central California between 1957 and 2009 in comparison to those of southern California collected in early times (1957–2009). Biological characterization also indicated a reduced number and less virulent SP isolates compared to SY isolates introduced to California.

Ambrós et al. (2013) have tried to extend their CTV genetic system, which is based on agroinfiltration of *Nicotiana benthamiana* with T36-based plasmids and results in systemic infection and production of enough CTV virions to infect citrus by slash inoculation. Using oncogenic *Agrobacterium* strains they have observed induction of tumors expressing GUS in different plant species, including citrus, but systemic infection only in *N. benthamiana*. Moreover, mechanical inoculation of CTV virions to *N. benthamiana* agroinfiltrated previously with a silencing suppressor resulted in systemic infection with T36, but not T318A, which replicates in protoplast of this plant to the same extent as T36. Finally, T36 was graft-transmitted from infected to healthy *N. benthamiana* plants agroinfiltrated previously with a silencing suppressor. These data indicate that extension of this genetic system still needs considerable improvement.

Harper (2013), by examining the complete genome phylogenies of 36 CTV sequences, tackles their classification into six strains or genotypes (T36, VT, T3, RB, T68, and T30 exhibiting a wide range of phenotypic characteristics) and dissects the major evolutionary processes that led to their formation: (i) ancestral diversification of the major CTV lineages, (ii) conservation and co-evolution of the major functional domains within, though not between CTV genotypes, and (iii) extensive recombination between lineages that have given rise to new genotypes. Knowledge of the selective pressures acting upon CTV strains is crucial to the development of cross-protection programs for synthesis of CTV-based viral vectors for field release, and for breeding of new resistant citrus cultivars.

Rubio et al. (2013) deal with a similar topic, but expanded to the family *Closteroviridae.* They conclude that the major factors that have shaped the genetic structure and diversity of this family of viruses comprise: (i) a strong negative pressure that seems responsible for the high genetic stability of some viruses, (ii) human transport of infected propagative material that has caused dispersion of genetically similar virus genotypes, (iii) recombination between divergent sequence variants resulting in generation of new genotypes, (iv) interactions between virus strains or between different viruses in mixed infections that may affect the final outcome, and (v) genetic drift caused by host change or insect transmission leading to changes in the viral population.

Bar-Joseph and Mawassi (2013) focus on the finding that the molecular characterization of CTV and other members of the *Closteroviridae* has revealed that, in addition to genomic and subgenomic RNAs, infected plants often contain one or more double-stranded defective RNAs (dRNAs) of various sizes, most of which contain diverse internal deletions flanked by the two genomic termini. The roles and biological functions of dRNAs remain *terra incognita,* but one possibility is that these abundant double-stranded RNAs are used as a buffering system to protect the large and fragile viral single-stranded RNA genomes from being targeted by the RNA silencing defense of the host.

Gushchin et al. (2013) report on an intriguing observation related to replication of *Beet yellows virus* (BYV), the type species of genus *Closterovirus.* Infection by eukaryotic viruses induces formation of membranous compartments, wherein replication occurs. Specifically, complexes from cell membranes of endoplasmic reticulum (ER) or mitochondrial origin appear in closterovirus infections. Computer-assisted analysis predicts several putative membrane-binding domains in the central region (CR) of the BYV polyprotein 1a. Transient expression in *N. benthamiana* of a hydrophobic segment of the CR results in reorganization of ER into ∼1-μm mobile globules, suggesting that this segment may be involved in the formation of multivesicular complexes in BYV-infected cells.

Melzer et al. (2013) have used pyrosequencing to characterize the genomes of closteroviruses infecting a single common green ti plant *(Cordyline fruticosa* L.) in Hawaii. Besides confirming the presence of *Cordyline virus 1* (CoV-1), sequence analysis has unveiled three additional closteroviruses (CoV-2 to -4), which based on the divergence of several viral proteins, represent four distinct closterovirus species. Phylogenetic reconstructions indicate that CoV-2, CoV-3, and CoV-4, together with *Little cherry virus 1* and *Grapevine leafroll-associated virus 7,* form a distinct clade within the family *Closteroviridae.*

The chapter by Dolja and Koonin (2013) summarizes advances in closterovirus research during the last several years, explores the relationships between virus biology and vector design, and outlines the most promising directions for future application of closterovirus-based vectors. These vectors offer high genetic capacity and stability, together with applicability to important woody plants such as citrus and grapevine. The description of the problems found (and their solutions) when designing vectors derived from the *Grapevine leafroll associated virus 2* is particularly illustrative.

Maree et al. (2013) provide an overview on *Grapevine leafroll-associated virus 3* (GLRaV-3), the type species for the genus *Ampelovirus,* which is regarded as the most important causative agent of grapevine leafroll disease (GLD). Complete genome sequencing of several isolates has revealed the existence of genetic variants, and characterization of the subgenomic RNAs has supplied insights into the replication strategy and the putative function of some viral proteins. Deep sequencing, apart from being a fine diagnostic tool, has furnished a more penetrating view of the complexity of viral infections and of the underlying plant pathogen interactions.

Almeida et al. (2013) discuss the ecology and management of GLD, focusing primarily on GLRaV-3, the most important virus species within the complex causing this disease. After introducing various aspects of GLD biology and ecology, the authors report on disease management case studies from four different countries and continents (South Africa, New Zealand, California-USA, and France), and end highlighting scientific gaps that must be filled for the development of knowledge-based sustainable GLD management practices.

Moving to the genus *Crinivirus* with a bipartite genome, Kiss et al. (2013) review replication and interactions with the host of the type species, *Lettuce infectious yellows virus* (LIYV). LIYV RNA1 encodes proteins involved in replication, which results in formation of vesiculated membranous structures where most likely this process occurs (see above). Four of the LIYV RNA2-encoded proteins, CP (major coat protein), CPm (minor coat protein), Hsp70h, and p59 are virion structural components, and CPm is a determinant of whitefly transmissibility. P5 is a small protein encoded at the 5′ end of RNA2 and its ortolog in BYV is localized to the ER and plays a role in cell-to-cell movement. The other small protein, p9, is unique to members of the genus *Crinivirus.*

A previous study using an AlexaFluor-based immunofluorescent localization assay has shown that retention of LIYV virions in the anterior foregut of its whitefly vector is required for virus transmission. Ng (2013), by incorporating photostable fluorescent nanocrystals, such as quantum dots (QDs), has improved the assay for the *in vitro* and *in situ* localization of LIYV virions. Immunoblot analyses resulted in a virus detection limit comparable to that of DAS-ELISA, and in membrane feeding experiments they revealed that specific virion retention in whitefly vectors corresponded with successful transmission.

Finally, the article by Tzanetakis et al. (2013) provides a detailed review on the epidemiology of the genus *Crinivirus,*

most of whose members have been characterized in the last 20 years. Criniviruses have emerged as a major agricultural threat to important horticultural crops—including tomato, potato, lettuce, and cucumber—at the end of the twentieth century with the establishment and naturalization of their whitefly vectors, belonging to genera *Trialeurode*s and *Bemisia,* in temperate climates around the globe.

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Received: 13 December 2013; accepted: 14 December 2013; published online: 27 December 2013.

Citation: Flores R, Moreno P, Falk B, Martelli GP and Dawson WO (2013) e-Book on Closteroviridae. Front. Microbiol. ***4****:411. doi: 10.3389/fmicb.2013.00411*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

GENERAL COMMENTARY
published: 03 February 2014
doi: 10.3389/fmicb.2014.00014

Closteroviridae: the beginning

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Keywords: closteroviridae, virus taxonomy, history, characterization, viruses

A commentary on

Closteroviridae
by eds R. Flores, P. Moreno, B. Falk, G. P. Martelli, and W. O. Dawson

Forty years ago, an unusual name—closterovirus—was coined for an unusual group of elongated plant viruses (Bar-Joseph and Hull, 1974). This essay reflects my personal encounter with these viruses between 1966 and 1986, a period that could be considered the beginning of the emergence of the *Closteroviridae* as an exciting complex virus family.

The first two viruses assigned to this group, *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV), have significant economic importance and, therefore, attracted considerable biological and epidemiological attention long before their molecular characterization. The seminal paper by Kitajima et al. (1964) reporting the association of long thread-like particles (TLP) with tristeza-expressing plants triggered much interest on the possibility of using those particles for diagnostic purposes. In 1966, I embarked on a Ph.D. project supervised by Prof. Gad Loebenstein that aimed to purify the TLP and develop a serological assay to be eventually used for the rapid detection of CTV-infected trees in case of an emerging epidemic. Isolating the long, thin, fragile TLP from woody tissue in the absence of a bioassay for quantitative estimation of the outcome of the numerous clarification, concentration and purification steps was a difficult and frustrating task. Indeed after almost three years, my attempts were still mostly unsuccessful. In retrospect, allowing me to continue the project at that stage was both remarkably generous and far-sighted. Improvements in TLP purification, including (i) the finding that young bark of only certain citrus species is the best source of TLP, (ii) the use of a careful extraction procedure and precipitation of TLP using polyethylene glycol, and (iii) the use of different combinations of buffers for extraction and resuspension allowed us to obtain sufficiently purified TLP particles to establish their viral-like composition and biophysical nature (Bar-Joseph et al., 1972), the infectivity of which was demonstrated by Garnsey et al. (1977).

The capable assistance of Mr. J. Cohen with electron microscopic analysis comparing the concentrations of TLP following the endless purification steps enabled me to complete my Ph.D. thesis in November 1972, almost six years after starting. I then took a post-doc position at the John Innes Institute (JII), Norwich. Shortly after my arrival, I realized that, 3 years earlier, a JII Ph.D. candidate had begun working on the characterization of BYV, but that work had been discontinued due to difficulties in obtaining purified BYV preparations. I asked for permission to use the CTV purification procedure for the isolation of BYV and, to the delight of the JII director, the late Prof. Roy Markham, with a few minor modifications this method was highly successful.

Working in cooperation with Roger Hull, we obtained sufficient amounts of BYV for the biophysical and molecular characterization of the virions and determined the sizes of their major coat protein subunits and RNA (Bar-Joseph and Hull, 1974). From these experiments we inferred that CTV and BYV shared not only similar particle structures, as revealed earlier by electron microscopy, but also closely similar RNA to coat protein mass ratios, thus providing direct virological support for their classification in a distinct taxonomic group.

However, because of the considerable variation in length, we suggested that the new group should be named *Closter virus* (*closter* is Greek for thread) to reflect the common morphological characteristic of its members, in contrast to previous groups of elongated plant viruses whose names were derived from their type members. Later analysis of *Carnation necrotic fleck virus* (CNFV), which shares several common biological features with BYV, further indicated the considerable degree of molecular and cytopathological similarity among closteroviruses as reported in the first review of this group (Bar-Joseph et al., 1979), which after a third of a century remains the main source of information on the biology of these viruses.

In 1980, Dr. Allan Dodds found large amounts of distinct dsRNA molecules in CTV-infected citrus tissues. The extension of his analyses to plants infected with BYV and CNFV revealed considerable similarities in the amounts of dsRNA they contained, as well as in their dsRNA profiles (Dodds and Bar-Joseph, 1983). It is interesting to note that years later the accumulation of large amounts of dsRNAs in plants infected by other members of the *Closteroviridae* was instrumental for their molecular cloning and genome

characterization, despite the absence of purified virions.

The first phase of my *Closteroviridae* work ended in 1986 with the realization that, despite advances in serological and molecular detection methods, the natural spread of CTV in Israel had developed into an epidemic that could not be controlled by eradication. Fortunately, most prevalent isolates induced only mild symptoms and even now, almost 30 years later, CTV remains a minor disease problem regardless of earlier projections that giving up on eradication would destroy the local citrus industry (see Bar-Joseph et al., 1989).

The other reasons for considering this period as the beginning has to do with considerable advances in *Closteroviridae* research mostly by new groups of molecular virologists whose excellent work is summarized in the present Frontiers series. Looking back, despite the difficulties and disappointments, I feel a great deal of satisfaction from friendships shared through these years with numerous dear colleagues and students that, unfortunately, space limits prevent listing.

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Received: 10 January 2014; accepted: 10 January 2014; published online: 03 February 2014.

Citation: Bar-Joseph M (2014) Closteroviridae: the beginning. Front. Microbiol. ***5****:14. doi: 10.3389/fmicb.2014.00014*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 14 May 2013
doi: 10.3389/fmicb.2013.00088

Citrus tristeza virus-host interactions

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Citrus tristeza virus (CTV) is a phloem-limited virus whose natural host range is restricted to citrus and related species. Although the virus has killed millions of trees, almost destroying whole industries, and continually limits production in many citrus growing areas, most isolates are mild or symptomless in most of their host range. There is little understanding of how the virus causes severe disease in some citrus and none in others. Movement and distribution of CTV differs considerably from that of well-studied viruses of herbaceous plants where movement occurs largely through adjacent cells. In contrast, CTV systemically infects plants mainly by long-distance movement with only limited cell-to-cell movement. The virus is transported through sieve elements and occasionally enters an adjacent companion or phloem parenchyma cell where virus replication occurs. In some plants this is followed by cell-to-cell movement into only a small cluster of adjacent cells, while in others there is no cell-to-cell movement. Different proportions of cells adjacent to sieve elements become infected in different plant species. This appears to be related to how well viral gene products interact with specific hosts. CTV has three genes (p33, p18, and p13) that are not necessary for infection of most of its hosts, but are needed in different combinations for infection of certain citrus species. These genes apparently were acquired by the virus to extend its host range. Some specific viral gene products have been implicated in symptom induction. Remarkably, the deletion of these genes from the virus genome can induce large increases in stem pitting (SP) symptoms. The p23 gene, which is a suppressor of RNA silencing and a regulator of viral RNA synthesis, has been shown to be the cause of seedling yellows (SY) symptoms in sour orange. Most isolates of CTV in nature are populations of different strains of CTV. The next frontier of CTV biology is the understanding how the virus variants in those mixtures interact with each other and cause diseases.

Keywords: Citrus tristeza virus, citrus, disease, host-interactions, stem pitting, seedling yellows

INTRODUCTION

Plant viruses are parasites that multiply and survive in plants. Their genomes are too small to effect their own replication and movement throughout plants alone. They must utilize a combination of virus-encoded genes working complementarily with host genes. Thus, viruses have evolved specific genes whose products interact with the host to replicate the virus, other viral gene products to interact with host to allow accumulation and distribution throughout the host plants, and other gene products to interact with vectors to allow transmission to other plants. Viral genes that are involved in replication tend to be conserved, suggesting that replication within a plant cell is rather generic. Indeed, many viruses are able to replicate in protoplasts from plants in which they are unable to systemically invade. In contrast, viral genes involved in spread within plants tend to be much less conserved. This observation suggests that different viruses use different strategies for invading their hosts. Members of the *Closteroviridae*, which consists of *Closterovirus*, *Crinivirus*, and *Ampelovirus* genera with mono-, bi-, or tripartite genomes, provide some of the better examples of combinations of conserved and unique genes. They all encode a mixture of conserved signature gene modules along with unique genes with no relationship found in other members of the family. The conserved gene products are involved primarily in replication and virion assembly. In fact, some domains and *cis*-acting elements involved in replication can be exchanged between different viruses. Additionally, members within a genus possess 1–5 unique genes. These gene products are thought to have evolved to interact exclusively with their specific hosts (Karasev, 2000; Dolja et al., 2006).

There are several unique features of the *Closterviridae*. First is that they have morphologically polar virions (Agranovsky et al., 1995; Febres et al., 1996; Tian et al., 1999), which is unique to this virus group. The second feature is that they encode proteins with similarities to molecular chaperones that are required for assembly (Peremyslov et al., 1999; Alzhanova et al., 2001) and possibly insect interactions (Tian et al., 1999). However, the most significant feature is that these viruses have evolved to be transmitted similarly, in a semi-persistent manner, but by at least three different types of insect vectors: aphids, whiteflies, and mealybugs. Based on sequence comparisons, they have two conserved gene modules. The first consists of replicase-associated genes including one or two protease (PRO) domains plus methyltransferase- (MT) and helicase- (HEL) like domains and an RNA-dependent

RNA polymerase (POL) domain, with the latter being translated by a +1 frame-shift. Although the order of these domains and the large intragenic regions are characteristic of this group of viruses, similar domains occur in most RNA viruses. These gene products are produced from the genomic RNA. The 3′ genes are expressed through subgenomic (sg) RNAs. The second signature gene module consists of five or six genes that encode the major coat protein (CP) and a related minor coat protein (CPm) that varies in size and genomic position among the different viruses plus three other proteins: a protein closely related to the ubiquitous HSP70 proteins (Karasev et al., 1992; Agranovsky et al., 1997), a small (6 kDa) hydrophobic protein proposed as a membrane anchor, and a protein of ∼60 kDa. As noted above, these viruses contain 1–5 non-conserved genes with no relationship to each other.

Citrus tristeza virus (CTV) has a 19.3-kb single-stranded positive-sense RNA genome (Bar-Joseph et al., 1979; Pappu et al., 1994; Karasev et al., 1995). The genomic RNA of CTV is organized into 12 open reading frames (ORFs), which potentially encode at least 19 final proteins (Karasev, 2000). Ten 3′ genes are expressed through a nested set of 3′ co-terminal sg mRNAs (Hilf et al., 1995), which consist of the signature ORFs (Pappu et al., 1994) plus 5 non-conserved genes (**Figure 1**).

REQUIREMENTS FOR REPLICATION

The ten 3′ genes are not required for replication of the genomic RNA (Satyanarayana et al., 1999). A replicon with only ORFs 1a and 1b plus the 5′ and 3′ non-translated regions (NTR) replicates well in protoplasts (Satyanarayana et al., 1999). The 5′ NTR is 107 nts and contains a precise structure with two stem loops. This was first noticed when López et al. (1998) analyzed the 5′ sequences of nine different CTV isolates that varied as much as 58%, yet all folded into the same structure. Gowda et al. (2003) found that the precise stem-loop secondary structures, in contrast to the primary sequence, are necessary for replication. In contrast to most other RNA plant viruses, the 3′ NTR does not contain a poly-A tract nor does it appear to fold as a tRNA mimic. Instead it is highly conserved among different CTV strains and is predicted to consist of 10 stem-loop structures with the replication signals within the 3′ 234 nts (Satyanarayana et al., 2002a). One of the 3′ genes, p23, although not essential, greatly affects the plus-strand to minus-strand ratio of CTV RNAs (Satyanarayana et al., 2002b). Mutants without a functional p23 gene produce almost equal amounts of negative and positive strands. The wild-type virus produces plus-stranded genomic and sgRNAs ∼10–50 times more than minus strands. The absence of a functional p23 gene also reduces or prevents protein production from 3′ genes apparently by preventing the production of single-stranded RNAs to serve as messenger RNAs.

REQUIREMENTS FOR ASSEMBLY

Although CTV virions had been semi-purified and characterized, only much later was it found that virions consisted of two coat proteins (Bar-Joseph et al., 1979; Agranovsky et al., 1995; Febres

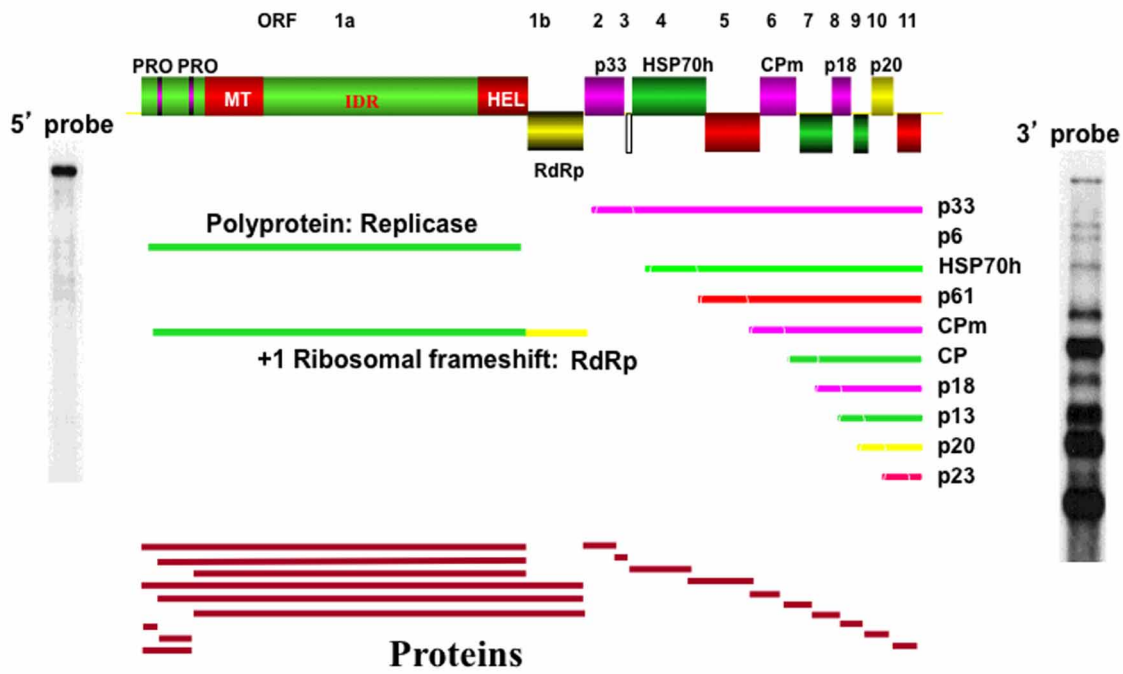

FIGURE 1 | A schematic diagram of the genetic organization of CTV showing ORFs (open boxes) of each gene. PRO, papain-like proteases; MT, methyl transferase-like domain; IDR, large interdomain region; HEL, helicase-like domain; RdRp, RNA-dependent RNA polymerase domain; HSP70h, analog to heat shock protein; CPm and CP, minor and major coat proteins. Below are expected protein products and the 10 subgenomic RNAs (the ORF translated is shown in parentheses). Left and Right are Northern hybridization analyses of total RNAs hybridized using a 5′-specific probe (left) or a 3′-specific probe (right).

et al., 1996). Most of the virion is encapsidated by coat CP, but ∼3% of the virion from the 5′ end is encapsidated by the minor coat CPm (Satyanarayana et al., 2004). Besides CP and CPm, the HSP70 homolog (p65) and p61 are involved in assembly of virions (Satyanarayana et al., 2000). Assembly of CPm is initiated at the stem-loop structures in the 5′ NTR and in the presence of HSP70h and p61 encapsidation stops at approximately nt 630 (Gowda et al., 2003; Satyanarayana et al., 2004). In the absence of HSP70h and p61, encapsidation occurs much more slowly and continues toward the 3′ terminus (Satyanarayana et al., 2004). Neither protein is active alone. Thus, these two proteins in combination enhance encapsidation by CPm and limit it to the 5′ end of the genomic RNA (Satyanarayana et al., 2004). Additionally, encapsidation by CPm in the absence of other assembly related proteins shows remarkably high specificity (Tatineni et al., 2010). Heterologous CPm's with 95–96% amino acid identity from related strains substituted into a CTV replicon with CPm as the only assembly related ORF, generally failed to initiate encapsidation. However, the heterologous CPm in combination with both HSP70h and p61 proteins, but not HSP70h or p61alone, encapsidated at wild-type levels, suggesting that non-specific interaction of CPm and its origin of assembly was mitigated by the combination of HSP70h and p61. Thus, in addition to enhanced virion formation and restriction of CPm encapsidation to the 5′ 630 nts of the genomic RNA, the HSP70h and p61 proteins facilitate encapsidation by heterologous CPm's.

MOVEMENT IN CITRUS HOSTS

To establish a productive infection in a host a plant virus needs to be able to move throughout a plant from an initially infected cell. Success depends upon compatible interactions between viral and host factors. Generally, systemic movement is thought to involve two distinct processes: cell-to-cell movement, which is a process that allows the virus to transverse the cell wall between adjacent cells, and long-distance movement, which is a process that allows the virus to enter the sieve element from an adjacent nucleated cell and rapidly move through the connected sieve elements, followed by its exit into another adjacent phloem-associated cell at a distal region of the plant. A major obstacle for the spreading virus is to cross the boundaries represented by the cell wall. For this purpose most viruses utilize specific virus-encoded movement proteins as well as some host proteins that facilitate their translocation through plasmodesmata channels. The viral proteins and their interactions with the host during cell-to-cell movement are fairly well-known (reviewed in Waigmann et al., 2004; Scholthof, 2005; Lucas, 2006). However, the mechanisms of long-distance transport and factors that aid virus entrance into phloem tissue, further vascular movement, and unloading from phloem are much less understood.

CTV generally follows the patterns described above, but the degrees of both cell-to-cell and long-distance movement are more limited than in most well-described systems, and this limitation varies depending on the citrus host. Since CTV infections are limited to phloem-associated cells, the infection can be most easily viewed by looking at fluorescence from green fluorescent protein (GFP)-tagged CTV in peeled bark that exposes phloem cells. In all citrus hosts, long-distance movement appears to be limited to relatively few initial infection sites. In the more susceptible hosts, *C. macrophylla* and Mexican lime, we estimated that only about 10–20% of the phloem-associated cells were infected (Folimonova et al., 2008). The number of fluorescent cells in grapefruit and sour orange bark patches was much less, with sweet orange being intermediate. Also, there was a difference in the size of the fluorescent areas. In the more susceptible species, *C. macrophylla* and Mexican lime, infection sites consisted of clusters of 3–12 cells. In the less susceptible species, sour orange, there were fewer infection sites and they usually were single cells (**Figure 2**). Sweet orange again tended to be intermediate between these two extremes. Our interpretation is that systemic invasion of CTV begins when the virus enters sieve elements of the phloem, which transport the virus from some distal position in the direction of sugar movement (source to sink), after which at some point the virus exits into an adjacent cell, usually in stems and leaf veins of a new flush. We assume that the adjacent cell is a companion or phloem parenchyma cell, but this differentiation in citrus phloem is not readily apparent, especially when using confocal microscopy of GFP-labeled virus. We refer to this process as "long-distance" movement. We consider the movement of virus to adjacent cells to fill the clusters of multiple cells as "cell-to-cell" movement. Apparently both long-distance and cell-to-cell movement mechanisms of CTV work differently in different citrus species.

In the more susceptible citrus species, CTV also has limited cell-to-cell movement that produces small clusters of infected cells. However, in less susceptible citrus species, it appears that little or no cell-to-cell movement occurs. The virus is able to exit sieve elements but cannot spread to adjacent cells, resulting in infection of isolated single cells. Thus, CTV provides a new pattern of systemic infection in which the virus appears to

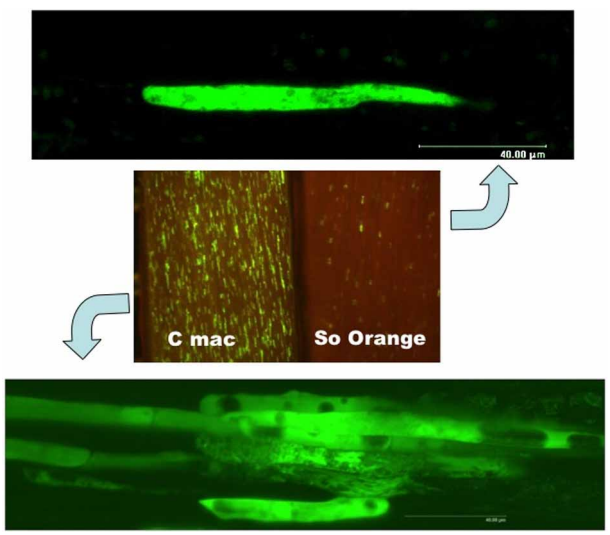

FIGURE 2 | Detection of GFP fluorescence in phloem-associated cells of *Citrus macrophylla* (C mac) and sour orange (So Orange) under a fluorescence-dissecting microscope (center) or a confocal laser scanning microscope showing single cell infections (top) and multiple cell infections (bottom).

function with only the long-distance movement mechanism, yet is able to survive in nature. Such a movement pattern has not been described previously. It is not known whether this pattern is characteristic of other members of the *Closterovirdae* or other phloem-limited viruses.

APHID TRANSMISSION

CTV generally has been moved long distances into new areas by transport of infected planting (or propagating) materials. Prior to the advent of rapid shipping in the nineteenth century, importation of citrus occurred only as seed, avoiding CTV spread as the virus is not transmissible by seed. However, as navigation improved, citrus was moved as plants or budwood, and so was CTV. Presently, the problem is that since even severe isolates are symptomless in some of their hosts, the virus often is spread by well-meaning individuals moving an infected but non-symptomatic plant or budwood from such a plant into a new area. Afterwards, local spread is by aphids, where transmission is in a semipersistent manner. This combination has effectively spread CTV (Moreno et al., 2008).

Factors affecting aphid transmission include isolate or strain differences of the virus, the aphid species, plant donor and receptor varieties, the environmental conditions, and the number of aphids involved (Roistacher and Moreno, 1990). In addition, specific isolates or strains of CTV in mixtures may not be equally distributed throughout the source plant, further reducing the likelihood of successful transmission (D'Urso et al., 2000). Finally, aphids show a marked preference for some citrus species over others, for example it has been observed in feeding choice experiments that *Aphis gossypii* preferentially infests mandarins or sweet oranges over lemons (Roistacher et al., 1984). Similarly, *A. gossypii* exhibited longer feeding periods on Mexican limes than sweet oranges (Backus and Bennett, 2009), suggesting that host preference can also affect transmission efficiency (Roistacher and Bar-Joseph, 1984; Hermoso-de-Mendoza et al., 1988; Cambra et al., 2000).

In addition, the observed movement and distribution of CTV correspond with observations of aphid transmissibility from and to specific citrus species. As mentioned earlier, there is a gradient of infection in citrus species, from frequent clusters of infected cells present in *C. macrophylla* to a scattered distribution of single cells in grapefruit and sour orange. By extrapolation one may suggest the scattered distribution of CTV in the latter species reduces the probability of virus acquisition by the aphid, and the lower titer reduces the chance of successful infection, which explains reports of grapefruit, sweet lime, sour orange, and lemon being both poor donor and receptor hosts (Bar-Joseph et al., 1977; Roistacher and Bar-Joseph, 1984; Hermoso-de-Mendoza et al., 1988). These differences in aphid transmission rates may have epidemiological consequences in the field (Moreno et al., 1988; Gottwald et al., 1996).

SILENCING OF RNAi

Not only must the virus have the capacity to produce proteins that interact with the host to allow cell-to-cell and long-distance movement, it must also have the ability to escape from the host's surveillance system. Plants have evolved an RNA silencing process, one function of which is to protect them against viruses (Dunoyer and Voinnet, 2005; Wang and Metzlaff, 2005). Viruses generally produce double-stranded RNA sequences that are subject to degradation resulting in production of small RNAs that, in turn, target the homologous sequences in the viral RNA, thus preventing systemic infection. Sometimes the result is a "recovery" phenotype. In turn, viruses generally encode proteins referred to as silencing suppressors that counteract the RNAi plant defense system to allow a systemic infection to be established and maintained (Voinnet et al., 1999; Roth et al., 2004; Qu and Morris, 2005). Mutations of viral suppressor genes generally result in reduction or prevention of systemic infection (Chu et al., 2000; Qu and Morris, 2002).

Citrus species utilize RNAi to reduce CTV titer and slow the progress of systemic infection. Thus, as with other viruses, over the course of its evolutionary history, CTV has acquired or adapted genes that exhibit suppression of silencing, namely p20, p23, and CP (Lu et al., 2004). The CP and p20 gene products function to suppress intercellular silencing, preventing the spread of the silencing signal, and it is presumed, activation of host defenses, while p20 and p23 suppress intracellular silencing and reduce viral degradation. Transgenic expression of p23 has been reported to increase the number and size of infection foci and thus the CTV titer in sour orange, and to release CTV from strict phloem-limitation in sour and sweet orange plants (Fagoaga et al., 2011). The p23 and CP genes also have additional roles in the viral replication cycle, respectively, control of negative strand accumulation and encapsidation. Even when the virus establishes a systemic infection, some degree of silencing and degradation of the CTV genome occurs, regardless of host species or viral strain (Ruiz-Ruiz et al., 2011; Harper, unpublished), which raises an important point to be made that host RNAi cannot completely inhibit or eliminate viral replication or infection, and the three suppressors of silencing cannot completely block the RNAi pathway. From an evolutionary perspective this competition has been likened to an "arms race" (Obbard et al., 2009), and although one would expect the rapidly evolving virus to overcome host RNAi, stabilizing selection may prevent further adaptation, and complete shutdown of the host RNAi pathway would prevent host-cell regulation, leading to severe symptoms and/or death of the plant.

SOME GENES ARE NOT NEEDED FOR SOME HOSTS

CTV contains five genes, p33, p18, p13, p20, and p23, in the 3′ half of the genome, which are not related to genes of other members of the *Closteroviridae*. We examined whether these genes are necessary for systemic infection of citrus trees by deleting single genes one at a time (Tatineni et al., 2008). The deletion of p20 or p23 prevented systemic infection. Apparently both are needed for counter action against the host RNAi resistance mechanism. Additionally, p23 affects replication of CTV RNA (Satyanarayana et al., 2002b).

However, we found that deletions within the p33, p18, or p13 ORFs individually resulted in no significant loss of ability of the virus to infect, multiply, and spread throughout our common laboratory hosts, *C. macrophylla* and Mexican lime (Tatineni et al., 2008). Furthermore, deletions in the p33, p18, and p13 genes in all possible combinations including deletions in all three genes

allowed the virus to systemically invade these plants. GFP-tagged CTV with deletions in the p33 ORF or the p33, p18, and p13 ORFs demonstrated that the movement and distribution of these deletion mutants were similar to those of the wild-type virus.

Because CTV was able to move in these hosts by both cell-to-cell and long-distance movement, it is expected that the virus has other genes that function as a minimal set of movement genes for these hosts. Yet, it was not expected that the virus would retain genes that it did not need. We further examined the roles of these expendable genes (p33, p18, and p13) in a wider range of citrus species and relatives within the CTV host range and found that they are needed for systemic infection of some of the hosts (Tatineni et al., 2011). However, different genes were required for systemic infection of different hosts. The p33 gene was required for systemic infection of sour orange and lemon trees. It would appear that the p33 is involved in interactions with host proteins of sour orange and lemon for successful long-distance transport of CTV. Either the p33 or the p18 gene was sufficient for systemic infection of grapefruit trees. Deletion of both genes prevented systemic infection, but deletion of either one did not. These results suggest that the p33 and p18 gene products provide similar or redundant functions in grapefruit. Similarly, the p33 or the p13 gene was sufficient for systemic infection of calamondin plants, again suggesting that these two gene products provide similar or redundant functions in this host. This property of either of two different genes providing the same function appears to be a rare property for viruses.

Thus, these three genes are required for systemic infection by CTV of its full host range, but different genes are specific for different hosts (Tatineni et al., 2011). These findings suggest that CTV acquired multiple non-conserved genes for movement and overcoming host resistance and some of these genes (p33, p18, and p13) were gained to extend its host range further.

INDUCTION OF DISEASE SYMPTOMS BY CTV

Although viruses of plants have been focused upon because of the diseases they cause, the ultimate interaction when a virus evolves with a host is likely "no disease" or "limited disease." Yet, as viruses interact with plant hosts, they do sometimes cause disease. When disease occurs in a plant, it is often accidental due to the virus moving to a new host presented to it by agricultural practices. Disease symptoms usually occur on portions of the plant that develop and grow subsequent to viral infection. Rarely do symptoms occur in areas of the plant that are fully developed at the time of infection. Disease often results from interference with differentiation or development. Yet, when diseases do occur, they can cause severe damage to plants, and in agricultural crops diseases cause economic losses, sometimes even preventing some crops from being grown.

Examination of a large number of virus isolates (which can be populations of different strains) on a series of different plants from the host range suggested that CTV has the largest number of distinct phenotypes of any plant viruses (Garnsey et al., 2005; Hilf et al., 2005; Moreno et al., 2008). The number of phenotypes is amplified by the specificity of the phenotypes in different plants. For example, some isolates cause specific symptoms in grapefruit but not other varieties, some in sweet orange and not other varieties, some in both and some in neither. This level of specificity occurs across the whole host range. Besides these disease symptoms seen in the field, vein clearing, leaf cupping, and temporary yellowing and stunting of young seedlings are phenotypes used in greenhouse diagnosis. Yet, it should be kept in mind that the most frequent phenotype is no symptoms.

However, CTV does cause or threaten to cause serious economic damage to all citrus industries. Depending on the virus isolate and the variety/rootstock combination, CTV can cause any of four distinct syndromes (Bar-Joseph et al., 1989; Bar-Joseph and Dawson, 2008; Moreno et al., 2008). "Decline" results in death of sweet orange, mandarin, or grapefruit varieties on sour orange rootstocks. During the last century, CTV-induced decline destroyed entire citrus industries worldwide, leading to the substitution of the most desirable sour orange rootstock by other rootstocks that are tolerant to CTV decline, but that are inferior for tree growth and fruit production in saline or alkaline soils, and also more susceptible to root pathogens. In contrast, the "stem pitting" (SP) disease caused by CTV results from aberrant phloem development, resulting in visible pits in the wood. This disease does not cause tree death, but substantially reduces vigor and yield of sweet orange and grapefruit trees resulting in chronic yield reductions and high cumulative economic losses. SP is not specific to any particular rootstock. The third CTV-induced syndrome, "seedling yellows" (SY) is characterized by stunting and leaf chlorosis when small sour orange, grapefruit, or lemon trees become infected (Fraser, 1952). Other varieties do not develop these symptoms. Sometimes, the stunting and chlorosis is so severe that there is a complete cessation of growth. Remarkably, the fourth CTV syndrome in citrus is a complete lack of symptoms in almost all varieties, even including the decline-sensitive sweet orange/sour orange rootstock combination, even though the virus multiplies to high titers. For instance, most citrus trees in Florida are infected with mild isolates that cause no disease symptoms.

STEM PITTING

Interference with differentiation or development results in numerous phenotypes induced by viruses. Lack of chloroplast development that causes chlorosis is probably the most common virus-induced symptom. The reduced photosynthesis causes reduced growth. SP is a common virus-induced phenotype of perennial woody plants that results from interference with stem growth. In healthy and in normally developed areas of infected trees, the cambium, which is between the phloem and xylem, divides and differentiates in opposite horizontal directions producing new xylem on the inward side and new phloem on the bark side resulting in increased girth of the tree trunk and branches. Stem pits develop in areas where development is disrupted. The surrounding areas grow normally leaving the disrupted areas as indented areas or pits. A range of different viruses distributed throughout the plant virus taxon induce SP in a range of plant species, including numerous Prunus species, apples, vinifera grapevines, citrus, and avocado, usually resulting in a slow decline of growth and poor yields. Although this disease phenotype is common in virus-infected perennial woody plants, there is little understanding of the processes that cause the stem pits.

CTV causes SP diseases that greatly limit production in many citrus industries around the world and areas that do not have isolates that cause this disease spend considerable effort to keep it out (Bar-Joseph et al., 1989; Moreno et al., 2008). Affected trees with severe SP grow poorly, lack vigor, and yield small, unmarketable fruit. Acid limes are very susceptible, sweet oranges and grapefruit also are susceptible, while mandarins are more tolerant. The disease is not associated with scion/rootstock interactions and pitting can occur on either scion or rootstock or both. Citrus production areas in which severe SP isolates are endemic can be productive only by using mild strain cross protection or by not growing susceptible varieties.

Brlansky et al. (2002) found that the formation of pits by CTV apparently is due to the inhibition of production of new xylem in the localized sites affected. The normally developing surrounding areas continue to grow leaving a depression or pit at the affected area. We examined the association of CTV with the formation of stem pits by tagging GFP to the mutants that induced this symptom (Tatineni and Dawson, 2012). Since CTV has three non-conserved genes (p33, p18, and p13) that are not required for systemic infection of some species of citrus (Tatineni et al., 2008), this allowed us to examine the effect of deletions of these genes on symptom phenotypes. In the most susceptible experimental host, *Citrus macrophylla*, the full-length virus causes only very mild SP symptoms. Surprisingly, we found that certain deletion combinations (p33 and p18 and/or p13) induced greatly increased SP, while other combinations (p13 or p13 plus p18) resulted in reduced SP (**Figure 3**).

One unexpected result was that in severely pitted areas, GFP fluorescence as a marker of virus replication was observed in regions normally made up of mature xylem or wood (Tatineni and Dawson, 2012). CTV was found in a group of cells that appeared to be on the woody side of the vascular system. In normally developing trees, most of the cells in this area differentiate into tracheary elements, which essentially consists of dead cells with thick walls connected into vessels for water transport. Interspersed in this area are live ray cells that transport nutrients from the phloem. In the full-length virus-infected trees, the fluorescence of GFP always was limited to the phloem ring outside of the cambium layer. However, increased SP was associated with virus-infected cells in areas not normally infected. Since CTV only multiplies and produces GFP in living cells and free GFP was not found in non-infected adjacent cells (Folimonov et al., 2007), it would not be expected that the virus could produce GFP in mature xylem cells without virus replication nor could GFP made in other cells accumulate in xylem. However, it should be kept in mind that this is a process that occurs over a period of time and the stem increases in girth as the plant grows in the presence of the viral infection. These results suggest that the process of forming a stem pit is not only the lack of producing new xylem in the affected area resulting in a depression in the wood, but also is affecting development and causing cells within the pitted area to continue living and to be susceptible to CTV invasion and replication.

FIGURE 3 | Stems of *Citrus macrophylla* infected with mutants of CTV with all combinations of deletions of the p33, p18, and p13 genes showing different degrees of stem pitting.

Previously, it was expected that a specific CTV gene product induced SP, and further this product could be used to identify isolates of virus that would cause this disease. In contrast, removal of sequences induced SP. How could deletions in CTV induce severe SP?

Deletion of the p13 ORF tended to be correlated with reduced SP. Thus, deletion mutants that retained the p13 gene (deletion of p33, p18, or p33 plus p18) tended to have the most SP, which might suggest that the p13 gene product was involved in induction of stem pits. However, the triple deletion mutant, which did not have the p13 gene, induced severe SP, demonstrating that interpretation is not so simple. In contrast, increased SP generally was associated with deletion of the p33 ORF. Mutants with the absence of the p33 ORF (deletion of the p33 plus the p18 ORFs, and the p33 plus the p18 and p13 ORFs) induced severe SP. Thus, mutants retaining the p33 gene (deletions of p13, p18, or p13 plus p18) had the least amounts of SP. These results suggest that the presence of the p33 protein could be correlated with reduced SP (its absence increases it). However, the mutant with the deletion of the p18 ORF (p33 and p13 retained) induced moderate SP. Overall, the production of stem pits or no stem pits appears to be related more to a balance between expression of the p33 and p13 and possibly p18 genes (Tatineni and Dawson, 2012).

In general, deletions in CTV resulted in a substantial increase in the SP disease of citrus. Yet, there are different phenotypes of SP. Some trees have large stem pits that are readily visible in tree trunks and limbs without removing the bark. Other trees exhibit "cheesy bark" SP, which is a high density of very small pits. There is a continuum of levels in between. Some cause rapid decline of tree growth and yield, while others cause little damage to the tree. Additionally, there is the extreme specificity between virus isolates and different citrus species and varieties. It should be noted that most of the other hosts examined did not form stem pits when infected with these mutants (Tatineni et al., 2008, 2011; Tatineni and Dawson, 2012). There is no reason to think that all of the

different SP phenotypes in different citrus hosts would be caused by the same virus-host interactions.

SEEDLING YELLOWS

The SY reaction is specific to only certain citrus hosts of CTV during the seedling stage, such as lemons, sour orange, and grapefruit, indicating that there are specific host factors involved in its expression in addition to isolate-specific viral factors. Mild SY symptoms are characterized by slight yellowing of new leaves and slight reduction in growth. Severe SY results in production of very small new leaves following infection. These leaves can be so chlorotic as to be almost white. The plants generally grow no more. Occasionally plants recover from SY and produce a new flush with normal leaves (Wallace and Drake, 1972).

In Florida, the decline isolate of CTV, T36, induces SY, whereas the widely distributed mild isolate, T30, does not. To delimit the viral sequences associated with the SY syndrome, we created a number of T36/T30 hybrids by substituting T30 sequences into different regions of the 3′ half of the genome of T36 (Albiach-Martí et al., 2010). Since T36 induces SY symptoms, the objective was to identify sequences that when substituted by T30 sequences would result in not inducing SY. T36/T30 hybrids were used to inoculate sour orange and grapefruit seedlings. Most of the T30/T36 hybrid constructs continued to induce SY symptoms identical to those of T36; however, two hybrids with T30 substitutions of the 3′-most gene (p23) and the 3′ NTR (nucleotides 18,394–19,296) failed to induce SY. This result suggested that the corresponding region of T36 (p23 to the 3′ end) was the determinant of this phenotype (Albiach-Martí et al., 2010).

DECLINE

Historically, decline has been the most devastating disease caused by CTV. It caused the death of almost 100 million trees, largely in the Americas early in the last century (Bar-Joseph et al., 1989; Moreno et al., 2008). It is a man-made disease based on propagation of sweet orange, grapefruit, and mandarins on the sour orange rootstock. This process was largely due to root rot caused by oomycetes of the genus *Phytophthora*. When growers learned that sour orange was resistant to this disease, industries were converted to this rootstock. This set up a disaster when CTV was brought into the areas in infected propagation materials. Remarkably, the virus does not cause decline in sour orange trees on their own roots, but causes an incompatibility at the graft union that kills other varieties grafted onto this rootstock. Sometimes death can occur in as short a period as a few days, providing the classic picture of a dead tree full of fruit but with no leaves. Yet, the disease easily can be controlled by using alternative rootstocks. However, there are soils in which all other rootstock choices are less desirable in terms of fruit quality and yield.

Decline has been the major problem caused by CTV in Florida because fortunately severe stem-pitting isolates have been kept out so far. Yet, there are soils in which other all other rootstock choices are deficient compared to the sour orange rootstock. Thus, one of our major projects has been to find a way to allow growers to use the sour orange rootstock. Florida has two predominant strains of CTV, a decline strain (T36) and a mild strain (T30). Remarkably the T30 strain does not induce decline. In comparing the two strains, it appears that T36 contains determinants that induce decline that T30 does not have. In an attempt to identify the decline determinants, we have made hybrids in T36 in which T36 sequences are removed and substituted by T30 sequences, similar to the mapping exercise to identify SY determinants. However, this project has lingered due to our inability to assay for decline in the greenhouse with small trees. Under these conditions, sweet orange on sour orange rootstocks grow normally. Apparently, the small trees replace phloem as fast as the virus causes damage to it. We now have a field test on which we await results.

The potential control strategy is to use cross protection (superinfection exclusion: see Folimonova in this series) to protect trees on the sour orange rootstock. Since T36 and T30 are from different strains, T30 cannot be used to protect trees from T36 (Folimonova et al., 2009). Yet, a non-decline inducing isolate of the T36 strain could be used to protect against the endemic T36 isolates. But we have never been able to find a non-decline isolate of the T36 strain. However, perhaps such an isolate could be made. If we can identify sequences in T36 that induce decline, it should be possible to substitute those sequences from the T30 strain resulting in a T36 hybrid that does not cause decline. This hybrid could be inoculated to the commercial nursery trees on the sour orange rootstock to protect against decline.

RNAi INDUCTION OF SYMPTOMS?

Is the viral counter-attack of the host RNAi system a component of disease induction? It has been shown that ectopic expression of one of the CTV suppressors of RNAi, p23, induces virus-like symptoms (Ghorbel et al., 2001; Fagoaga et al., 2005; see Flores et al., 2013). In addition to intense vein clearing in leaves, transformed Mexican lime plants develop chlorotic pinpoints in leaves, stem necrosis, and collapse (Ghorbel et al., 2001), which usually are not symptoms associated with CTV infection. Transgenic sour orange plants expressing p23 also develop vein clearing, leaf deformation, defoliation, and shoot necrosis (Fagoaga et al., 2005). These transgene-induced symptoms differ from the virus-induced symptoms in sour orange. In transgenic limes, symptom severity parallels the accumulation levels of p23, regardless of the source or sequence of the transgene (Ghorbel et al., 2001; Fagoaga et al., 2005), whereas the symptom intensity in CTV-infected limes depends on the pathogenicity characteristics of the virus isolate. Yet, this difference in the host response could be related to the fact that, in transgenic plants, p23 is produced constitutively in most cells, whereas, in nature, p23 expression associated with virus infection is limited to phloem tissues.

In non-citrus species is has been shown that ectopic expression of viral suppressors of silencing alters mRNA expression levels and induces symptoms (Soitamo et al., 2011), therefore it may be speculated that suppression of host RNAi defenses alters that plant's small RNA regulatory pathways, resulting in symptom expression (Pacheco et al., 2012). It frequently has been observed that virus infections trigger an enrichment of both miRNA and passenger miRNA* (Bazzini et al., 2011; Du et al., 2011; Hu et al., 2011). Virus infections have also been observed to initiate the expression of novel classes miRNA-like small RNAs (ml-sRNA)

produced from the stem-loop precursors of conventional miRNAs (Hu et al., 2011). Changes in the expression of these small RNAs can lead to up or down regulation of their target mRNA (Pacheco et al., 2012). In virus-infected plants, changes in miRNA expression have been observed to up or down regulate genes involved in regulation of growth and cell differentiation (Hu et al., 2011; Pacheco et al., 2012). Changes in the accumulation patterns of sRNAs, including miRNAs, have been reported in CTV-infected citrus plants (Ruiz-Ruiz et al., 2011). Similarly, in citrus there are significant differences in the expression of miRNAs involved in transcription and hormone responses between healthy and CTV-infected plants, although their link to symptom expression remains unknown (Harper, unpublished). Thus, it appears likely that suppression of the host RNAi processes affects symptom production by CTV in at least some of its hosts, but remains an area of future research.

CONCLUDING REMARKS

CTV non-conserved genes apparently evolved to allow systemic infection of its hosts. These are genes involved in cell-to-cell and long-distance movement and in counter surveillance. Some are not needed for all hosts. These non-conserved genes can also be involved in induction of disease symptoms. A specific region was mapped to be involved in the SY syndrome. In contrast, deletion of genes was involved in induction of SP in *C. macrophylla*, apparently causing gene product ratios that induced abnormalities. In both cases, the symptoms resulted from an alteration of development. Interestingly, both of these disease symptoms are non-continuous. SY symptoms usually are transient. Plants respond only briefly and new growth does not exhibit the symptoms. SP is spatially sporadic. Some infected areas develop abnormally resulting in pits, but most other infected areas continue to develop normally.

Viruses evolve to survive in hosts with which they are presented. This involves acquiring and modifying genes to interact precisely with their hosts. A range of potential host species creates a bewildering array of selective factors; each species will differ to some degree in physiology, gene expression, metabolism, and antiviral defenses, and an isolate at an adaptive peak in one host may be less fit in another. The process of adaption to one host may also create the potential to cause disease in another. In citrus for example, most isolates are mild to asymptomatic in pomelo, mandarin, and citron (Garnsey et al., 1996), which are the three ancestral Citrus species (Nicolosi et al., 2000) and likely those in which CTV evolved. These same isolates, however, cause an array of symptoms on commercial citrus species, all of which are hybrids of the three ancestral species.

However, the results described above come from simple systems—a pure culture—a single strain of virus from a cDNA clone. Yet, most virus infections in the field are complex populations of mixtures of different strains and defective RNAs. Little is known concerning how these populations equilibrate and which components of the population interact with the host to elicit or prevent disease symptoms. Do components of the population complement to induce disease symptoms? Do some components counteract other components? The next frontier in plant virology is developing an understanding of populations.

ACKNOWLEDGMENTS

We especially thank all of the people past and present in our laboratories who made this review possible. Funding has been provided by an endowment from the J.R. and Addie Graves family, the Florida Citrus Research and Development Foundation, and the University of Florida Agricultural Experiment Station.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 January 2013; paper pending published: 01 March 2013; accepted: 28 March 2013; published online: 14 May 2013.

*Citation: Dawson WO, Garnsey SM, Tatineni S, Folimonova SY, Harper SJ and Gowda S (2013) Citrus tristeza virus-host interactions. Front. Microbiol. **4**:88. doi: 10.3389/fmicb.2013.00088*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 03 May 2013
doi: 10.3389/fmicb.2013.00098

Citrus tristeza virus p23: a unique protein mediating key virus–host interactions

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The large RNA genome of *Citrus tristeza virus* (CTV; ca. 20 kb) contains 12 open reading frames, with the 3′-terminal one corresponding to a protein of 209 amino acids (p23) that is expressed from an abundant subgenomic RNA. p23, an RNA-binding protein with a putative zinc-finger domain and some basic motifs, is unique to CTV because no homologs have been found in other closteroviruses, including the type species of the genus *Beet yellows virus* (despite both viruses having many homologous genes). Consequently, p23 might have evolved for the specific interaction of CTV with its citrus hosts. From a functional perspective p23 has been involved in many roles: (i) regulation of the asymmetrical accumulation of CTV RNA strands, (ii) induction of the seedling yellows syndrome in sour orange and grapefruit, (iii) intracellular suppression of RNA silencing, (iv) elicitation of CTV-like symptoms when expressed ectopically as a transgene in several *Citrus* spp., and (v) enhancement of systemic infection (and virus accumulation) in sour orange and CTV release from the phloem in p23-expressing transgenic sweet and sour orange. Moreover, transformation of Mexican lime with intron-hairpin constructs designed for the co-inactivation of p23 and the two other CTV silencing suppressors results in complete resistance against the homologous virus. From a cellular point of view, recent data indicate that p23 accumulates preferentially in the nucleolus, being the first closterovirus protein with such a subcellular localization, as well as in plasmodesmata. These major accumulation sites most likely determine some of the functional roles of p23.

Keywords: closteroviruses, nucleolar proteins, RNA silencing suppressor, small interfering RNAs, zinc-finger domain

INTRODUCTION

Citrus tristeza virus (CTV) – a member of the genus *Closterovirus*, family Closteroviridae – features singular biological and physical properties. In natural infections it is confined to the phloem of most species of the genera *Citrus* and *Fortunella* within the subfamily *Aurantioideae* (Bar-Joseph et al., 1989; Moreno et al., 2008), although when inoculated experimentally via *Agrobacterium tumefaciens* it may incite systemic infection and symptoms into the presumed non-host *Nicotiana benthamiana* (Ambrós et al., 2011). CTV may cause three different syndromes depending on the virus isolate, the citrus genotype, and the scion/rootstock combination. Tristeza, a bud-union problem causing phloem necrosis and decline of most citrus species propagated on sour orange (*Citrus aurantium* L.) rootstock, was the first syndrome observed in the 1930s' decade, after this rootstock species became massively used worldwide to avoid root rot caused by oomycetes of the genus *Phytophthora*. In addition, some CTV isolates incite yellowing, stunting, and occasional growth arrest of sour orange, lemon (*C. limon* (L.) Burm. f.), and grapefruit (*C. paradisi* Macf.) seedlings [referred to as the seedling yellows (SY) syndrome], and/or stem pitting (SP; uneven radial growth with local depressions) on different citrus species irrespective of the rootstock used for their propagation, reducing the vigor, production, and fruit quality (**Figure 1**; Moreno et al., 2008).

CTV is also peculiar from a physical standpoint because its monopartite, single-stranded RNA (ssRNA) (+) genome (19.3 kb), the largest reported for a plant virus, is organized in 12 open reading frames (ORFs) potentially encoding at least 17 proteins, confined between 5′ and 3′ untranslated regions (UTRs; **Figure 2**; Karasev et al., 1995; Mawassi et al., 1996; Vives et al., 1999; Yang et al., 1999). Remarkably, while the 3′ moieties of the genomic RNA (gRNA) from distinct CTV isolates display high sequence identity (89–97%), the corresponding 5′ moieties differ significantly (60–70%), with the difference increasing at the 5′UTR (Mawassi et al., 1996; López et al., 1998; Bar-Joseph and Dawson, 2008). The two 5′-proximal ORFs encoding components of the replicase complex are translated directly from the gRNA, with the remaining ORFs being expressed via ten 3′ co-terminal subgenomic RNAs (sgRNAs) differing in their accumulation and time course appearance during the infection process (Hilf et al., 1995; Navas-Castillo et al., 1997). Prominent among the resulting proteins, which mediate different aspects of the virus biology, is p23, encoded by the 3′-terminal ORF (**Figure 2**). The absence

FIGURE 1 | Syndromes incited by CTV. (A) Quick decline of sweet orange grafted on sour orange rootstock. **(B)** Seedling yellows expressed in Duncan grapefruit (right) compared with a non-infected control (left). **(C)** Stem pitting in sweet orange.

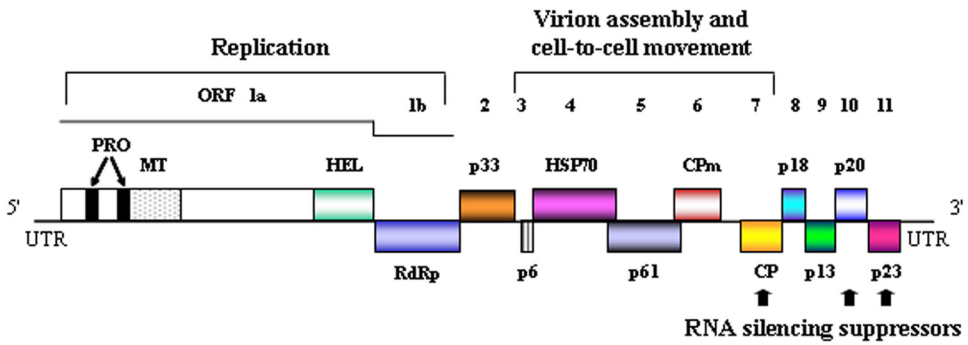

FIGURE 2 | Genomic organization of CTV. Schematic representation of the genomic CTV RNA with boxes denoting open reading frames (ORFs) flanked by untranslated regions (UTRs). ORFs 1a and 1b contain several domains: PRO, papain-like protease; MT, methyltransferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase. The functional role of some of the protein products is indicated. HSP70, CPm, and CP refer to a homolog of the plant heat shock protein 70 and to the minor and major coat proteins, respectively.

of homologs in other closteroviruses, including the type species of the genus *Beet yellows virus* (BYV) with which CTV has many homologous genes (Dolja et al., 2006), suggests that p23 might have evolved for regulating specific interactions of CTV with its citrus hosts.

In the following sections, we will review the structural and biochemical properties of p23, the different known roles in CTV biology played by this multifunctional protein, and its major subcellular accumulation sites. Some of these roles are most likely interconnected with one another and dependent on the intracellular locations of p23.

STRUCTURAL AND BIOCHEMICAL PROPERTIES

Analysis of many different CTV isolates has revealed a constant size for p23 of 209 amino acids, resulting in a molecular mass of approximately 23 kDa. Although no significant similarity was observed between p23 and other amino acid sequences deposited in databases (Pappu et al., 1994), a basic motif and some conserved cysteines, also found in proteins with RNA-binding properties encoded at the 3′-proximal region of the gRNA of some filamentous viruses (see below), suggested that p23 might also have RNA-binding properties and be engaged in regulating viral gene expression (Dolja et al., 1994). A closer inspection revealed the conserved motif CVDCGRKHDKALKTERKC between amino acids 68 and 85, with the underlined cysteines and histidine – and their relative positions – making highly feasible the coordination of a Zn ion by adopting a tetrahedral zinc-finger domain (**Figure 3**; López et al., 1998). Subsequent examination has confirmed that the three cysteines and the histidine that potentially coordinate the Zn ion are strictly conserved, as well as most of the flanking basic amino acids between positions 50 and 86, and some additional basic motifs (Sambade et al., 2003). Furthermore, while the N-terminal region of p23 has a net positive charge (the p*I* of the segment delimited by amino acids 29–155 is close to 11), the C-terminal region has a net negative charge (the p*I* of the segment corresponding to the last 54 amino acids is 4.35). This asymmetrical charge distribution, and the putative zinc-finger domain, have been observed in the transactivating domains of some transcription factors (López et al., 1998).

The predicted RNA-binding properties of p23 were confirmed following its expression in *Escherichia coli*, fused to the maltose-binding protein, and purification by affinity chromatography (López et al., 2000). Gel retardation and UV crosslinking experiments showed that p23 binds ssRNA cooperatively and in a non-sequence-specific manner. Even if formation of the p23–RNA

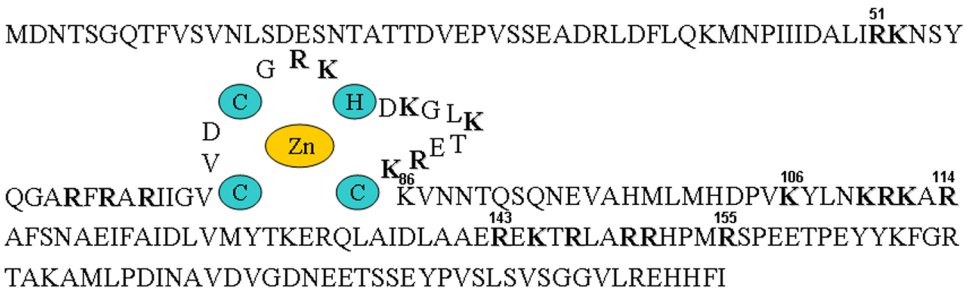

FIGURE 3 | Biochemical and structural properties of p23. Outline of the amino acid sequence of p23 from strain T36, with the putative zinc-finger domain and the coordinating histidine and cysteines highlighted with colored background, and the arginines and lysines forming part of motifs rich in basic amino acids denoted with bold fonts.

complex depends on the conformational state of p23 and on the presence of at least one basic motif, this complex is stable at high salt concentrations, suggesting the involvement of interactions other than those between the basic motifs of p23 and the negatively charged RNA. Competition assays showed a clear higher affinity of p23 for ssRNA and double-stranded RNA (dsRNA) than for their DNA counterparts, although the affinity for both RNA types was similar. Mapping studies with deletion mutants demarcated the RNA-binding domain of p23 to the segment between positions 50 and 86, containing the putative zinc-finger domain and motifs rich in basic amino acids. Additional p23-derivatives lacking the conserved amino acids predicted to coordinate the zinc ion displayed RNA-binding activity, although with an apparent dissociation constant higher than the wild-type protein, suggesting that those amino acids might provide increased binding stability or specificity *in vivo* (López et al., 2000).

REGULATION OF THE ASYMMETRICAL ACCUMULATION OF CTV RNA STRANDS

Analysis of the accumulation kinetics of CTV RNAs in *N. benthamiana* protoplasts revealed that the gRNA increased to a maximum at 3–5 days after transfection, with the full-length minus RNA increasing with similar kinetics but at approximately one-tenth the level of the plus strand RNA. Accumulation of most sgRNAs paralleled that of the gRNA, but their levels differed considerably. Interestingly, the sgRNA corresponding to p23 reached the highest level (similar to that of the gRNA), and increased earlier than other sgRNAs (Navas-Castillo et al., 1997), an observation consistent with a regulatory role for p23.

The finding that CTV infection results in the production of more plus than minus strands (of both genomic and subgenomic length) is typical of ssRNA viruses. However, this asymmetrical proportion was perturbed in a CTV mutant replicon with all of the 3′ genes deleted, which replicated efficiently in *N. benthamiana* protoplasts but resulted in an almost equal ratio of plus to minus strands (Satyanarayana et al., 2002). Because a frameshift mutation in one of the first codons of the 3′-proximal ORF caused essentially the same effect, p23 itself, an not the RNA fragment coding for it, appeared to control the asymmetrical balance of CTV strands. Data obtained with additional in-frame deletion mutants of this ORF indicated that both terminal regions (delimited by amino acids 5–45 and 181–209) were dispensable, while the central region (from amino acid 46 to 180, containing the zinc-finger domain and RNA-binding motifs; **Figure 3**), was required for asymmetrical RNA accumulation. Further alanine substitution mutants mapped the conserved cysteines in the zinc-finger domain as critical for this regulatory activity of p23. Moreover, whereas non-functional p23 essentially resulted in a significant overaccumulation of minus strands, functional p23 induced a decrease in the accumulation of the minus-stranded sgRNA corresponding to the major coat protein (p25) but not in the accumulation of this protein (which was substantially increased), suggesting that the availability of the corresponding plus-stranded sgRNA as a messenger is blocked by the excess of its minus-stranded counterpart. In summary, by downregulating minus-stranded RNA accumulation (and increasing indirectly expression of the 3′ genes), p23 would control the unbalanced accumulation of CTV RNAs (Satyanarayana et al., 2002).

ASSOCIATION WITH THE SEEDLING YELLOWS SYNDROME IN SOUR ORANGE AND GRAPEFRUIT

As indicated above, some CTV isolates incite the SY syndrome in sour orange, lemon, and grapefruit (**Figure 1**), which occasionally may be transitory with the plants resuming normal growth after some time. Although economically not relevant, this syndrome can be studied under greenhouse conditions more easily than decline and SP. The construction of an infectious CTV-cDNA clone (Satyanarayana et al., 1999, 2001) opened the possibility of dissecting the pathogenicity determinant of SY. Instrumental for this purpose were an isolate of the decline strain T36, which additionally induces SY, and an isolate of the mild strain T30, which does not. By replacing in an infectious full-length cDNA of T36 different regions of its 3′-moiety with the corresponding T30 sequences, eleven T36/T30 recombinant clones were obtained able to replicate in protoplasts of *N. benthamiana.* Five of these hybrids produced virions that were inoculated mechanically to alemow (*C. macrophylla* Wester), a sensitive CTV host, resulting in systemic infections. When tissues from these infected plants were graft-inoculated into sour orange and grapefruit seedlings, three of the T30/T36 recombinants incited SY symptoms identical to those of T36, whereas the other two – with T30 substitutions in the p23-3′UTR – did not. Moreover, the presence of a non-SY

p23-3′UTR recombinant in sour orange seedlings resulted in their protection against challenge inoculation with the parental T36 virus, thus showing the potential for cross-protection of the engineered CTV constructs (Albiach-Martí et al., 2010). Because the 3′UTR is highly conserved among all CTV genotypes (Mawassi et al., 1996; López et al., 1998), p23 is a plausible candidate for the pathogenicity determinant of SY (and perhaps for the decline syndrome). Moreover, p23 is an intracellular suppressor of RNA silencing and, as such, with potential for disrupting developmental pathways mediated by small RNAs (see next section).

INTRACELLULAR SUPPRESSION OF RNA SILENCING

RNA gel-blot analyses have revealed that, in addition to the gRNA and sgRNAs, CTV infection of Mexican lime [*C. aurantifolia* (Christm.) Swing.], another particularly sensitive host, results in the high accumulation of virus-derived small RNAs (vsRNAs) (Fagoaga et al., 2006; see also below). The latter belong to a broader class of small RNAs (sRNAs) – including micro RNAs (miRNAs) of 21 and 22 nt and small interfering RNAs (siRNAs) of 21, 22, and 24 nt – that are the hallmark of RNA silencing, a regulatory mechanism for modulating host gene expression and protecting plants and most other eukaryotes against invading nucleic acids, both foreign (viruses and transgenes) and endogenous (transposons). RNA silencing is triggered by dsRNAs and snap-folded ssRNAs that are processed into sRNAs by particular RNase III isozymes (Dicer or Dicer-like, DCL, in plants; Carthew and Sontheimer, 2009; Chen, 2009). The sRNAs subsequently load and guide specific Argonaute (AGO) proteins, at the core of the RNA inducing silencing complex, for inactivating their cognate DNAs and RNAs at the transcriptional and post-transcriptional level (Mallory and Vaucheret, 2010). To cope with this mechanism, viruses have evolved to encode in their genomes RNA silencing suppressors (RSS) that perturb one or more steps thereof (Csorba et al., 2009; Ding, 2010). Since the defensive branch of RNA silencing overlaps to some extent with another branch regulating plant homeostasis via miRNAs and siRNAs, the developmental alterations caused by viruses are deemed lateral effects of their RSS acting on both branches (Kasschau et al., 2003; Jay et al., 2011), although not all viral symptoms must inevitably have this origin (Díaz-Pendón and Ding, 2008).

To identify and characterize the RSS in CTV, nine of the proteins encoded in the 3′ moiety of the gRNA were co-agroexpressed, together with the green fluorescent protein (GFP) in a transgenic line of *N. benthamiana* (16c) expressing GFP constitutively (Lu et al., 2004). Analysis of GFP in the infiltrated leaves by fluorescence and RNA gel-blot hybridization revealed strong and moderate RSS activity for p23 and p20, respectively, as well as occasional partial suppression of systemic silencing in plants co-infiltrated with the p25 construct (despite this protein not functioning as a RSS in the infiltrated leaves). Further examination was performed in an independent silencing system based on the GUS (beta-glucuronidase) transgene in a tobacco line (6b5), wherein silencing of the transgene takes place autonomously and persistently in each generation. Following their introduction by genetic crosses into this line, the effect of p23, p20, and p25 on the intracellular and intercellular silencing of the GUS transgene was analyzed in the F1 progeny: abundant accumulation of the GUS-mRNA was detected in p20 × 6b5 and, particularly, in p23 × 6b5 plants, but not in their p25 × 6b5 counterparts, thus indicating that the two former, but not the latter, function as intracellular RSS. Subsequent grafting experiments in which the tobacco line T19 expressing GUS was propagated onto control 6b5 and p23 × 6b5 rootstocks showed that the corresponding scions became silenced (as illustrated by the accumulation of GUS-specific siRNAs and the decrease of GUS-mRNA), hence indicating that p23 did not interfere with the production or export of the GUS-specific silencing signal from the 6b5 rootstock locus into the T19 scions. Conversely, accumulation of GUS-mRNA and lack of GUS-siRNAs were observed in T19 scions grafted onto either P20 × 6b5 or CP × 6b5 rootstocks. Altogether these findings showed that p23 is a suppressor of just intracellular silencing, p25 of just intercellular silencing, and p20 of both types of silencing, thus unveiling that CTV has evolved an elaborated viral counter-defense directed against several steps of the silencing antiviral route, a strategy most likely needed for protecting such a large gRNA (Lu et al., 2004). The RSS activity of p23 has been examined in more detail recently (see below).

To complement these studies and getting a deeper insight into the CTV-induced RNA silencing response, as well as on the counter defensive reaction mediated by its three RSS, the sRNA patterns were analyzed by gel-blot hybridization, deep sequencing (Solexa-Illumina), and bioinformatic approaches in young bark of three natural hosts infected by a severe CTV isolate, as well as in their corresponding mock-inoculated controls. The data obtained (Ruiz-Ruiz et al., 2011) show that CTV sRNAs: (i) are very abundant (more than 50% of the total sRNAs, indicative of a strong antiviral response) in Mexican lime and sweet orange [*C. sinensis* (L.) Osb.; in which the virus titer is relatively high], but only amount to 3.5% in sour orange (with a considerably lower CTV titer), (ii) have a predominant size of 22- 21-nt, with an uneven balance in their 5′ nucleotide and a moderate over-accumulation of those of (+) polarity, and (iii) result from all the CTV genome, permitting its entire assembly from viral sRNA contigs, but display an asymmetrical profile characterized by a major hotspot corresponding to the 3′-terminal 2500 nt. These observations suggest that the genesis of the 22- and 21-nt CTV sRNAs is most likely catalyzed by the citrus homologs of DCL 2 and 4, respectively, and that these two ribonuclease isoenzymes operate on the gRNA as well as on the 3′ co-terminal sgRNAs, and, particularly, on their double-stranded forms. In the three mock-inoculated controls, the sRNA pattern was very similar and dominated by the 24-nt sRNAs. This pattern remained essentially unaffected by CTV infection in sour orange, while a clear reduction of the 24-nt sRNAs was detected in Mexican lime and sweet orange. In addition, CTV influences the accumulation of some miRNAs. For instance, miRNA 168 is upregulated by CTV in the three hosts (Ruiz-Ruiz et al., 2011). This miRNA targets the mRNA coding for AGO1, which mediates miRNA-directed (Baumberger and Baulcombe, 2005) and vsRNA-directed silencing (Morel et al., 2002). While AGO1 accumulation results from the host defensive response, miR168 induction – detected in distinct plant–virus combinations – appears a counter-defense reaction of the virus (Varallyay et al., 2010).

INDUCTION OF CTV-LIKE SYMPTOMS WHEN EXPRESSED TRANSGENICALLY IN SEVERAL CITRUS SPECIES

Before their role as RSS was elucidated, many viral proteins of this class were identified as pathogenicity determinants, because in addition to blocking the host defensive response RSS often interfere with host developmental pathways mediated by endogenous sRNAs (see above). This was also the case with p23, the deduced amino acid sequence of which correlated in phylogenetic reconstructions with mild and severe strains (Pappu et al., 1997; Sambade et al., 2003). Interestingly, ectopic expression in Mexican lime of p23 (strain T36) under the control of the 35S promoter from *Cauliflower mosaic virus* (CaMV) resulted in phenotypic aberrations – including intense vein clearing, general epinasty and chlorotic pinpoints in leaves, pitting and necrosis in the stems, and collapse – even more pronounced than those caused by CTV in non-transgenic plants (vein clearing, epinasty of emerging leaves that later evolves in leaf cupping, and SP), whereas Mexican limes expressing transgenically a truncated version of p23 were normal (**Figure 4**). Therefore, induction of CTV-like symptoms was associated with the expression of p23, and its accumulation level paralleled symptom intensity (Ghorbel et al., 2001). An extension of this study to other citrus hosts revealed that transformation with p23 of the CTV-susceptible sour and sweet orange, and of the CTV-resistant trifoliate orange [*Poncirus trifoliata* (L.) Raf.], also incited CTV-like leaf symptoms (vein clearing, epinasty, and stunting in sour and sweet orange, and chlorosis, leaf abscission, stem necrosis, stunting, and apical necrosis in trifoliate orange), which again were not expressed in the controls transformed with a truncated p23 version (**Figure 4**). However, in contrast with the situation observed in Mexican lime, p23 was nearly undetectable in transgenic sour, sweet, and trifoliate orange, although symptom intensity correlated with levels of the p23 transcript. Because p23

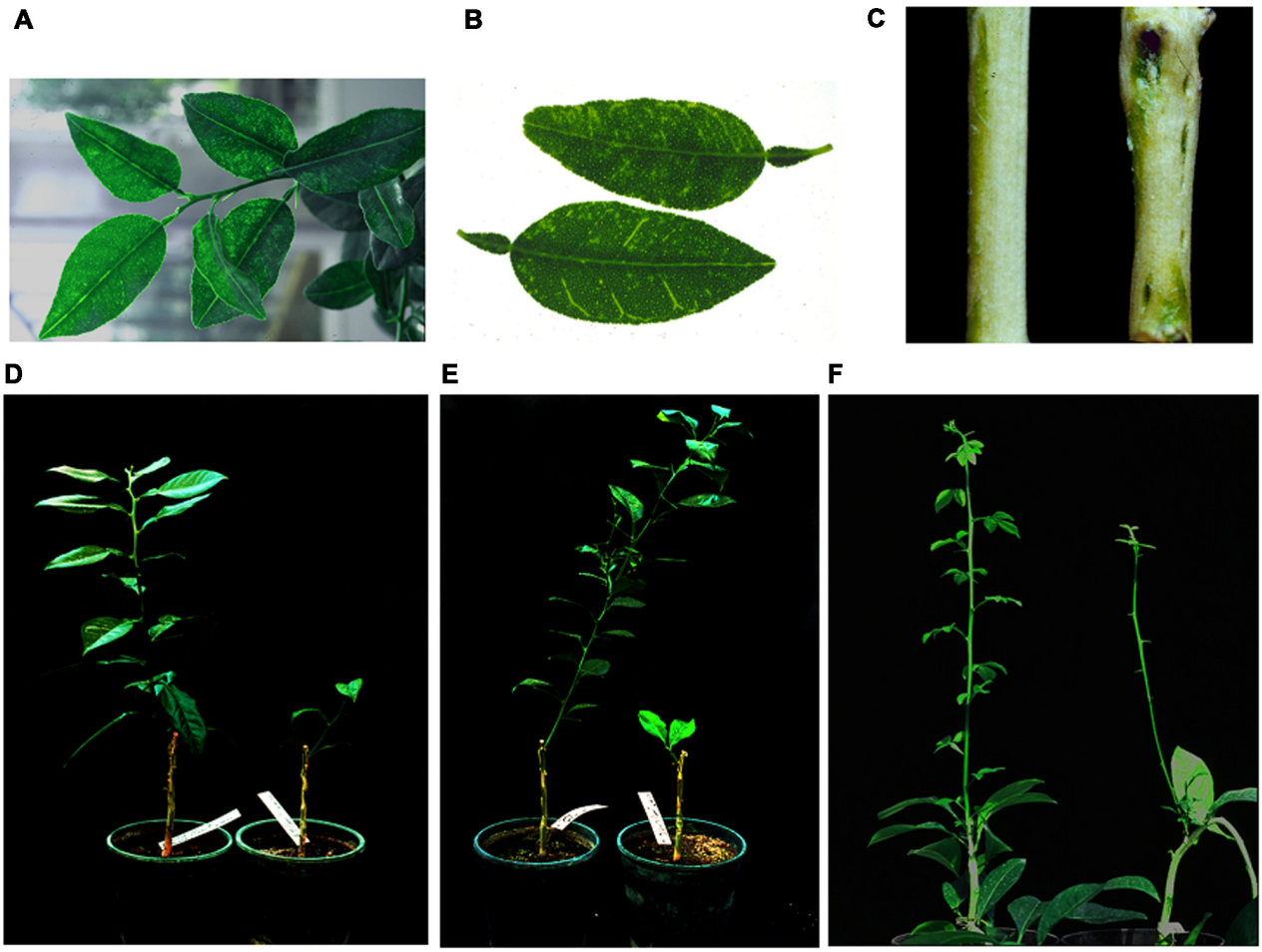

FIGURE 4 | Phenotypic aberrations resulting from the transgenic expression of p23 in citrus plants. (A) Vein clearing and chlorotic pinpoints in Mexican lime expressing p23. **(B)** Leaves from a non-transgenic Mexican lime inoculated with a severe CTV strain (top) and from a non-inoculated transgenic Mexican lime expressing p23 (bottom). **(C)** Stem pitting and normal appearance in Mexican lime transformed with a vector expressing p23 (right) and with the empty vector control (left), respectively. **(D)** Leaf distortion and stunting of a sour orange plant expressing transgenically wild-type p23 (right) compared with the normal appearance of a plant expressing a truncated version of this protein (left). **(E)** The same as in **(D)** but referred to sweet orange. **(F)** Developmental aberrations, including leaf abscission and stunting, of a trifoliate orange plant expressing transgenically wild-type p23 (right) compared with the normal appearance of a plant expressing a truncated version of this protein (left).

also accumulates poorly in non-transgenic sweet and sour orange inoculated with CTV in comparison with Mexican lime, these results suggest that even minimal levels of p23 may induce toxic effects in the two former species. On the other hand, transgenic expression of p23 in *N. benthamiana* and *N. tabacum* resulted in the accumulation of p23 without visible phenotypic aberrations, indicating that p23 interference with plant development is citrus-specific (Fagoaga et al., 2005).

Also in this context, examination of the phenotypic aberrations resulting from the transgenic expression of p23 under the control of a phloem-specific promoter should better mimic the symptoms incited by CTV, which is a phloem-confined virus. Indeed, it will be interesting to see whether restricting the expression of p23-derived transgenes to phloem-associated cells of Mexican lime by using a promoter from *Commelina yellow mottle virus* (Medberry et al., 1992) results in phenotypes more closely resembling symptoms induced by virus infection, because the other abnormalities observed previously may well be pleiotropic effects derived from p23 accumulation in non-phloem cells. There are experiments in progress aimed at examining this and another two related questions: (i) whether some of the CTV-like symptoms induced by the phloem-specific expression of p23 from strain T36 are not induced by p23 from the milder strain T317, in contrast with the similar effects observed when both protein variants are expressed constitutively (Fagoaga et al., 2005) and, (ii) whether expression in phloem tissues of the p23 fragment comprising the zinc-finger domain and flanking basic motifs is sufficient to induce CTV-like symptoms, corroborating that the N-terminal region (delimited by amino acids 1 and 157; **Figure 3**) determines, at least in part, CTV pathogenesis in Mexican lime.

TRANSGENIC EXPRESSION OF p23 INCREASES CTV ACCUMULATION IN SOUR ORANGE AND PROMOTES VIRUS RELEASE FROM THE PHLOEM IN SWEET AND SOUR ORANGE

Analysis of CTV distribution in two citrus species after inoculation with the virions resulting from transfecting protoplasts of *N. benthamiana* with the transcript from an infectious CTV-cDNA clone (strain T36) expressing the GFP as an extra ORF (Satyanarayana et al., 2001; Folimonov et al., 2007) revealed that the infection foci comprise clusters of multiple cells in the highly susceptible host alemow, in contrast to the single-cell foci usually observed in the less-susceptible host sour orange. These results suggest lack of intercellular movement in sour orange, in which CTV infection would represent an extreme situation, with the virus apparently relying only on the long-distance movement (Folimonova et al., 2008).

To get a deeper insight into this question, transgenic plants of sweet orange (a highly susceptible host) and sour orange expressing ectopically p23 (Fagoaga et al., 2005) were graft-inoculated with the mild CTV isolate T385 or with the GFP-tagged CTV clonal strain derived from isolate T36. While CTV accumulation in p23-expressing and control (transformed with an empty vector) sweet orange was similar, the viral load was several times higher in transgenic sour orange expressing p23 than in the corresponding control plants. Moreover, in contrast with the few single-cell infection foci detected in the phloem of CTV-infected sour orange controls, in p23-expressing sour orange the number of foci was higher and included generally two to six cells, thus indicating intercellular movement of the virus. On the other hand, CTV infection in p23-expressing plants was not restricted exclusively to phloem tissues, since GFP-derived fluorescence was observed in some mesophyll protoplasts and cells from infected sour and sweet orange expressing p23, but not in similar protoplasts and cells from infected controls (Fagoaga et al., 2011).

Altogether these results show that, when expressed ectopically, p23 facilitates CTV phloem escaping and additionally enhances systemic infection of the less-susceptible sour orange host. Moreover, the distinct reaction observed in sweet and sour orange implies a multifaceted interaction between p23 and other virus- and host-encoded proteins for traversing diverse cell boundaries. In this context, the possibility that CTV exit from the vascular system in sweet and sour orange could be mediated by a p23 function unrelated to silencing suppression should be entertained. The finding that p23 accumulates preferentially in plasmodesmata, in a addition to the nucleolus (see below), is consistent with the involvement of this protein in CTV cell-to-cell and long-distance movement (Fagoaga et al., 2011).

TRANSGENIC PROTECTION MEDIATED BY RNA SILENCING

The initial aim of overexpressing p23 in transgenic citrus (see above) was to test whether, under such conditions, this regulatory protein could interfere with CTV replication and provide resistance. Although most transgenic lines showed phenotypic aberrations similar to CTV symptoms (see above), certain lines of Mexican lime transformed with gene *p23* appeared normal and manifested the typical features of RNA silencing: multiple copies and methylation of the transgene, presence of *p23*-specific siRNAs, and low accumulation of the corresponding mRNA. CTV inoculation, by grafting or aphids, of propagations from these latter lines resulted in three responses: some were immune (they neither expressed symptoms nor accumulated the virus), others were partially resistant (with delayed and attenuated symptoms compared to controls transformed with the empty vector), and a third group was susceptible (with symptoms and virus titer similar to the controls). This erratic response of clonal propagations denoted that RNA-mediated resistance is also influenced by factors other than the transgenic background, like the developmental stage or growing conditions of each plant (Fagoaga et al., 2006).

It has been known for some time that intron-hairpin constructs containing virus sequences induce a strong antiviral reaction; this is because the transcribed dsRNA triggers RNA silencing that ultimately results in transgene-derived siRNAs and inactivation of the cognate viral ssRNA (Smith et al., 2000). Based on this finding, a 549-nt CTV sequence comprising part of gene *p23* and the 3′UTR of the gRNA, a segment highly conserved (>90% identity) in different virus isolates, was used to transform Mexican lime in intron-hairpin, sense, and antisense formats. When graft-inoculated with CTV, all propagations from sense, antisense, and empty-vector transgenic lines were virus-susceptible, except one (out of seven) from a sense-line showing transgene-derived siRNAs, low accumulation of the transgene-derived transcript, and an intricate transgene integration profile. In contrast, 9 of 30 intron-hairpin lines were partly resistant to CTV: 9–56% of

their propagations (depending on the line) remained uninfected, with the others being susceptible. Focusing on intron-hairpin lines with a single-transgene integration (to facilitate comparison between lines), the low accumulation of the transgene-derived transcript was a better predictor of CTV resistance than the high accumulation of the transgene-derived siRNAs, possibly because only a part of the latter are competent for RNA silencing (López et al., 2010).

These and results from other groups (Batuman et al., 2006; Roy et al., 2006; Febres et al., 2008) illustrate that developing RNA silencing-based resistance against CTV has remained elusive. To move forward, Mexican lime was transformed with an intron-hairpin construct containing full-length, untranslatable versions of genes *p25*, *p20*, and *p23* (clonal strain T36) to silence concurrently the expression of the three RSS of CTV in infected cells. Graft-inoculation with an isolate of the same viral strain, in the scion or in the rootstock, revealed that three transgenic lines were completely resistant: all their propagations remained asymptomatic and virus-free, with the accumulation of transgene-derived siRNAs being necessary but insufficient for CTV resistance. However, resistance was only partial following inoculation with an isolate of a severe SP strain (T318A), with nucleotide identities with T36 of 91–92% for the three genes, thus showing the involvement of a sequence-dependent mechanism. Apart from representing a step ahead in the quest for developing full transgenic resistance to CTV, these results show that the simultaneous inactivation of the three viral RSS is crucial for this aim, although the participation of other concomitant RNA silencing mechanisms cannot be dismissed (Soler et al., 2012).

NUCLEOLAR LOCALIZATION

Because determining the subcellular localization site of a protein is central for understanding its biological role, the fusion p23–GFP was agroexpressed in leaves of *N. benthamiana*. Analysis of the infiltrated halos by confocal laser-scanning microscopy revealed the preferential accumulation of this recombinant protein in the nucleolus and in nucleolar bodies resembling Cajal bodies, as well as in punctuated structures at the cell wall similar to plasmodesmata (**Figure 5**). These results, confirmed by coexpression experiments with proteins marking specifically the nucleolus (fibrillarin) and plasmodesmata (the movement protein of an ilarvirus), strongly suggested the presence in p23 of a nucleolar localization signal (NoLS) and of a plasmodesmatal localization signal (PLS). Because NoLS (as well as nuclear localization signals) are formed by short motifs rich in basic amino acids, the possibility that motifs of this class identified previously in p23 might form part of its NoLS was examined. Assay of seven truncated versions of p23 fused to GFP showed that regions 50–86 and 100–157 (excluding fragment 106–114), both with basic motifs and the first with a predicted zinc-finger domain (**Figure 3**), contain what appears a bipartite NoLS. Additional data obtained with 10 alanine substitution mutants confirmed and delimited this signal to three cysteines of the zinc-finger domain and to some basic amino acids. It is also worth noting that, even if a fine dissection of the PLS was not carried out (in part because the molecular features of these signals are not well established), all deletion mutants (except one lacking the C-terminal fragment delimited by amino acids 158 and 209) lost their PLS (Ruiz-Ruiz et al., 2013).

As already indicated, p23 behaves as an RSS when co-agroexpressed with GFP – both under the control of the 35S promoter – in the transgenic line of *N. benthamiana* 16c expressing GFP also constitutively (Lu et al., 2004). However, while in leaves co-infiltrated with plasmids 35S-p23 and 35S-GFP the fluorescence remained intense 6–7 days later, it became almost undetectable in leaves infiltrated with just plasmid 35S-GFP, or co-infiltrated with either the empty plasmid or with any of the 17 plasmids expressing the individual p23 deletion and substitution mutants (with the exception of that affecting the histidine of the predicted zinc-finger domain). In accordance with these observations, gel-blot hybridizations with a

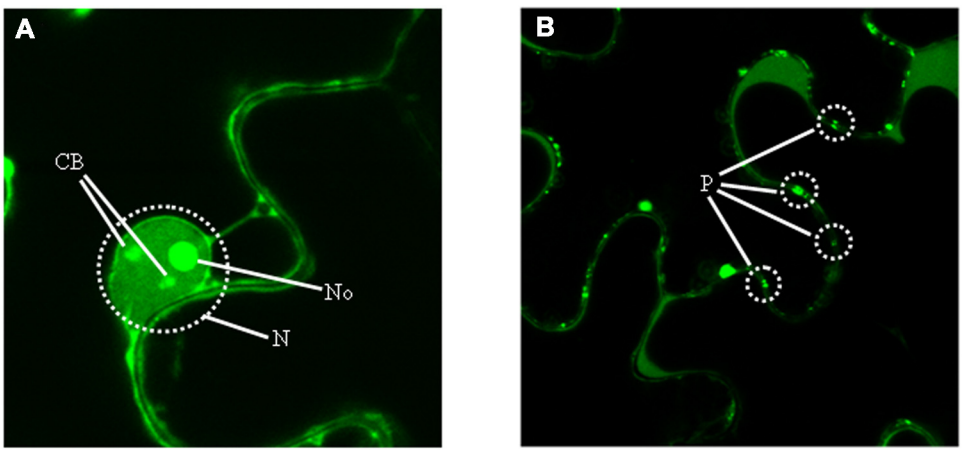

FIGURE 5 | Predominant subcellular accumulation of p23. Examination with confocal laser-scanning microscopy of leaves from *Nicotiana benthamiana* agroinfiltrated with a construct expressing the recombinant protein p23–GFP, which accumulates predominantly in **(A)** two nuclear (N) compartments, the nucleolus (No) and Cajal bodies (CB), and in **(B)** plasmodesmata (P).

GFP-specific riboprobe and RNA preparations from leaves emitting strong fluorescence revealed high and low accumulation of GFP-mRNA and GFP-siRNAs, respectively, whereas the reverse situation occurred using RNA preparations from leaves with undetectable fluorescence. Collectively these results showed that the RSS activity of p23 involves most regions of this protein (Ruiz-Ruiz et al., 2013).

Although p23 does not incite phenotypic aberrations when expressed transgenically in *N. benthamiana* (see above), the protein was additionally expressed as a sgRNA of *Potato virus X* (PVX) because different RSS act as pathogenicity determinants in this experimental context (Voinnet et al., 1999). Indeed, p23 induced a necrotic reaction in *N. benthamiana*, with this ability being preserved only in the deletion mutant lacking the C-terminal fragment delimited by amino acids 158 and 209 and in the substitution mutant affecting the histidine of the predicted zinc-finger domain. Therefore, this domain and its flanking basic motifs form part of the pathogenicity determinant. Ectopic expression of p23 and three deletion mutants thereof in transgenic Mexican lime delimited a similar determinant for symptom expression, suggesting that similar regions of p23 are associated with pathogenesis in *N. benthamiana* and citrus (Ruiz-Ruiz et al., 2013).

CONCLUDING THOUGHTS AND PERSPECTIVES

Despite not being part of the virion, p23 has been more deeply analyzed than most other CTV-encoded proteins, with these studies unveiling a plethora of effects on the accumulation of CTV RNA strands, RNA silencing suppression, pathogenesis, and virus movement. The preferential location of p23 in the nucleolus (and Cajal bodies), as well as in plasmodesmata, is most likely instrumental for these effects. Considerable progress has been also achieved in dissecting the structural motifs of p23 associated with its functions. However, many facets of this singular protein await a deeper analysis, including its potential interactions with other proteins from the virus (like those catalyzing replication or mediating movement) and the host (including different AGOs involved in silencing), as well as with nucleic acids also of viral and host origin (for instance, the sRNAs that mediate silencing). These interactions should be ultimately mapped to specific p23 motifs/domains, as recently reported for other multifunctional virus proteins (Duan et al., 2012).

As noted above, p23 is unique to CTV within closteroviruses. However, the gRNAs of some filamentous viruses – from genera *Vitivirus*, *Carlavirus*, and *Benyvirus* – encode in their 3′-proximal region proteins containing basic amino acid motifs and a putative zinc-finger domain (Chiba et al., 2006, 2013). Some of these proteins, like p23, accumulate in the nucleolus (or in the nucleus), and display RNA-binding activity, suppression of RNA silencing, and induction of necrosis in *N. benthamiana* when launched from PVX (Lukhovitskaya et al., 2009). Why viruses from different genera have evolved proteins with similar structural and, apparently, functional roles, while only one member of the genus *Closterovirus* (CTV) has followed this evolutionary pathway, remains an intriguing conundrum.

ACKNOWLEDGMENTS

Research on CTV has been supported by a grant (Prometeo/2008/121) from the Generalitat Valenciana (GV), and by a grant (AGL2009-08052) from the Ministerio de Ciencia e Innovación (MICINN)-Fondo Europeo de Desarrollo Regional. Susana Ruiz-Ruiz has been additionally supported by a postdoctoral contract from the GV (APOSTD/2012/020, Program VALi+d), Nuria Soler by a predoctoral fellowship from the IVIA, and Carmen Fagoaga by a postdoctoral contract "Ramón y Cajal" from the MICINN.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 February 2013; paper pending published: 06 March 2013; accepted: 06 April 2013; published online: 03 May 2013.

Citation: Flores R, Ruiz-Ruiz S, Soler N, Sánchez-Navarro J, Fagoaga C, López C, Navarro L, Moreno P and Peña L (2013) Citrus tristeza virus p23: a unique protein mediating key virus–host interactions. Front. Microbiol. ***4****:98. doi: 10.3389/fmicb.2013.00098*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 04 April 2013
doi: 10.3389/fmicb.2013.00076

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

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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

INTRODUCTION

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; Karasev, 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 several 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* L.) 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. aurantifolia* (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 (Brlansky 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 prevents a secondary infection with the same or closely related virus, was first demonstrated by McKinney (1926, 1929) between two genotypes of *Tobacco mosaic virus*. Since then, cross-protection has been observed often for viruses of different taxonomic groups, including bacteriophages and animal viruses, for which the phenomenon was commonly referred to as homologous interference or superinfection exclusion (Salaman, 1933; Bennett, 1951; Dulbecco, 1952; Visconti, 1953; Steck and Rubin, 1966a,b; Bratt and Rubin, 1968; Hull and Plaskitt, 1970; Fulton, 1978; Adams and Brown, 1985; Delwart and Panganiban, 1989; Lecoq et al., 1991; Wen et al., 1991; Strauss and Strauss, 1994; Karpf et al., 1997; Singh et al., 1997; reviewed by Hull, 2002; Lee et al., 2005; Gal-On and Shiboleth, 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 Shiboleth, 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 Shiboleth, 2006). The practical aspect of the cross-protection phenomenon is reflected in the more focused definition of the phenomenon used by Gonsalves 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 plants 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 Ollennu, 1994), *Tomato mosaic virus* in tomato and pepper (Tien and Zhang, 1983), and *Papaya ringspot virus* in papaya (Yeh 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

sweet orange, two for Galego lime, and one for Ruby Red grapefruit. Results, however, varied depending on the source of the isolate as well as the variety of the plants tested. Thus, mild isolates from Pera sweet orange did not provide protection in lime or grapefruit trees. Similarly, the best isolates for Pera sweet orange were collected from trees of the same cultivar (Costa and Müller, 1980). Furthermore, similar mild isolate protection approaches had minimal or no success in other regions or with other varieties. In South Africa a search for protecting isolates to preserve profitability of the Star Ruby grapefruit industry was initiated in the late 1970s and is still continuing (van Vuuren and Manicom, 2005; Roistacher et al., 2010). Mild isolates that were initially selected for the interim protection proved unsuitable in field trials over several years. Host specificity of cross-protection efficiency was also noticed, even to a much greater extent. Most mild isolates derived from grapefruit cultivars other than Star Ruby performed poorly in this cultivar, with one exception of an isolate collected from a Redblush grapefruit tree. The latter isolate is the present pre-immunizing isolate for grapefruit in South Africa. Mixed results were obtained in Australia. Trials using a few mild isolates were conducted over a period of 20 years in two distinct field sites. In some cases the degree of protection appeared to be affected by climate, with breakdown in cross-protection being less in the hotter inland site than on the coast (Broadbent et al., 1991). 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; Zhou et al., 2002). 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 of the virus that would 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 were 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 (Dolja et al., 2006; Tatineni et al., 2008); p20 and p23, which along with CP are suppressors of RNA silencing (Lu et al., 2004); and p33, p13, 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, T30, T36, 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., 2005; Roy et al., 2005; Harper et al., 2010). This compares to a range of 89–94.8% identity found in more conserved 3′ half regions of the genomes of isolates from different CTV strains. Each strain is composed of isolates with minor sequence divergence, generally less than 5% throughout the entire genome (Hilf et al., 2005; Moreno et al., 2008; Harper et al., 2010). Isolates of a strain, however, may have significant variations in symptoms and symptom severity. Remarkably, field trees usually contain complex populations of CTV, which are often composed of mixtures

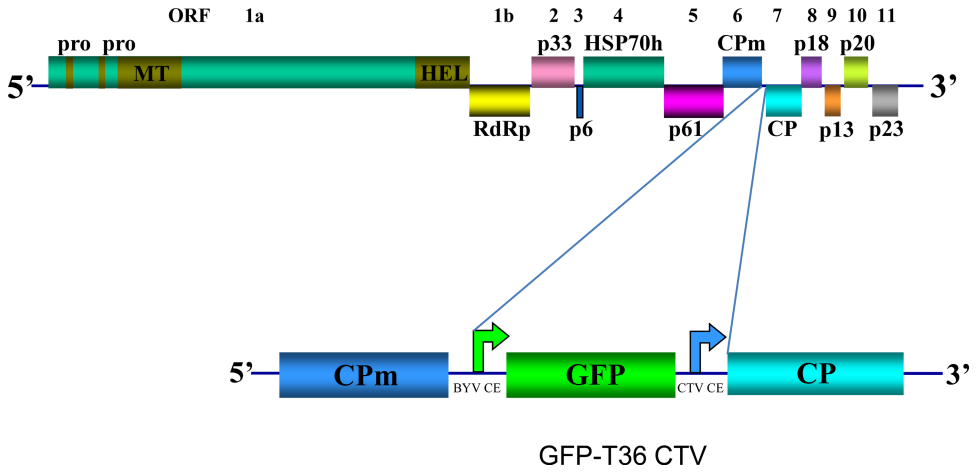

FIGURE 1 | Schematic diagram of the genome organization of wild type CTV and its derivative GFP-T36 CTV encoding green fluorescent protein (GFP). The open boxes represent ORFs and their translation products. PRO, papain-like protease domain; MT, methyltransferase; HEL, helicase; RdRp, an RNA-dependent 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).

of different genotypes and recombinants between these genotypes (Grant and Higgins, 1957; López et al., 1998; Kong et al., 2000; Rubio et al., 2001; Vives et al., 2005; Weng et al., 2007; Martín 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 CP ORFs under the control of the CP sgRNA controller element (CE) from *Beet yellows virus* (**Figure 1**). The biological characteristics of GFP-T36 CTV in citrus trees were nearly identical to that of the wild type T36. Both viruses showed similar time intervals for developing systemic infections and produced similar symptoms in infected plants (Folimonov et al., 2007). Multiplication of GFP-T36 CTV in different citrus varieties produced GFP fluorescence, observation of which allowed visualization of virus distribution in phloem-associated cells of those hosts (Folimonova et al., 2008). The engineered GFP-tagged T36 CTV has been used as a tool in examination of the relationships between different isolates of CTV in terms of cross-protection.

The definition of cross-protection has evolved over time. It was first used to describe the phenomenon of the inability of a second virus to establish infection in a host that is already infected with another isolate of the same virus. Cross-protection also has been viewed as amelioration of symptoms of a severe virus isolate 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 (T30, 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 interference 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 prevented superinfection by the GFP-tagged virus of the same T36 strain. The results were "black and white." The isolates from heterologous strains conferred no protection. The isolates from the same strain protected totally. Additional experiments in which interactions of several different combinations of primary and challenging virus isolates were evaluated using reverse transcription polymerase chain reaction (PCR)- or serology-based differentiation 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). Remarkably, 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. Furthermore, 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 initially 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. Additionally, when trees were initially infected and later challenged with isolates belonging to the same strain, there was no evidence

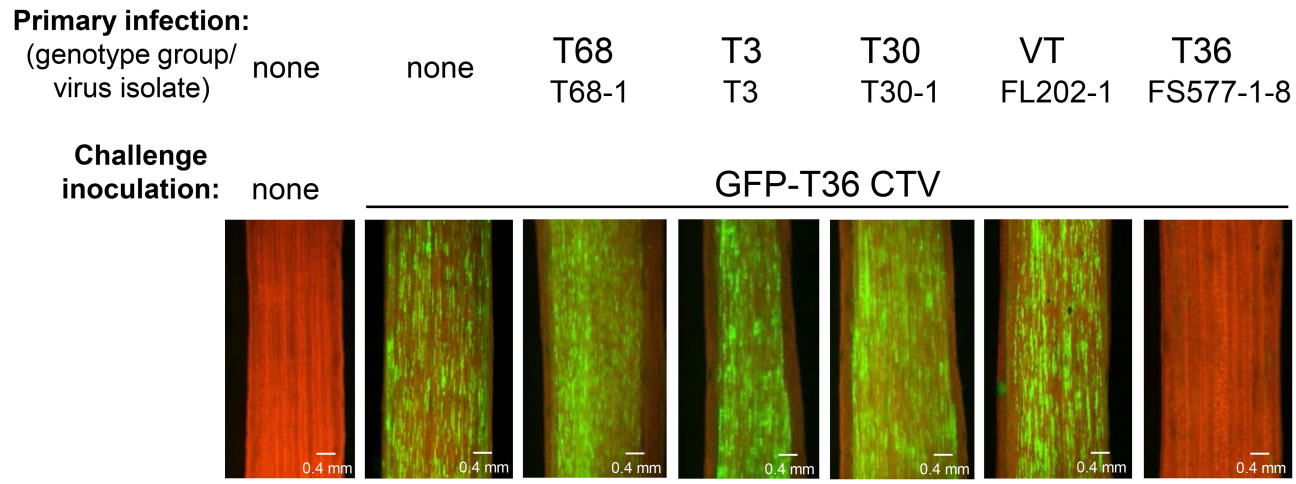

FIGURE 2 | Observation of GFP fluorescence in phloem-associated cells of *C. macrophylla* trees upon challenge with GFP-T36 CTV. Left panel represents a non-inoculated healthy tree. Other panels represent trees with no primary infection (second panel) or pre-infected with isolates belonging to five CTV 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).

of infection and replication of the challenge isolate in any of the trees.

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., 2005), and induction of RNA silencing by the protector virus that leads to sequence-specific degradation of the challenge virus RNA (Ratcliff 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; Baulcombe, 2004; Li and Ding, 2005). RNA silencing can be triggered systemically: 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 Shiboleth, 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; Hilf et al., 1995). 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 (Tatineni 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 sgRNA or a frameshift mutation within the p33 ORF failed to exclude the wild type virus. The plants pre-infected with the p33 mutants 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 (Albiach-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 (Rubio et al., 2001; Hilf et al., 2005; Roy and Brlansky, 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; Coetzee et al., 2010).

For the second objective, non-symptomatic trees in which CTV is detected will be of particular interest, since such trees may contain desirable mild CTV isolates. The genotype composition of those isolates could be characterized using the same approaches as described above. The basic rule for selection of a protecting isolate is that the mild isolate has to have a similar genotype composition as the severe one that needs to be controlled. If a severe isolate contains a mixture of several different genotypes, then a mild isolate that contains a similar genotype mixture needs to be found. Additionally, knowledge needs to be obtained about what genotype in the severe isolate is responsible for disease symptoms. For this purpose, attempts to separate individual genotypes by single aphid transmission or passaging through selective citrus hosts should be conducted, followed by biological characterization of the resulting isolates using indicator hosts coupled with their molecular characterization. Once it becomes known which genotype causes the disease, a mild isolate containing the same genotype could be put in use to trigger exclusion of the former variant.

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 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.

ACKNOWLEDGMENTS

The author thanks William O. Dawson and Stephen M. Garnsey for insightful discussions, encouragement, and critical reading of the manuscript. Some of the research discussed here was supported by grant from the National Science Foundation (Grant number 1050883 to Svetlana Y. Folimonova).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2013; accepted: 15 March 2013; published online: 04 April 2013.

Citation: Folimonova SY (2013) Developing an understanding of cross-protection by Citrus tristeza virus. Front. Microbiol. ***4****:76. doi: 10.3389/fmicb.2013.00076*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 06 September 2013
doi: 10.3389/fmicb.2013.00259

Mild strain cross protection of tristeza: a review of research to protect against decline on sour orange in Florida

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Tristeza, caused by *Citrus tristeza virus* (CTV), has long been present in Florida but outbreaks of decline on sour orange rootstock were occasional events until the late 1970s. Sour orange rootstock was valued for the high quality of fruit produced and was widely used because of its tolerance of citrus blight, a disease of unknown etiology. Research was directed towards the selection and screening of mild strains of CTV which could protect against sour orange decline strains. Following the introduction of *Toxoptera citricida* (also known as the brown citrus aphid) in 1995 there was a greater concern for maintaining production of existing blocks of citrus on sour orange rootstock. Availability of the CTV genome sequence around the same time as well as molecular characterization of *in planta* CTV populations led to the selection of mild CTV isolates which when inoculated into existing field trees, extended the productive life of the groves and enabled a more graduate replanting of trees on CTV-tolerant rootstocks. The history of CTV in Florida and the methods developed to select mild isolates for use for mild strain cross protection will be reviewed.

Keywords: biological indexing, strain differentiation, serology, stem pitting, mild isolate selection

TERMINOLOGY

For purpose of this review, we refer to strains of CTV as causing a specific biological activity consistently; e.g., mild strains of CTV will always produce mild symptoms even on susceptible hosts, decline strains will consistently cause decline on sour orange rootstock. The term isolate is used to describe the viral population of CTV obtained from a field source, and the isolate may be composed of a mixture of strains. Genotypes of CTV are identified by the use of specific methods using molecular markers that are dependent on genome sequence.

INTRODUCTION

Citrus tristeza virus (CTV) is the most important viral disease of citrus worldwide. Since the first outbreaks in South America in the 1940s, CTV has killed more than 85 million trees worldwide (Lee et al., 1992; Bar-Joseph et al., 2010). CTV occurs as strains which cause a variety of biological symptoms on various hosts. Mild strains of CTV (CTV-M) cause no detectable symptoms in common scion/rootstock combinations and produce very slight to no symptoms in Mexican lime indicator plants under ideal conditions. Some CTV strains cause decline of scions on sour orange rootstock (CTV-D); these strains are apparent in areas which use sour orange as rootstock. The decline on sour orange due to CTV-D may be rapid, within weeks, or more gradual taking up to 18–24 months for the trees to gradually decline (Lee and Brlansky, 1990). Some strains of CTV produce stem pitting symptoms in scions (CTV-SP). CTV strains which induce stem pitting on grapefruit (CTV-SPg) are the most commonly seen stem pitting strains, but some strains also induce stem pitting on sweet orange scions (CTV-SPs). Some CTV-SP strains will stem pit only grapefruit and not sweet orange, others stem pit only sweet orange and not grapefruit (Lee and Rocha-Pena, 1992), and others will stem pit both grapefruit and sweet orange. The symptomatology of stem pitting can be varied as well as the effect the stem pitting has on the tree vigor and yield. The most severe stem pitting are the very small pits produced in the bark with corresponding fine pegs in the wood of stems and branches, with a gumming occurring in the pitted areas. Often ropey-like large pits occur on the trunk of trees, especially in grapefruit trees, and while detrimental to tree vigor over time, the economic impact on the tree is usually less than trees showing the small pitting with gum deposits in the pitted area (Bar-Joseph et al., 1989). Another symptom associated with CTV is seedling yellows (CTV-SY) which is expressed by a yellowing on lemon, grapefruit, or sour orange followed by a stunting of growth in these hosts. A standardized host range using Mexican lime, sour orange, Madame vinous sweet orange, and Duncan grapefruit seedlings and a sweet orange budded onto sour orange as standard indicator plants has been developed and used as a method for determining the biological activity of a specific CTV isolate and to enable comparison of biological activities of CTV isolates from various areas of the world (Garnsey et al., 1987, 2005).

Once CTV becomes established in a citrus growing area, what methods are available for control of the disease? If CTV-D is the predominate CTV strain in an area that has mostly trees on sour orange rootstock, the disease may be managed by simply replanting trees on a rootstock tolerant to CTV decline. For CTV-SP strains, options can include implementation of mild strain cross protection, development of CTV resistance in commercially desirable cultivars via genetic engineering methods, and/or development of CTV resistance by conventional plant breeding

method using the immunity against CTV that is present in *Poncirus trifoliata*.

Genetic engineering resistance to CTV in commercially desirable varieties may take 15–20 years while the use of conventional plant breeding is a longer term endeavor requiring several decades. Mild strain cross protection may be implemented immediately if protective mild strains have been selected ahead of time, or in less than a decade if no pre-selection of mild strains has been done.

USE OF MILD STRAIN CROSS PROTECTION IN VARIOUS CITRUS AREAS

Mild strain cross protection is defined as the phenomenon which occurs when a mild isolate of a virus is introduced into a plant and that virus prevents the expression of the symptoms of a severe isolate of the same virus that is later introduced into the same plant (Lee et al., 1987). Lee et al. (1987), based on observations in Florida, suggested that an ideal CTV strain for cross protection should occur in relative high virus concentration in the plant tissue, should express only mild symptoms in all hosts which may be planted in the region, have the ability to quickly move into new growth flushes, and should be easily aphid transmitted so that once established in a tree or area, it would spread to become the predominate strain in other trees in the area. Mild strain cross protection should not be viewed as a permanent cure to protect against the economic losses caused by severe isolates of CTV, and it is not a form of virus resistance. Rather, mild strain cross protection is a means to extend the economic life of citrus (Lee et al., 1992). Management of CTV by mild strain cross protection should be considered only as a last resort where no other management options are available.

Since mild strain of CTV is a relative term, we will define a mild strain of CTV according to Florida standards: a mild strain of CTV is a strain of CTV that produces only very mild or slight vein clearing and stem pitting on Mexican lime, a very sensitive indicator plant, under optimal cool temperature which favors CTV symptom development. Under most climatic conditions, Mexican lime and other sensitive indicator plants for CTV need to be tested by serological assays to verify the presence of CTV. CTV-M isolates selected in Florida have proven to be the mildest upon comparison with other mild strains from other countries based on host range testing conducted at the USDA ARS Exotic Citrus Pathogen Quarantine, Beltsville, MD, USA (Garnsey et al., 1987, 1991).

Mild strain cross protection has been used successfully in many citrus growing areas to continue production of citrus despite the presence of severe isolates of CTV. In Australia, severe stem pitting on grapefruit was a problem beginning in the 1940s (Fraser and Broadbent, 1979). Apparent mild isolates of CTV were collected from surviving trees and evaluated in field trials (Thornton and Stubbs, 1976). Following aphid transmissions, selection of mild isolates from the aphid transmitted isolates and further evaluations, for the past 35 years all commercial grapefruit trees in Australia have been inoculated with mild isolate PB61 (Zhou et al., 2002).

In Brazil, a similar approach was used in the 1960s where CTV isolates from surviving trees were selected to protect against stem pitting of Pera sweet orange. The IAC selection of Pera has been used for more than 30 years with little breakdown of cross protection. More than 80 million Pera trees have been propagated from this source since its selection in the 1960s (Müller and Costa, 1987; Müller and Rezende, 2004). Two isolates selected in the 1960s for protection of Galego lime against CTV stem pitting and decline have performed well, with protected trees yielding up to five times that of the unprotected trees (Müller and Costa, 1972).

In Peru, Satsuma mandarin budwood was imported from Japan in the 1950s, and this importation is thought to be the source of the severe stem pitting strains of CTV that are present in Peru at the present time (Roistacher, 1988). Screening was performed in a nursery setting where budwood from CTV affected trees was propagated at a single location, and selections were made for trees which grew well despite the presence of severe CTV (Bederski et al., 2005; Roistacher et al., 2010). Additionally, mild attenuated strains of CTV derived by passage through *Passiflora* species, were imported from California (Roistacher and Bar-Joseph, 1987). Using budwood sources infected with the protective strains of CTV, the Navel orange and lime production has increased in the coastal production area of Peru (Bederski et al., 2005; Roistacher et al., 2010).

In South Africa, stem pitting on grapefruit was discovered in the 1940s and presented a production problem (Oberholzer et al., 1949). Selections of CTV were made from surviving grapefruit trees, and these were evaluated for protection against stem pitting when the Citrus Improvement Programme was started in the 1970s (von Broembsen and da Graça, 1976). One of the mild isolates selected came from a Marsh grapefruit planted in 1926 but still producing well when the selection was made in the mid-1970s; this isolate was originally referred to as the Nartia isolate but was later named GFMS12, and was used universally beginning in 1984 to protect grapefruit (Kotzé and Marais, 1976; Marais, 1994). A selection made from lime, later named lime mild strain 6 (LMS6), was found to be effective in lime (van Vuuren et al., 1993), and also was used in sweet orange and mandarin propagations to protect against CTV-SP (Luttig et al., 2002). Later trials also indicated that another CTV selection from grapefruit, named GFMS35, was better at protecting Star Ruby grapefruit and other pigmented grapefruit varieties than the GSMS12 (Marais and Breytenbach, 1996; da Graça and van Vuuren, 2010).

In Japan, Hassaku dwarf disease, caused by CTV, severely affects production on Hassaku, *Citrus hassaku*, causing dwarfing and severe stem pitting. An apparently healthy Hassaku, which was later found to be infected with a mild isolate of CTV and citrus vein enation virus, was used as a budwood source (Sasaki, 1979). Trees propagated from this source have grown well, although about 20% of the protected trees showed stem pitting symptoms after 16 years. Other mild isolates of CTV have been identified that protect against stem pitting in Navel orange (Ieki et al., 1997).

HISTORY OF CTV IN FLORIDA

The first confirmation of CTV occurring in Florida was by Grant (1952); CTV was reported in Orange, Lake, and Highland counties and confirmed by indexing on Mexican lime. Cohen and Knorr (1953) reported the presence of CTV in 27 counties of Florida. At that time, there was no substantial occurrence of decline on sour orange rootstock. It is probable that severe decline strains were

prevented from becoming widespread because the predominate rootstock in use in the citrus industry in the early days was sour orange, and a CTV-D isolate would probably have killed the tree, or it would perform so poorly that it would not be propagated. Several nurserymen indicated that some introductions made before the Florida Budwood Registration Bureau existed would not grow on sour orange rootstock (Lee et al., 1997). Following the first confirmed report of CTV in Florida in 1952, there were occasional outbreaks of decline on sour orange rootstock; an outbreak was reported in the Ft. Pierce area in 1956 and other reports of outbreaks in Orange and Polk counties in the 1960s (Norman et al., 1961; Brlansky et al., 1986) A severe outbreak of CTV decline on sour orange was reported in western Orange and southern Lake counties in 1974 (Garnsey and Jackson, 1975). In a survey of registered budwood source trees being used for propagation on sour orange in 1979, 87% of the sweet orange source trees tested positive for CTV and 9% of the grapefruit sources (Garnsey et al., 1980). In the 1980s, CTV decline on sour orange was becoming widespread in the Central and South Ridge growing areas, and in the Indian River on the East Coast and the Flatwoods in Southwest Florida (Brlansky et al., 1986).

The Florida Budwood Registration Bureau (FBRB) was started as a volunteer program in 1953 (Rucks, 1994; Kesinger, 2003). When the Bureau began, registered scion trees had to be tested and found free from CTV and the program allowed trees held by nurseries to be registered as budwood source trees. However, CTV was being naturally spread by aphids within Florida. By 1964, immediately following a severe freeze in December 1962 which increased the demand for budwood, there was concern that if registered scion trees continued to be removed from registered status due to presence of CTV, there would be a severe shortage of budwood. Beginning in 1964, trees were no longer removed from status as budwood sources trees because of CTV (Rucks, 1994); CTV had not been a severe problem at this time in Florida. While budwood source trees infected with CTV could be used for propagations, trees in the FBRB foundation planting at Dundee, FL, USA, were removed when they became CTV infected. This policy of removing CTV infected trees in the Dundee foundation planting was abandoned in 1968 because too many trees were being removed. In 1967–1968, the FBRB established a foundation planting at the Ona Range Cattle Station in Hardee County. The Ona foundation planting was about 1 km away from the nearest commercial citrus planting. By 1972 the Ona foundation planting was no longer used because of the spread of CTV through the planting. In 1989 a 20 acre foundation planting was established at the University of Florida's Immokalee Research and Education Center, Immokalee. The foundation planting at Immokalee had 28 different registered selections replicated on 22 different rootstocks. CTV began spreading through the foundation planting and in 1992 the foundation trees were inoculated with three different mild isolates of CTV (T30, T26, and T55) so that the industry would be provided with cross protective mild strains. By 1996, the CTV incidence in the Immokalee foundation planting was 37.5% as determined by MCA-13 ELISA to selectively detect severe strains of CTV. Budeyes were no longer cut from the field planting, and subsequently budeyes cut at Immokalee were from a screenhouse. More than 1.1 million budeyes were cut from the Immokalee foundation planting from 1992 to 1998. Registered budwood source trees held by nurseries were located in the field and used to cut budeyes up until the mandatory budwood certification program began in January 1, 1997 (Kesinger, 2003).

IMPACT OF CTV ON THE ROOTSTOCK USAGE IN FLORIDA

Figure 1 shows the rootstock usage in Florida by percent of propagations since the inception of the Florida program and also the dates of historic freezes as freeze occurrences increase the demand of propagated trees to replace trees lost (Kesinger, 2011). Historically, sour orange was the prevalent rootstock when citrus was grown mainly on the ridge area in Central Florida and the Indian River production area. When new groves were brought into production in the Flatwoods production areas in the 1960s and 1970s, rough lemon was the preferred rootstock. However, a disease of unknown etiology, called citrus blight, began taking trees on rough lemon rootstock out of production beginning when they were 5–7 years of age (Derrick et al., 1992). By the late 1970s, blight was killing about 15% of the trees on rough lemon rootstock each year, and the epidemic of blight spread from the Flatwoods production areas in Southeast and Southwest Florida to the older citrus production areas. While many trees were propagated on Carrizo, and later on the Kuharske selection of Carrizo rootstock (popular because of its tolerance to burrowing nematode), these trees were still subject to losses due to blight. Sour orange continued to be a popular rootstock through the 1980s and lost favor only because the brown citrus aphid (BrCA), *T. citricida*, became established in Florida following its introduction in 1995 (Halbert et al., 2000). Sour orange displays a field tolerance to blight, and produces high quality fruit (Rocha-Pena et al., 1995). The Indian River production area traditionally used sour orange rootstock, and this rootstock has contributed to building the Indian River's reputation for high quality grapefruit and citrus fruit for the fresh fruit market. Swingle citrumelo became a popular rootstock when the use of sour orange rootstock decreased, because of Swingle's field tolerance to citrus blight and tolerance to tristeza decline (**Figure 1**). In recent years following the introduction and establishment of Huanglongbing (HLB) in Florida in 2005 (Halbert, 2005), some growers have reverted to sour orange rootstock, especially for grapefruit. HLB had shortened the productive economic life of trees so much that the growers think they would be better off utilizing the advantages of sour orange to get good tree growth and high quality fruit, and also thinking that since the trees are treated with insecticide so often to protect against psyllid infestations, the BrCA, the aphid vector of CTV, should also be less of a problem.

Severe freezes occurred in Florida in January 1977, 1981, December 1983, January 1985, and December 1989 (**Figure 1**; Kesinger, 2011). Each nurseryman had favorite registered trees which produced vigorous budlings when propagated on sour orange rootstock. Following freeze years when demand for trees to replace freeze losses was high, budwood from favored trees was not enough to meet the demand, so budwood from other registered scion trees was used for propagation (Rucks, 1994; Kesinger, 2011). Some of these propagations did not grow well on sour orange rootstock, but if the propagation was on a CTV tolerant rootstock, the effect of CTV was not apparent. This created an ideal situation

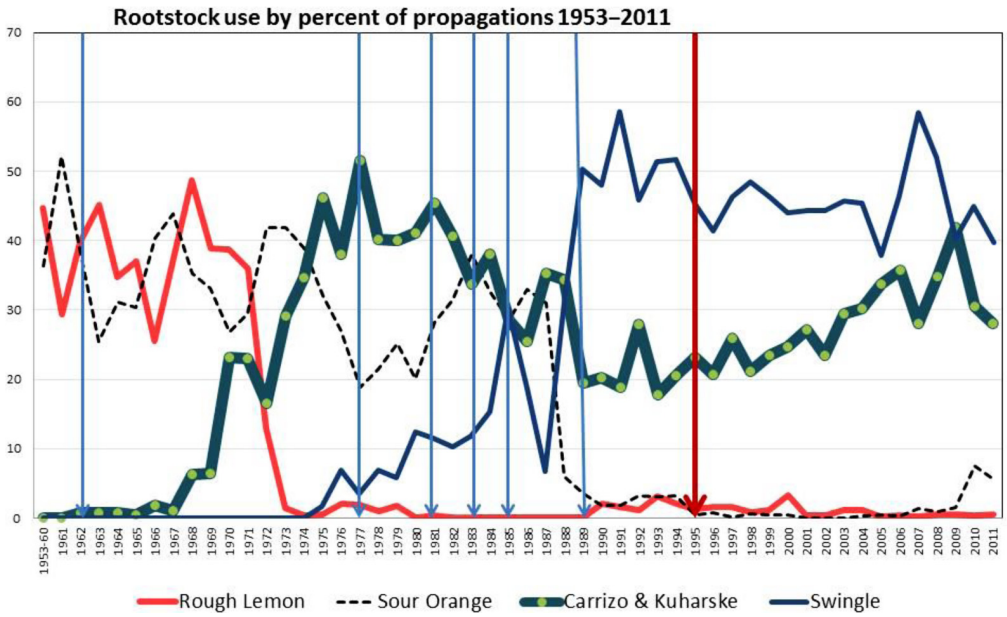

FIGURE 1 | Rootstock usage in Florida expressed as a percent of the total propagations since the beginning of the Florida Budwood Registration Bureau in 1953. Major freeze occurrences are indicated by blue vertical lines. In 1995, the program became mandatory (red line) while from 1953 to 1994 the program was voluntary. Data summarized from Kesinger (2011).

to distribute CTV-D throughout the Florida citrus industry and created the circumstance for the epidemic of CTV decline which occurred in the 1980s (Brlansky et al., 1986). In 1984, 655 scion sources that were being propagated on sour orange rootstock were biologically indexed on sour orange liners in cooperation with several nurseries (Yokomi et al., 1992). Ten trees were propagated from each budwood source and healthy, mild CTV and CTV-D inoculated controls were included. The stem diameters were measured, and if the diameter was less than 70% of the healthy controls, the plant was declared stunted. Of the 655 scion trees indexed, 18% were stunted. This indicated that many plants were coming from nurseries already infected with CTV-D strains, and the spread of the CTV-D strains was aided by the aphid vectors once the trees were planted (Yokomi et al., 1992).

The BrCA was found in Florida in 1995, and within 2 years was present in all of Florida's citrus production area (Halbert et al., 2004). A survey was conducted in Southeast Florida in 1994–1995 prior to the BrCA being found in Florida, and 2 years after the BrCA had been found, these same trees were re-sampled to determine changes in incidence, distribution, and severity of CTV isolates in Florida. Serological and molecular assays were conducted, and selected isolates were biologically indexed on sweet orange and grapefruit. A severe CTV-SP was found near Delray Beach, with the incidence of severe strains increasing more than that of the mild strains. With the use of strain group specific probes (Halbert et al., 2004), some trees were found to be infected with up to five different CTV strains. This was the first report of severe CTV-SP occurring in Florida. In 2002–2003, the presence of CTV-SP, causing mild stem pitting on sweet orange and mandarins, was reported in Central Florida and a subsequent survey indicated this stem pitting strain was spreading (Sieburth and Nolan, 2005).

METHODS USED TO DIFFERENTIATE STRAINS OF CTV

For selection of mild CTV isolates for use for cross protection and for the evaluation of mild strain cross protection experiments, methods are needed to determine and/or predict the biological activity of the isolates. Several methods have been developed, albeit here we will include only the methods actually used in our research to select and screen for mild protective strains of CTV on a timely basis as reported below.

One of the first methods developed for differentiating mild from CTV-D or CTV-SP strains was by the use of monoclonal antibody MCA-13 which recognizes the severe strains of CTV but not the CTV-M strains (Permar et al., 1990). Pappu et al. (1993) demonstrated that the critical amino acid in the MCA-13 epitope was at position 124, with this residue being phenylalanine in the MCA-13 reactive severe CTV strains, but tyrosine in the non-reactive mild strains. This antibody has been used extensively to test registered budwood source trees in the Florida Budwood Registration Bureau since it became mandatory in 1997 (Rucks, 1994; Kesinger, 2003). Trees in Florida which tested positive with MCA-13 could not be used as a source of budwood. In the Central California Tristeza Eradication Agency, the MCA-13 antibody is now used as a pre-screen test to flag CTV infected trees for further molecular and biological testing (Yokomi et al., 2012).

Analyses of the coat protein (CP) gene sequences of several CTV isolates having different biological activities led to the discovery that often CTV strains having similar biological activities show group-specific nucleotides at certain positions of the CP gene. This resulted in the development of the strain group specific probes (SGSP; Cevik, 1995; Niblett et al., 2000). Eight hybridization probes were designed: Probe 0 contains a sequence conserved in the CP gene of all known CTV isolates, and it

serves as a universal probe to detect all CTV strains. Probe I hybridizes with CTV strains expressing decline and seedling yellows (T36, T66 are type isolates). Probe II hybridizes with CTV strains expressing decline, seedling yellows, and stem pitting on grapefruit and sweet orange (B1, B53 are type isolates). Probe III hybridizes with CTV strains expressing decline, seedling yellows, and stem pitting on grapefruit and sweet orange (B165, B185 are type isolates). Probe IV hybridizes with CTV strains expressing decline on sour orange, seedling yellow, and stem pitting on sweet orange (T3, B220 are type isolates). Probe V hybridizes with CTV strains expressing decline on sour orange, seedling yellows, stem pitting on grapefruit and sweet orange (B128, B249 are type isolates). Probe VI hybridizes with CTV strains which are very mild, such as found in Florida (T26, T30 are type isolates). Probe VII hybridizes with CTV strains which are mild, but commonly found in the Orient (B188, B215 are type isolates). Probe VIII hybridizes with all CTV strains that are mild, regardless of origin (T26, T30, B188, and B215 are the type isolates). The SGSP analyses have been useful in field surveys (Halbert et al., 2004), and in evaluation of CTV isolates being considered for mild strain cross protection (Ochoa et al., 2000). More information on the CTV isolates beginning with B (for Beltsville collection) may be found in Garnsey et al. (2005) and their biological activity as determined by biological indexing is summarized in **Table 1**.

The single-stranded conformation polymorphism (SSCP) method is a useful approach to detect single base mutations in genes. This method has been applied to the CP gene of CTV (Rubio et al., 1996) and to the p18, p20, and p27 genes of CTV (Febres, 1995; Ayllón et al., 1999; Rubio et al., 2001). The amplified RT-PCR products are denatured and then electrophoresed on non-denaturing polyacrylamide gels. The denatured DNA strands form intra-molecular hydrogen bonds when entering the non-denaturing gel instead of annealing to their complementary strands, and are separated based on their relative conformations. After silver staining, there are usually two bands that can be visualized; multiple strains of CTV in the plant being tested produce a more complex band pattern. This often is used as a screen to select clones for sequencing of the samples.

THE FLORIDA PROTOCOL FOR TIMELY SELECTION OF MILD STRAINS OF CTV FOR CROSS PROTECTION

When the BrCA arrived in Florida in 1995 and it was realized that the spread of CTV, including severe strains, was increasing (Halbert et al., 2004), we began efforts to select potentially useful mild strains for future use for mild strain cross protection. We will summarize this protocol as it was helpful to select promising mild isolates more rapidly than the empirical approach used previously.

The starting point was to pick isolates from groves being decimated with CTV, and in our instance the decimation was occurring in trees on sour orange rootstock. Budwood was collected from the best looking surviving trees in the field (see example in **Figure 2**). When the budwood was taken to the laboratory, the first test was to conduct MCA-13 ELISA and broad spectrum detection ELISA from each piece of budwood collected. The broad spectrum ELISA confirms the presence of CTV, and we were looking for mild isolates that are non-reactive in the MCA-13 ELISA.

The budwood from surviving trees which had low reactivity in MCA-13 ELISA as compared to the broad spectrum detection ELISA were then propagated into eight sweet orange budlings propagated on sour orange rootstock using blind buds (no bud eyes; **Figure 3**). These budlings were held in small pots to minimize space required. Once the success of CTV graft transmission was confirmed by use of broad spectrum ELISA (usually 3 months), half of the budlings from each budwood source were challenged by graft inoculation of four severe CTV isolates (T36, T68, T66, and T3800) into each of the four plants. The bud take was monitored at two weeks after graft challenge, and plants reinoculated if buds had died. The plants were then held for 3–6 months, and the growth of the challenged plants compared to the growth of the four budlings which had only the CTV isolate recovered from the field. If the challenged plants continued to grow well, the four unchallenged budlings were retained, and the challenged plants discarded. If the challenged plants did not grow, or exhibited yellowing, all of the plants were discarded, the unchallenged plants and the challenged plants. This is a severe early test to select for potentially useful cross

Table 1 | Summary of the biological activities of the type strains of CTV used in the strain group specific probe assays and other isolates of CTV referred to.

Isolate	Mexican lime	Sour orange	Grapefruit	Sweet orange	Sweet orange on sour orange
T36	2.0	2.0	1.0	0.0	1.5
T66	1.0	1.0	0.5	0.0	1.0
B1	3.0	3.0	2.0	0.0	3.0
B53	2.5	2.5	2.8	1.2	2.5
B165	2.5	2.2	3.0	2.5	2.5
B185	2.5	2.0	3.0	1.3	2.5
T3	2.5	2.0	1.0	0.5	2.0
B220	2.0	2.5	2.2	0.0	2.5
B128	2.5	0.5	2.2	0.5	1.0
B249	2.0	3.0	2.5	1.0	3.0
T26	0.5	0.0	0.0	0.0	0.0
T30	0.5	0.0	0.0	0.0	0.0
B188	1.2	0.0	0.0	0.0	0.0
B215	0.8	0.0	0.0	0.0	0.0
T3800	3.0	2.5	3.0	0.5	3.0
T55	0.5	0.0	0.0	0.0	0.0
T56	0.5	0.0	0.0	0.0	0.0
T60	1.0	0.0	0.0	0.0	0.0
T68	2.5	2.0	1.0	0.0	2.0

Biological activities were determined as reported by Garnsey et al. (1987, 2005) using five different indicator plants, Mexican lime, sour orange, Duncan grapefruit, Madam vinous sweet orange, and sweet orange grafted onto sour orange rootstock. The plants are rated using a scale where 0 is healthy and 3 is most severe. The Mexican lime was rated for chlorosis, stunting, and stem pitting; sour orange for seedling yellows symptoms; Duncan grapefruit for seedling yellow, stem pitting, and stunting; Madam vinous sweet orange for chlorosis, stunting, and stem pitting, and the sweet orange on sour orange for decline.

FIGURE 2 | An example of a surviving tree (red arrow) in a grove of Valencia sweet orange on sour orange rootstock. Missing trees result in empty tree spaces or recently planted new trees. The trees surrounding the surviving tree show decline, general chlorosis in the tree canopy, and thinning of the canopy. The tree shown here was the source of one of the mild isolates evaluated under greenhouse conditions in Dekkers and Lee (2002).

protecting CTV isolates, and for each isolate retained for further clean up and evaluation, probably 100 were discarded.

The next step was to do single aphid transmissions from the sources which had been selected. From a colony of BrCA maintained on healthy citrus, the aphids were transferred to tender young tissue on the source plants and allowed to remain for 24 h, after which the aphids were transferred to the young receptor plants, usually Madam vinous sweet orange seedlings, with one aphid per plant. After 24 h, the aphids were removed, the plants sprayed with an insecticide, and then placed back into the greenhouse. After 12–15 weeks, the receptor plants were checked by MCA-13 and broad spectrum detection ELISA to see if they were infected with CTV and if severe isolates were present. Most of the times the plants testing positive after the first round of single aphid transmission were subjected to another round of single aphid transmission as before.

The single aphid transmitted isolates were then subjected to molecular testing, using SGCP and SSCP of the p27 as well as retesting by MCA-13 and broad spectrum detection ELISA (Ochoa et al., 2000). Selection of isolates for host range testing was then made from isolates which appeared to consist of a single strain and not a mixture of strains based on either SGSP and SSCP analyses.

The host range testing was performed on five indicator plants: Mexican lime, sour orange, Duncan grapefruit, Madam vinous sweet orange, and Hamlin sweet orange grafted onto sour orange using the protocol described by Garnsey et al. (1987; 1991; 2005). The results of indexing on all of the hosts except sweet orange on sour orange can be completed in 6–8 months. The biological host range test is important to make sure the selected mild strains are, in fact, mild.

The selected isolates are then ready for greenhouse testing and evaluation (Dekkers and Lee, 2002), after which the most promising isolates would go out to field trials. The selection of mild strains should be a continuous process as the dynamics of the CTV strains and populations in the field will be constantly changing, especially in areas where the BrCA has become established.

USE OF MILD STRAINS OF CTV IN FLORIDA TO PROTECT AGAINST SEVERE CTV STRAINS

The decision of when to implement mild strain cross protection as a management strategy to limit CTV losses normally is made after the severe strains of CTV have become endemic and are causing economic losses, and there is little risk in widespread dissemination of mild strains. CTV decline on sour orange rootstock may be managed effectively by growing trees on a CTV-tolerant rootstock, however sour orange is a desirable rootstock because of the high fruit quality that it induces on grafted varieties. The Indian River production area in Florida is known for the high quality fruit, and most of the fresh fruit originates in this area. This quality is due in part to the use of sour orange rootstock. It was because of the demand for high quality fruit grown on sour orange rootstock that we began research to empirically select mild isolates of CTV which would protect against CTV decline on sour orange rootstock. In Florida, it was not until 2002 that the occurrence of a stem pitting isolate of CTV was reported in commercial citrus and shown to be spreading (Sieburth and Nolan, 2005). Most of the evaluation of Florida mild isolates for protection against CTV stem pitting strains has been done with foreign cooperators (van Vuuren et al., 1991; Ochoa et al., 1993; Vegas et al., 1995).

Several mild CTV isolates (T26, T30, and T55; **Table 1**) that are useful for mild strain cross protection against CTV-D have been selected empirically in Florida. Two different approaches have been used to protect against CTV-D strains which became common in Florida in the 1970s and 1980s. The first approach is to introduce the mild strain into budlings in the nursery, either by blind bud inoculation or by the use of budwood sources already infected with

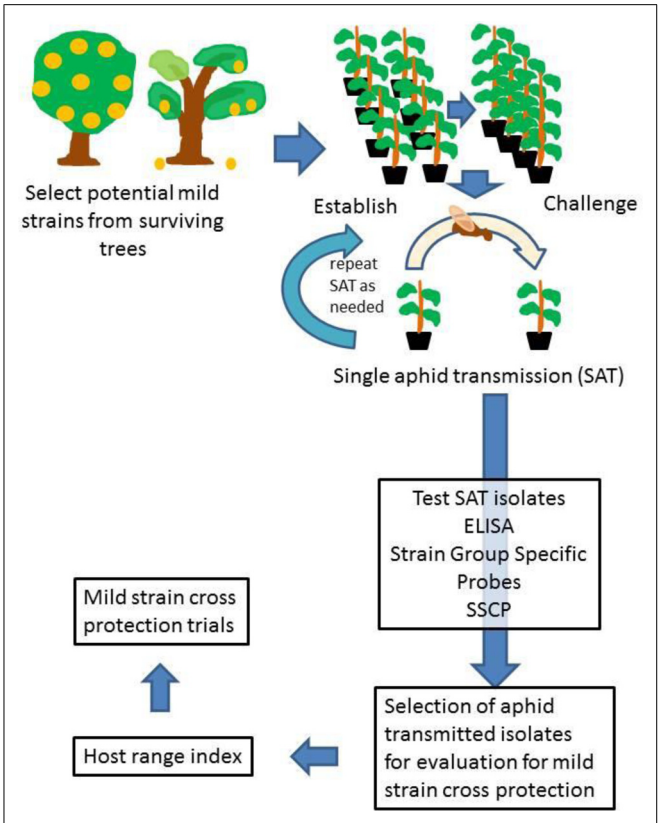

FIGURE 3 | Diagram showing the scheme followed to select mild isolates of CTV for use for mild strain cross protection.

the desired mild strains. The performance of these cross protected budlings has been monitored by greenhouse trials and by field trials (Yokomi et al., 1987; Rocha-Pena and Lee, 1991; Rocha-Pena et al., 1991, 1992; Ochoa et al., 2000; Dekkers and Lee, 2002). The second approach is to introduce the mild CTV strain into mature (7–25 years old) trees on sour orange rootstock using blind buds or leaf piece inoculations, even though the trees are already infected with common and/or CTV-D strains (Lee and Brlansky, 1990; Lee et al., 1995; Lee, 2009). This approach was used in field situations where up to 20% tree loss was occurring annually. While trees on sour orange continue to decline, the rate of decline is slowed so that the production stays at a more consistent level, rather than having all the trees decline at once, then waiting 3 years before the newly planted trees on a CTV tolerant rootstock start to come into production.

Most of the data obtained on the effectiveness of mild strain cross protection has come from experiments which incorporate the mild strain into the budlings at the nursery level. More information has been obtained on the use of Florida mild strains to cross protect against stem pitting strains of CTV than their long term ability to cross protect against CTV-D strains. This is due to severe freezes in Florida in December 1983, January 1985, and December 1989 which destroyed most field plot experiments prior to their completion. Greenhouse evaluations have been utilized to obtain preliminary evaluation of the effectiveness of mild strain cross protection (Rocha-Pena et al., 1991, 1992; Dekkers and Lee, 2002).

Data obtained from cross protection experiments established in Florida suggests that cross protection is possible against CTV-D strains. One experiment was established in the DPI Foundation Grove, Dundee, FL, USA in 1985, exposed only to the natural challenge in that location. Before it was killed in the December 1989 freeze, blocks inoculated with three mild isolates (T30, T49, and T50a) had no declining trees, blocks inoculated with three other mild isolates (T55a, T56, and T60a) had only 10% decline, while those planted virus-free had 50% decline (Yokomi et al., 1992). Companion experiments were established at the Citrus Research and Education Center, Lake Alfred, FL, USA; one plot was challenged with the CTV-D isolate T36 using aphids, and in 1986 the second plot was graft challenged with CTV-D isolates T36 and T66. Testing with ELISA using the MCA-13 monoclonal antibody that reacts specifically with severe CTV isolates indicated severe strains were present in the trees, but the trees continued to grow well except for the occurrence of stunting in some trees (Rocha-Pena et al., 1991; Lee and Niblett, 2000) up until the freeze of 1989.

The inoculation of mild strains for cross protection into mature trees was studied beginning in 1987 (Lee and Brlansky, 1990). Earlier studies had indicated that some mild strains of CTV were able to spread throughout a tree canopy when inoculated into mature trees (Lee et al., 1988). Preliminary trials indicated that if branches at the four compass points on the canopy were inoculated, the T30 isolate of CTV, which is easily identified by a unique double stranded RNA pattern, was distributed throughout the tree canopy within 6 months (Lee et al., 1988, 1992). The first field trial was in a 12 year old grove of pineapple sweet orange on sour orange rootstock in the flatwoods production area where 20% of the trees were being killed annually due to CTV-D (Lee and Brlansky, 1990; Lee et al., 1992). There were seven single tree replications of mild strain T30, mild strain T26, and no mild strain inoculated trees. The inoculations were performed on the compass points of the tree using leaf piece inoculum. At the end of one year, the trees were rated on a 1–4 scale where 1 was healthy and 4 was dead, and the average value is for the trees still living. The control treatment (no mild strain) was 3.3 with 2 trees dead; the T30 treatment was 2.3 with 1 tree dead, and the T26 treatment was 1.8 with no trees dead. In 1987, a block of Navel sweet orange on sour orange rootstock was selected in the flatwoods area near Avon Park, FL, USA. Mild isolates T26, T30, T55, and T11 were inoculated into the 7 year old trees where CTV was causing the demise of 5% of the trees per year, using 5 by 5 tree blocks and four replications per treatment. By monitoring selected trees by double stranded RNA analyses, it became apparent that the non-inoculated trees had acquired mild strain T30 within the 1 year. When this was realized, in 1988, we selected a neighboring block in the same grove to use as a control block, separated by two roads and an irrigation canal; this control block of 500 trees had 2% missing trees due to CTV-D in 1989. In 1999, 11 years after the mild isolates had been introduced into the treatment plot, 89% of the original trees on sour orange rootstock still remained while in the control block, where mild strains were not introduced, had only 21% of the original trees on sour orange rootstock remaining (Lee, 2009). In 1993, trees in the FBRB Foundation Planting at Immokalee, FL, USA were inoculated with mild isolates T11, T26, T30, and T55 (Kesinger, 2003). Over 1 million budeyes were cut from this foundation block from 1989 to

1998 and used for propagations and budwood increase blocks by commercial citrus nurseries in Florida. While the performance of these budeyes/propagations were not monitored for performance in protecting against severe CTV isolates, this management practice did distribute a lot of budlings into the Florida citrus industry which were infected with mild CTV isolates. From inoculum provided by the University of Florida Citrus Research and Education Center, Lake Alfred, FL, USA to growers in Northern Lake, Orange, and Marion Counties from 1999 to 2003, an estimated 13,000 ha of existing citrus on sour orange rootstock were inoculated with mild CTV isolates (Lee, 2009).

The Florida mild isolates have been exported to Brazil, South Africa, and Venezuela as freeze dried infectious preparations (Garnsey et al., 1981). The freeze dried preparations were slash inoculated into receptor hosts (Müller et al., 1990). In each country, the CTV cultures were established *in planta* and then graft inoculated into a wide host range of citrus commonly grown in that country as well as hosts commonly used for biological indexing of CTV (Garnsey et al., 1987). Once all interested parties were satisfied that the introduced mild CTV isolates were in fact mild, cross protection evaluations were established under quarantine conditions, first in a greenhouse or screenhouse for a preliminary evaluation, then in a small scale field plot in an isolated location. The field plots were established on sour orange, a rootstock that normally would not allow trees surviving more than a few months because of the presence of severe CTV isolates in those countries. In South Africa, Florida mild strains T26, T55, T32, T33, T54, T30, and an Israel isolate, Micveh T, provided the best protection in Valencia on sour orange rootstock, both in tree growth and yield (van Vuuren et al., 1991). The same Florida mild isolates performed well on Mexican lime and grapefruit in other evaluation trials (van Vuuren et al., 1991). In Brazil, an experiment was established with Marsh grapefruit, Galego lime, Ponkan mandarin, and Pera sweet orange scions, all on sour orange rootstock, with other plants of the same scions on Rangpur lime as a CTV tolerant rootstock for comparison (Vegas et al., 1995). Nine Florida mild isolates, T11a, T26, T30, T30-132, 37-T4b, 49-T59, 50-T4, 53-T35b, and 58-T37, along with two Brazil isolates, no. 50 and SP-Brazil Satsuma, were inoculated to six trees of each scion in a replicated block. All plants tested positive by MCA-13 ELISA indicating the presence of severe CTV strains. After one year in the field on sour orange rootstocks, Florida isolates 30-T4, T11a, and T30 provided the best growth on Pera sweet orange; T11a and T30a provided the best growth on Galego lime; isolates T26, and 53-T35b provided the best growth on Marsh grapefruit along with a Brazilian isolate no. 50. With the Ponkan mandarin scions, the two Brazil isolates, no. 50 and SP-Brazil Satsuma, provided the best growth. At the end of 3 years in the field, the Pera, Galego lime, and Ponkan mandarin scions were all unthrifty, the Marsh grapefruit scions on sour orange preimmunized with no. 50 CTV isolate from Brazil were still growing and producing, but much smaller than the same scion on Rangpur lime rootstock. In another trial in Brazil, Ponkan mandarin, Marsh grapefruit, Galego lime, Pera, Folha Murcha, and Hamlin sweet oranges were propagated on GouTouCheng sour orange hybrid rootstock and preimmunized with Florida mild isolates T26 and T30 and Brazil mild isolate no. 50. After 9 years, all the preimunized trees were still growing satisfactorily with little stem pitting and bearing good crops. The authors concluded that the Florida mild isolates may provide good protection against CTV-SP strains in the presence of the BrCA if the trees are on a CTV-tolerant rootstock (Vegas et al., 1995). In Venezuela, a field trial was established to evaluate the performance of Valencia sweet orange on sour orange rootstock preimmunized with three Florida mild isolates, T26, T30, and T30a, and eight Venezuela isolates of CTV. After 3 years of evaluations, trees preimmunized with Florida T30 mild isolate continued to grow satisfactorily whereas the other trees were stunted and showed vein corking and stem pitting (Ochoa et al., 1993).

WHAT DOES THE FUTURE HOLD FOR MILD STRAIN CROSS PROTECTION OF CTV?

Citrus tristeza virus with a single stranded RNA genome of ∼19.3 kb presented a challenge in construction of an infectious clone, but in 1999 the infectious clone of CTV isolate T36 was reported by Satyanarayana et al. (1999) Since that time, with the use of the infectious clone of T36, much has been learned about the expression strategies of CTV, genetic variability, and the infectious clone with a green fluorescent protein (GFP) label has been useful for evaluating transgenic plants for resistance to CTV (Dawson, 2010). As the molecular biology of CTV has been studied, better and more sensitive diagnostic procedures have been developed and applied for studies on cross protection and epidemiology. The resistance in *P. trifoliata* has been identified and characterized, and this may be useful in developing resistance at least to most of the isolates of CTV in the future (Mirkov et al., 2010). However, *P. trifoliata* resistance breaking isolates of CTV have been reported, first in New Zealand and later in other locations (Dawson and Mooney, 2000). Transgenic resistance has been reported in grapefruit, but this has not been used on a commercial scale (Febres et al., 2008). More recently, transformation of Mexican lime with an intron-hairpin construct expressing untranslatable versions of the genes coding for the three silencing suppressors of CTV (Lu et al., 2004) conferred complete resistance to the same genotype of CTV (Soler et al., 2012). There are many things still to be learned about CTV; where are the pathogenicity factors located and what interactions do they have with the host to impart resistance or tolerance? We now know that defective RNAs commonly occur with CTV, but it is still to be discovered what role they play in the biology and replication of CTV in various hosts. We now know that CTV has three potentially gene silencing suppressors (Dawson, 2010); as the regulation of these genes becomes better understood, they may be useful for protecting against severe isolates of CTV in commercial crops.

It was recently reported that infection with one strain of CTV excludes infection by another isolate of the same strain of CTV (Folimonova et al., 2010). Using CTV isolates generated from the infectious clone, the inoculation first with T36 strain prevented subsequent infection when the same plants were inoculated with T36 labeled with GFP, but when other CTV strains were used, there was no apparent effect on replication or movement of the challenge virus. This discovery may be useful in the future where possibly the severe CTV isolate could be genetically characterized and a mild variant created using an infectious clone. The mild variant

could then be used as the protecting isolate, providing protection against later infection by the severe isolate. There will need to be some breakthrough in technology before this becomes a reality. We know now that even well characterized isolates of CTV may contain "hidden" severe strains that may become apparent later, usually by aphid transmission (Albiach-Marti et al., 2000; Tsai et al., 2000; Brlansky et al., 2003). While the cost of fully sequencing the genome of a CTV isolate has become very reasonable, the resulting sequence is often a consensus of the population present in the sample, and probably would not detect the presence of minor, but potentially severe, strains of CTV in the isolate. Also, it is not easy to construct an infectious clone, and the T36 infectious clone is the only one reported to date. While many of the 3′ end genes have been substituted for those of other CTV isolates, the report by Folimonova et al. (2010) suggests they will not work to prevent superinfections.

In the future, technology will be developed which will allow identification of severe strains of CTV in a given isolate, and with a much better understanding of how superinfections can be prevented, cross protection may be applied using a much more intelligent approach with molecular tools. For the foreseeable future, the empiric approach, coupled with improved diagnostic ability to quickly and accurately detect and differentiate among CTV strains, will still be the most productive approach for developing mild strain cross protection against CTV as additional citrus production areas experience the introduction and spread of severe CTV which limits production.

ACKNOWLEDGMENTS

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 March 2013; accepted: 14 August 2013; published online: 06 September 2013.

Citation: Lee RF and Keremane ML (2013) Mild strain cross protection of tristeza: a review of research to protect against decline on sour orange in Florida. Front. Microbiol. ***4****:259. doi: 10.3389/fmicb.2013.00259*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 December 2013
doi: 10.3389/fmicb.2013.00366

Past and future of a century old *Citrus tristeza virus* collection: a California citrus germplasm tale

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Citrus tristeza virus (CTV) isolates collected from citrus germplasm, dooryard and field trees in California from 1914 have been maintained *in planta* under quarantine in the Citrus Clonal Protection Program (CCPP), Riverside, California. This collection, therefore, represents populations of CTV isolates obtained over time and space in California. To determine CTV genetic diversity in this context, genotypes of CTV isolates from the CCPP collection were characterized using multiple molecular markers (MMM). Genotypes T30, VT, and T36 were found at high frequencies with T30 and T30+VT genotypes being the most abundant. The MMM analysis did not identify T3 and B165/T68 genotypes; however, biological and phylogenetic analysis suggested some relationships of CCPP CTV isolates with these two genotypes. Phylogenetic analysis of the CTV coat protein (CP) gene sequences classified the tested isolates into seven distinct clades. Five clades were in association with the standard CTV genotypes T30, T36, T3, VT, and B165/T68. The remaining two identified clades were not related to any standard CTV genotypes. Spatiotemporal analysis indicated a trend of reduced genotype and phylogenetic diversity as well as virulence from southern California (SC) at early (1907–1957) in comparison to that of central California (CC) isolates collected from later (1957–2009) time periods. CTV biological characterization also indicated a reduced number and less virulent stem pitting (SP) CTV isolates compared to seedling yellows isolates introduced to California. This data provides a historical insight of the introduction, movement, and genetic diversity of CTV in California and provides genetic and biological information useful for CTV quarantine, eradication, and disease management strategies such as CTV-SP cross protection.

Keywords: bioindexing, diversity, stem pitting, seedling yellows, virus exclusion

INTRODUCTION

Citrus tristeza virus (CTV) isolates from citrus materials introduced in California between 1914 and 2009 have been maintained *in planta* under quarantine in the Citrus Clonal Protection Program (CCPP) at the University of California, Riverside (UCR). Therefore, this unique assemblage of CTV isolates has preserved the genetic profile of CTV collected over time and space in California and provides unique spatiotemporal materials to examine CTV genetic and biological attributes that are relevant for current management and detection strategies.

The CTV isolates in the CCPP collection represent two distinct CTV periods in California. The first CTV period can be defined from the late 1800's, when citrus was first introduced in the state, until 1957, when the Citrus Variety Improvement Program (CVIP), the precursor of the CCPP, was established. For example, in the 1870s, the Parent Washington navel and other citrus varieties were introduced from Brazil and Far East, respectively, for commercial use. In 1907, the Citrus Experiment Station (CES, UCR's precursor) was established and citrus materials began to be introduced systematically to California for experimental use. At that time the viral nature of tristeza (quick decline) disease was unknown and the graft-transmissible nature of citrus diseases was not discovered until much later (i.e., 1933, citrus psorosis). As a result, these early commercial and CES citrus introductions were performed without any specific disease screening (Hiltabrand, 1959; Wallace and Drake, 1959; Hodgson, 1967; Soost et al., 1977; Calavan et al., 1978; Wallace, 1978; Roistacher et al., 1981; Lawton and Weathers, 1989; Kahn et al., 2001). Scientific developments at the CES and Brazil between 1946 and 1951 (Meneghini, 1946; Wallace and Drake, 1951) suggested a viral etiology for tristeza (quick decline) disease, a hypothesis later supported by observation of virus-like particles associated with diseased plants (Kitajima et al., 1964). Indexing on Mexican lime (*Citrus aurantifolia* Christm. Swing.) showed a CTV association with the tristeza quick decline (QD) epidemics which decimated citrus on sour orange rootstock in southern California (SC) at that time (Fawcett and Wallace, 1946; Bar-Joseph et al., 1981; Roistacher, 1995; Agranovsky, 1996; Karasev, 2000; Lee and

Bar-Joseph, 2000; Gottwald et al., 2002; Garnsey et al., 2005). Based on the knowledge obtained with tristeza (quick decline), as well as parallel discoveries on bioindexing of citrus virus and virus-like pathogens, the CVIP was inaugurated at UCR in 1957. From that point on, systematic indexing of citrus introductions to California led to the discovery of various CTV isolates and the establishment the collection used in this study (Weathers and Calavan, 1959; Calavan et al., 1978).

The second CTV California period represented in the CCPP collection is from 1957–2009. During this period, citrus plantings in central California (CC) were developed using QD-tolerant trifoliate (*Poncirus trifoliata* L. Raf.) and trifoliate-hybrid rootstocks. Since southern vs. CC citrus-growing regions are separated by the Tehachapi Mountains, which range from 1.5–2.1 km in elevation and stretches for a distance of 60–80 km, this separation isolated a revitalized citrus industry from the QD-affected areas of SC (Calavan et al., 1978; Barnier et al., 2010). Furthermore, in 1963, a CTV eradication program, managed by the CC Tristeza Eradication Agency (CCTEA), was established in CC where ∼200,000 acres or 76% of the California citrus industry is located today (Gottwald et al., 2002; Usda-Nass, 2012). In 2009 and after localized high CTV incidence in some survey areas, the Citrus Pest Detection Program (CPDP) of the CCTEA adopted a CTV suppression program focused on a selective removal of CTV positive trees from CC based on serological and genotypic criteria (Permar et al., 1990; Yokomi and Deborde, 2005; Barnier et al., 2010; Yokomi et al., 2011a,b).

The CTV isolates from the two spatiotemporal periods described above are represented in the CCPP collection as derived from: (i) CES and CCPP introductions from exotic sources, (ii) citrus trees of various ages that became naturally infected with strains of CTV by vectors from urban landscapes and commercial citrus groves primarily in SC; (iii) interceptions by the CPDP of citrus illegally propagated or topworked using CTV infected budwood as well as isolates being spread by aphid vectors in CC.

In this study, genotypes of 48 CTV isolates, representing approximately 90% of the CCPP CTV collection, were characterized using multiple molecular markers (MMM) assays (Hilf et al., 2005; Yokomi and Deborde, 2005; Moreno et al., 2008; Folimonova et al., 2010; Roy et al., 2010; Roy and Brlansky, 2010). The MMM genotype characterization was complimented by CTV phylogenetic analysis of the major coat protein (CP) gene. Finally, the genetic data of specific CTV isolates were correlated with spatiotemporal information and biological activity on indicator plants (Garnsey et al., 1987, 1991; Polek et al., 2005). These findings serve as a reference for CTV genetic profiles collected over the past century in California and provide a valuable database for CTV management strategies such as CTV strain differentiation and mild strain cross-protection as well as regulatory actions.

MATERIALS AND METHODS

CTV SOURCES

CTV isolates were obtained from the CCPP *in planta* Citrus Pathogen Collection at Riverside, CA. Source plants were graft-inoculated Madam Vinous sweet orange (*C. sinensis* L.) and represent isolates from imported citrus propagations from abroad as well as field sources collected in California over the past century. The CCPP CTV were separated in categories according to the following; location: SC; CC; excluded from California (XC) i.e., intercepted in California and eliminated so there was no field spread; CTV period: 1907–1957 (1), 1957–2009 (2); type of original source: A = CES and CCPP introductions, B = infected trees from urban landscapes or commercial groves; and C = interceptions by the CPDP (**Table 1**).

MULTIPLE MOLECULAR MARKERS (MMM) ANALYSIS

The MMM genotype analysis of CTV was performed using reverse transcription polymerase chain reaction (RT-PCR) with specific primers for the genotypes T30, VT, T36, T3, and B165/T68, as previously described (Hilf et al., 2005; Roy and Brlansky, 2010; Roy et al., 2010) (**Table 2**). Total RNA from ∼0.2 g of bark tissue was extracted using the Spectrum Plant Total RNA kit (Sigma, Saint Louis, Missouri, USA) according to the manufacturer's instructions and eluted in 30 μl of RNase-free water. RT-PCR was performed using AMV Reverse Transcriptase Kit and GoTaq Hot Start Green Master Mix Kit (Promega, Madison, WI, USA) or Qiagen One-Step RT-PCR Kit (Qiagen, Germantown, MD, USA) using proper positive and negative controls as previously described (Hilf et al., 2005; Sharma et al., 2011). The RT-PCR products were analyzed using electrophoresis on 1% agarose gel and visualized over a UV transilluminator after ethidium bromide staining. All MMM reactions were repeated twice and at least two RT-PCR amplicons, per CTV isolate, were sequenced in order to verify homology with the corresponding CTV genome regions. Sequence analysis was performed with ClustalX (1.81), BioEdit (7.0.5.3), and GeneDoc (2.7.000) software (Nicholas and Nicolas, 1997; Thompson et al., 1997; Hall, 1999). The frequencies of the CTV genotypes were calculated as the sum of genotype counts in single, double, and triple mixtures.

COAT PROTEIN GENE PHYLOGENETIC ANALYSIS

A set of primers for universal CTV detection was designed from the genomic region of the CP gene (CP-Universal, CP-U) using the Primer 3 software (Rozen and Skaletsky, 2000) (**Table 2**). The RT-PCR products were purified using Zymo Research DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA), and then sequenced directly using the CP-U forward and reverse primers as previously described (Hajeri et al., 2011). For each of the 48 CTV isolates, the CP-U RT-PCR amplified products were sequenced in order to obtain the complete sequence of CTV CP gene (**Table 2**). Consensus sequences were assembled using DNA Dragon software (http://www.dna-dragon.com) with 2–3 × coverage per strand yielding a sequence of 672 nt in length corresponding to the CP gene. The CTV CP gene sequences acquired were deposited in GenBank (GenBank accession numbers KC841779-KC841826).

Thirty-four full-length CTV genome and CP gene sequences produced at previous studies available in the GenBank were analyzed phylogenetically (data not shown). Subsequently, eleven GenBank CTV sequences representatives of the identified phylogenetic clades, namely, T30 (AF260651), T36 (U16304), NUagA (AB046398), NZ-M16 (EU857538), VT (U56902), B165/T68 (EU076703), NZRB-M12 (FJ525431), A18 (JQ798289), SY568

Table 1 | *Citrus tristeza virus*(CTV) isolates in the Citrus Clonal Protection Program (CCPP) collection, University of California, Riverside.

Name	Isolation year	Origin	Location	Period	Type	Genotype	CP Gene GenBank	CP Gene phylogenetic clade	Biological characterization score sum
SY550	1963	P. R. China	SC	2	A	VT	##798	7	18
SY551	1967/1917[a]	Riverside, CA	SC	1	A	T30+T36	##822	3	15
SY553	1960/1917	Riverside, CA	SC	1	A	VT+T36	##823	3	10.7
SY554	1963	Riverside, CA	SC	2	A	VT	##799	5	14
SY555	1971	Riverside, CA	SC	2	A	VT	##800	7	10
SY556	1972	Waiakea, Hawaii	XC	2	A	VT	##801	5	18.9
SY557	1971	Waiakea, Hawaii	XC	2	A	VT	##802	5	21.8
SY558	1968/1914	Honolulu, Hawaii	SC	1	A	T30+VT+T36	##826	7	22
SY560	1978/1914	Riverside, CA	SC	1	A	T30+VT	##813	5	16.7
SY561	1978/1918	Riverside, CA	SC	1	A	T30	##793	1	14
SY563	1976/1914	Brazil: Bahia	SC	1	A	T30	##794	1	17.6
SY565	1978/1914	Australia	SC	1	A	VT	##803	4	15.5
SY566	1978/1914	Honolulu, Hawaii	SC	1	A	VT	##804	5	23.1
SY568	1978/1961	Riverside, CA	SC	2	A	T30+VT	##814	6	28.3
SY575	1980	San Bernardino, CA	SC	2	B	T30+VT	##815	5	16
SY576	1982	San Bernardino, CA	SC	2	B	T30+VT	##816	5	15
SY577	1979/1914	Miami, Florida	SC	1	A	T30+VT	##817	5	23.3
SY578	1979/1948	Riverside, CA	SC	1	A	T30	##795	1	14
SY579	1979	Orange, CA	SC	2	B	T30	##796	1	13.5
SY580	1980/1963	Riverside, CA	SC	2	A	T30+VT	##818	7	7
SY581	1983	Riverside, CA	SC	2	A	T30+VT	##819	1	nt
SY583	1979/1914	Florida	SC	1	A	VT+T36	##824	7	25
SY584	2009	Argentina: Tucuman	XC	2	A	T30+VT	##820	7	nt
T19	1975	Riverside, CA	SC	2	A	T30+VT	##805	1	nt
T500	1968	Riverside, CA	SC	2	A	T30	##779	1	4
T505	1971	Central CA	CC	2	C	T30	##780	1	7
T506	1971	Ventura, CA	SC	2	B	T30	##781	1	2.5
T508	1971	Ventura, CA	SC	2	B	T30	##782	1	6
T509	1972	Orange, CA	SC	2	B	T30+VT	##806	1	nt
T510	1972	San Bernardino, CA	SC	2	B	T30+VT	##807	1	7
T511	1972	Ventura, CA	SC	2	B	T30	##783	1	4
T514	1974	Tulare, CA	CC	2	C	T30	##784	1	6
T515	1977	Calaveras, CA	CC	2	C	T30+VT	##808	1	8.5
T517	1979	Orange, CA	SC	2	B	T30	##785	1	nt
T518	1979	Orange, CA	SC	2	B	T30+VT	##809	1	nt
T519	1978	Riverside, CA	SC	2	A	T30+VT	##810	6	nt
T520	1978	Tulare, CA	CC	2	C	T30	##786	1	nt
T521	1980	Orange, CA	SC	2	B	T30+VT	##811	1	nt
T522	1981	Ventura, CA	SC	2	B	T30	##787	1	nt
T524	1981	Tulare, CA	CC	2	C	VT	##797	7	nt
T525	1975	Orange, CA	SC	2	B	T30+T36	##821	2	3.5
T528	1990	Tulare, CA	CC	2	C	T30	##788	1	nt
T529	1990	Tulare, CA	CC	2	C	T30	##789	1	nt
T530	1990	Tulare, CA	CC	2	C	T30	##790	1	nt
T531	1992	Florida	XC	2	A	T30	##791	1	nt
T532	1996	Australia: Victoria	XC	2	A	T30+VT	##812	5	nt
T534	2008	San Diego, CA	SC	2	C	T30	##792	1	nt
T535	2000	Japan	XC	2	C	T30+VT+T36	##825	3	25

[a] The year of the original CTV record is reported if it is different from the isolation year; SC, CC, and XC: South, Central, and Excluded, California, respectively; 1, first and 2, second CTV California periods; A, Citrus Experiment Station/CCPP introductions; B, urban/commercial areas infected trees; and C, Citrus Pest Detection Program interceptions; CP, coat protein, ## KC841-GenBank contain additional notes on original host and history, nt: not tested.

Table 2 | Multiple molecular markers (MMM) and primers.

MMM[a]	Reference sequences	Primer names	Primer sequences
CP-U	AF260651	CP-U-F-16054-16075	CWTGAGCRCTGCTTTAAGGGTC
		CP-U-R-16836-16814	GATGAAACTCCACCATCCCGATA
T30-5′-H	AF260651	T30-5′-F-6-26	CGATTCAAATTCACCCGTATC
		T30-5′-R-600-580	TAGTTTCGCAACACGCCTGCG
T30K17-H	AF260651	T30K17-F-4848-4870	GTTGTCGCGCCTAAAGTTCGGCA
		T30K17-R-5256-5235	TATGACATCAAAAATAGCTGAA
T30POL-H	AF260651	T30POL-F-10772-10791	GATGCTAGCGATGGTCAAAT
		T30POL-R-11467-11448	CTCAGCTCGCTTTCTCACAT
T30-R	AF260651	T30-F-588-613	TGTTGCGAAACTAGTTGACCCTACTG
		T30-R-793-769	TAGTGGGCAGAGTGCCAAAAGAGAT
VT-5′-H	U56902	VT-5′-F-1-22	AATTTCTCAAATTCACCCGTAC
		VT-5′-R-492-472	CTTCGCCTTGGCAATGGACTT
VTK17-H	U56902	VTK17-F-4824-4846	GTTGTCGCGCTTTAAGTTCGGTA
		VTK17-R-5232-5211	TACGACGTTAAAAATGGCTGAA
VTPOL-H	U56902	VTPOL-F-10745-10764	GACGCTAGCGATGGTCAAGC
		VTPOL-R-11440-11421	CTCGGCTCGCTTTCTTACGT
VT-R	U56902	VT-F-1945-1972	TTTGAAAATGGTGATGATTTCGCCGTCA
		VT-R-2246-2222	GACACCGGAACTGCYTGAACAGAAT
T36-5′-H	U16304	T36-5′-F-1-20	AATTTCACAAATTCAACCTG
		T36-5′-R-500-481	CTTTGCCTGACGGAGGGACC
T36K17-H	U16304	T36K17-F-4871-4892	GTTTTCTCGTTTGAAGCGGAAA
		T36K17-R-5279-5258	CAACACATCAAAAATAGCTAGT
T36POL-H	U16304	T36POL-F-10797-10816	TGACGCTAACGACGATAACG
		T36POL-R-11511-11490	ACCCTCGGCTTGTTTTCTTATG
T36-R	U16304	T36-F-1775-1799	TTCCCTAGGTCGGATCCCGAGTATA
		T36-R-2610-2585	CAAACCGGGAAGTGACACACTTGTTA
T3K17-H	EU857538	T3K17-F-4846-4867	GTTATCACGCCTAAAGTTTGGT
		T3K17-R-5254-5233	CATGACATCGAAGATAGCCGAA
T3-R	EU857538	T3-F-4846-4873	GTTATCACGCCTAAAGTTTGGT**ACCACT**[c]
		T3-R-5254-5231	CATGACATCGAAGATAGCCGAA**GC**[c]
B165/T68-R[b]	EU076703	B165/T68-F-1885-1912	GTCAAGATTTTGATGATTTGTGCCACTC
		B165/T68-R-2633-2607	AAAATGCACTGTAACAAGACCCGACTC

[a] *Two MMM methodologies were developed independently by Hilf et al. (2005)(-H) and Roy and Brlansky (2010)(-R) and Roy et al. (2010). CP-U: Coat protein universal primer was developed in this study and was used as positive internal control for CTV detection.*

[b] *Genotypes B165 (EU076703) and T68 (JQ965169) represent the same genotype (Folimonova et al., 2010; Roy and Brlansky, 2010).*

[c] *Extra nucleotide sequences of T3-R compared to the T3K17-H sequences are bold and underlined.*

(AF001623), HA16-5 (GQ454870), and T3 (KC525952) were selected for analysis with the CTV isolates in this study.

All topologies were reconstructed with neighbor-joining, maximum parsimony and maximum likelihood methods using MEGA5.1 software. The confidence level in tree topology was examined using bootstrap with 10,000 replicates (Tamura et al., 2011). The topologies from the three phylogenetic methods were similar so only the neighbor-joining phylogenic tree is presented.

BIOLOGICAL CHARACTERIZATION

CTV biological characterization was performed between 1970 and 2012 by graft-inoculation of Mexican lime [*C. aurantifolia* (Christm.) Swing.], Duncan grapefruit (*C. paradisi* Macf.), Eureka or Lisbon lemon (*C. limon*L. Burm.f.), sour orange (*C. aurantium* L.), and Madam Vinous or Pineapple sweet orange seedlings as previously described (Roistacher, 1991; Garnsey et al., 2005; Polek et al., 2005). CTV isolates were inoculated using at minimum six seedlings of each plant indicator, including negative and positive controls. Indicator plants were maintained under standard greenhouse conditions (24–28°C day and 17–21°C night temperatures), infection was confirmed by enzyme-linked immunosorbent assay (ELISA) and symptoms were rated 6–9 months post-inoculation. Symptoms were evaluated on a 0–5 scale (0 = negative; 1 = very mild; 2 = mild; 3 = moderate; 4 = severe; 5 = very severe) over several replicated experiments and the average score for each indicator among different experiments was used (**Table 4**). Finally, the sum score of the seedling yellows (SY) (0–15) and SP (0–10) indicators as well as the total score for all indicators (0–30) for each isolate were calculated and used for statistical analysis.

The biological characterization data were tested for normality using the Kolmogorov–Smirnov test. Normally distributed

data were analyzed by One Way ANOVA, otherwise, the non-parametric Kruskal–Wallis test on ranks (ANOVA on Ranks) was conducted. Statistically significant differences among means were identified by the Holm-Sidak method. Normality test, ANOVA, and tests of significance were performed at $p < 0.05$ using the Sigma Plot 11.00 software (Copyright© 2008 Systat Software, Inc.; San Jose, CA, USA).

RESULTS

CTV GENOTYPES DETERMINATION

The MMM analysis is presented in **Table 3**. The identification of the T30 and VT genotypes in single infections were in general agreement (25 out of 26) between MMM-H and MMM-R (one isolate reacted differently). In contrast, seven CTV isolates reacted differently for MMM-H and MMM-R in the mixtures of T30 and VT genotypes (**Table 3**). The T36 genotype was identified only in mixtures with T30 and VT genotypes (**Table 3**).

Genotype T3 markers developed by Roy et al. (2010) are located in the exact same CTV genome area and contain the identical sequence of that of Hilf et al. (2005) except having a few extra nucleotides at the 3′ end of primers (**Table 2**). Interestingly enough, the T3 markers reacted differently for the CTV isolates tested by MMM-H versus MMM-R. There was a general agreement (42 out of 48) on isolates identified as non-T3 but the two MMM systems identified six different isolates as T3 genotype (**Table 3**). Sequence analysis of the T3 MMM-H and MMM-R PCR products showed 85–89% similarity with the k17 region of ORF 1a of the newly characterized T3 representative (Harper, 2013). The highest sequence similarity (95–97%) was observed with the k17 region of the Indian isolates BAN-1 (AY285670), BAN-2 (AY285668), and B226 (AY285669) which have been reported as various mixtures of T30, VT, T36, and T3 genotypes (Roy and Brlansky, 2004). Hence, the determination of T3 genotypes in the CCPP CTV collection was inconclusive and the T3 genotype was excluded from further analysis.

Eighteen CTV isolates contained the single genotype T30 (37.5%) and eight isolates contained the VT (16.7%) genotype. No CTV isolate was found solely with the T36 genotype (**Figure 1**). The remaining 22 CTV isolates contained mixtures of two or three of T30, T36, and VT genotypes (45.8%). The most common genotype mixture was T30+VT identified in 16 isolates (33.3%). The genotype mixtures of T30+T36, VT+T36, and T30+VT+T36 represented 4.2% of the CTV isolates (**Figure 1**).

CTV GENOTYPES SPATIOTEMPORAL ANALYSIS

The CTV genotype frequencies and spatiotemporal distribution is presented in **Figure 1**. T30 and VT were identified at the same frequency for period 1. In period 2, the T30 genotype frequency increased while VT and T36 frequencies decreased (**Figure 1**). The T30 genotype frequency was similar for SC and CC (i.e., 79.4% and 87.5%, respectively). On the contrary, the VT genotype frequency was reduced to less than half in CC in comparison to SC while the T36 genotype was not detected in the CC isolates. All three genotypes were identified amongst the six California excluded (XC) isolates intercepted in the state but eliminated before field spread (**Figure 1**).

The diversity of the SC-2 CTV genotypes was reduced in comparison to the SC-1. Two genotype mixtures identified in SC-1 (VT+T36 and T30+VT+T36) were not identified in SC-2. In addition, the genotype frequency for T30 increased and T36 decreased in SC-2 in comparison to SC-1 (**Figure 1**). T30 and VT were the only genotypes identified in CC-2. The CC-2 genotypic diversity was not as big as the SC-1. For example, three genotype mixtures identified in SC-1 (T30+T36, VT+T36 and T30+VT+T36) were not identified in CC-2 while VT and T36 frequencies were reduced (**Figure 1**). Finally, the T30 genotype frequency was stable (∼87%) for SC-2 and CC-2, however, VT and T36 frequencies were reduced in CC-2. All three genotypes were identified in CTV isolates originated from CES/CCPP introductions, urban/commercial groves, and CPDP interceptions (**Figure 1**).

CTV CP GENE PHYLOGENETIC AND SPATIOTEMPORAL ANALYSIS

GenBank contains a plethora of CTV sequences. In order to select appropriate CTV representative accessions for a meaningful analysis with the CCPP CTV isolates, GenBank CTV accessions were analyzed phylogenetically on their own. The analysis identified five distinct clades associated with the T30, T36, VT, B165/T68, and T3 CTV genotypes and three additional clades with isolates not related to any standard CTV genotype (data not shown). The CP gene sequence of 11 GenBank CTV accessions representing the eight identified phylogenetic clades was selected for analysis with the 48 CCPP CTV isolates in this study (**Figure 2**).

The 48 CCPP CTV isolates were clustered into seven distinct clades (**Figure 2**). SC and XC isolates were present in seven and four clades, respectively. In contrast, CC isolates were limited in two clades. California CTV isolates from periods 1 and 2 were well-distributed and present in five and six clades, respectively. CTV isolates from type A were present in six clades while types B and C were limited in three clades (**Figure 2**).

The majority of the CCPP CTV isolates (34 out of 48) clustered with representatives of the T30 and VT genotypes (clade 1 and 5). A total of six CCPP CTV isolates clustered with the representatives of T36, T3, and B165/T68 representatives (clade 3, 4, and 6). The remaining isolates clustered with the non-standard CTV genotypes NZRB-M12 (clade 2) and HA16-5 (clade 7) (**Figure 2**). It is worth noting here that NZRB-M12 and HA16-5 were strongly associated (bootstrap >85%) with CCPP CTV isolates of T30+T36 and VT genotype, respectively. (Vives et al., 2005; Harper et al., 2010; Melzer et al., 2010; Harper, 2013).

CTV BIOLOGICAL CHARACTERIZATION, SPATIOTEMPORAL AND GENOTYPIC ANALYSIS

Results of biological indexing per isolate are presented in **Table 4**. Mexican lime reacted with all CTV isolates tested; three isolates did not induce SY; and 16 isolates did not induce SP reactions. Four CTV isolates reached the maximum SY sum score of 15 while nine additional isolates scored 12 and above. In contrast, no CTV isolate reached the maximum SP sum score of ten and only two isolates scored above seven (**Table 4**).

The biological characterization data were analyzed in relation to the location (SC, CC, and XC), period (1 and 2), type of original source (CES/CCPP introductions, urban/commercial groves,

Table 3 | Multiple molecular markers (MMM) analysis of the *Citrus tristeza virus*(CTV) isolates in the Citrus Clonal Protection Program collection.

Name	CP-U[b]	T30				VT				T36				T3		B165/T68	Genotype
		H[a]	H	H	R	H	H	H	R	H	H	H	R	H	R	R	
		T30-5	T30K17	T30POL	T30	VT-5	VTK17	VTPOL	VT	T36-5	T36K17	T36POL	T36	T3K17	T3	B165/T68	
T500	1[c]	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T505	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T506	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T508	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T511	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	T30
T514	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T517	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T520	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T522	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T528	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T529	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	T30
T530	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	T30
T531	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
T534	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
SY561	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	T30
SY563	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	T30
SY578	1	0	0	0	1[d]	0	0	0	0	0	0	0	0	0	0	0	T30
SY579	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	T30
T524	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	VT
SY550	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	VT
SY554	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	VT
SY555	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	VT
SY556	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	VT
SY557	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	VT
SY565	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	VT
SY566	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	VT
T19	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	T30+VT
T509	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	T30+VT
T510	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	T30+VT
T515	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	T30+VT
T518	1	0	1	1	1	0	1	0	1	0	0	0	0	0	0	0	T30+VT
T519	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	T30+VT
T521	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	T30+VT
T532	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	T30+VT

(Continued)

Table 3 | Continued

Name	CP-U[b]	T30				VT				T36				T3		B165/T68	Genotype
		H[a]	H	H	R	H	H	H	R	H	H	H	R	H	R	R	
		T30-5	T30K17	T30POL	T30	VT-5	VTK17	VTPOL	VT	T36-5	T36K17	T36POL	T36	T3K17	T3	B165/T68	
SY560	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	T30+VT
SY568	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	T30+VT
SY575	1	1	1	1	1	1	0	1	1	0	0	0	0	0	1	0	T30+VT
SY576	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	T30+VT
SY577	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	T30+VT
SY580	1	1	1	1	1	0	1	1	0	0	0	0	0	0	1	0	T30+VT
SY581	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	T30+VT
SY584	1	0	1	0	1	1	0	1	1	0	0	0	0	1	0	0	T30+VT
T525	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	T30+T36
SY551	1	1	0	1	0	0	0	0	0	0	0	1	1	0	0	0	T30+T36
SY553	1	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	VT+T36
SY583	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	VT+T36
T535	1	1	1	1	1	0	0	1	1	0	0	1	1	0	0	0	T30+VT+T36
SY558	1	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0	T30+VT+T36

[a] Two MMM methodologies were developed independently by Hilf et al. (2005) (H) and Roy and Brlansky (2010) (R) and Roy et al. (2010). [b] CP-U: Coat protein universal primer was used as positive internal control for CTV detection. c0 and1: negative and positive reaction, respectively. dBoxed areas indicate result differences between MMM-H and MMM-R.

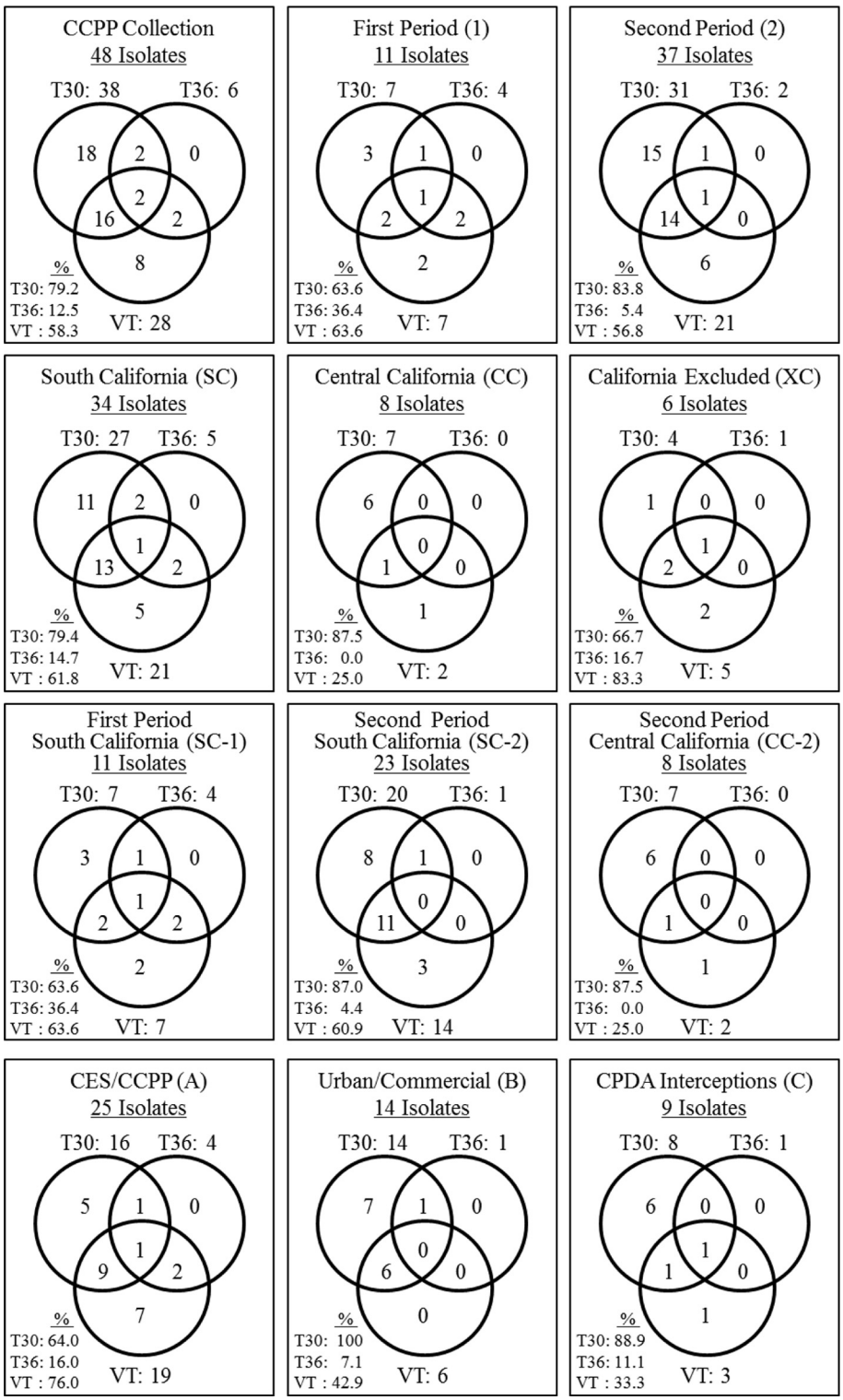

FIGURE 1 | Summary of the genotype frequencies of the *Citrus tristeza virus* (CTV) isolates in the Citrus Clonal Protection Program (CCPP) collection and specific CTV genotype frequencies for different isolation periods (first and second), locations (South, Central, and Excluded, California), and types of CTV sources (Citrus Experiment Station-CES/CCPP introductions, urban/commercial areas, and Citrus Pest Detection Program (CPDP) interceptions). The second period Excluded California genotype frequencies were identical to the Excluded California frequencies thus, is not presented separately. The CTV genotypes frequencies were calculated as the sum of genotype counts in single, double, and triple mixtures.

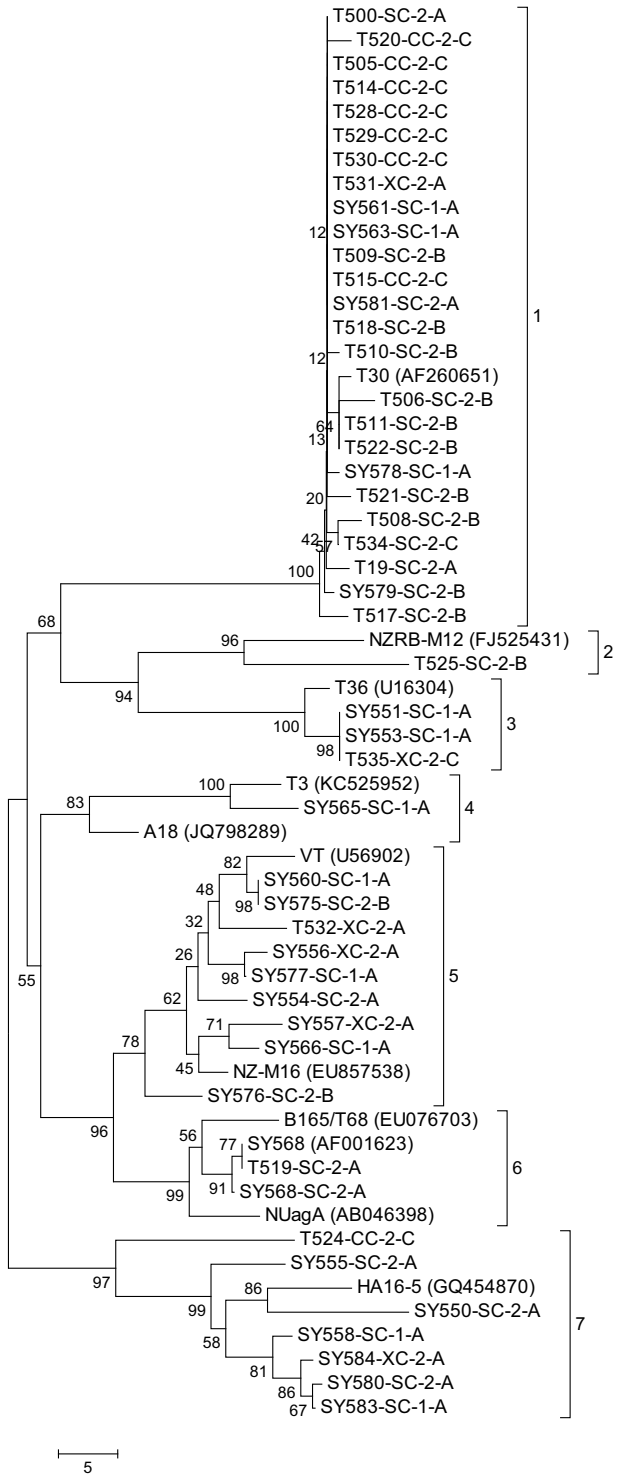

FIGURE 2 | Neighbor joining tree of the complete sequence of the major coat protein gene of the *Citrus tristeza virus* (CTV) isolates in the Citrus Clonal Protection Program (CCPP) collection including 11 representative CTV isolates previously deposited in GenBank (accession numbers in parenthesis). Bootstrap values (10,000 replicates) are shown next to the branches and identified phylogenetic clades are labeled 1–7. The name of the CTV isolate is followed by the geographic

(Continued)

FIGURE 2 | Continued

location; SC, CC, and XC: South, Central, and Excluded, California, respectively; the CTV isolation period: 1: early and 2: second; and the types of isolations: A, Citrus Experiment Station/CCPP introductions; B, urban/commercial areas infected trees; and C, Citrus Pest Detection Program (CPDP) interceptions.

and CPDP interceptions), and genotype (T30, T36, and VT) of the CCPP CTV isolates (**Tables 1**, **4**). Geographical location, time period, source type, genotype and CP gene phylogeny of the CTV isolates had no significant effect on the Mexican lime reactions ($p > 0.05$, ANOVA). In contrast, XC isolates had significantly higher biological activity scores than CC isolates ($F_{30} = 3.656$, $p = 0.039$). Similarly, CTV isolates from period 1 induced more severe biological reactions than those from period 2 ($F_{30} = 5.802$, $p = 0.023$). When geographical location and time period were co-analyzed, CTV isolates SC-1 and XC-2 induced significantly more severe biological reactions than SC-2 and CC-2 ($F_{30} = 6.324$, $p = 0.002$). CTV isolates from CES/CCPP (type A) were significantly more virulent than the isolates naturally spreading in urban and commercial groves (type B) ($F_{30} = 5.008$, $p = 0.014$). CTV isolates with T30 genotype induced significantly milder biological activity scores in comparison to VT and T30+VT+T36 ($F_{30} = 3.123$, $p = 0.025$). The milder biological activity of T30 genotypes compared to VT genotypes was also supported by the significantly different biological score of CTV isolates that clustered in clade 5 (VT representatives) versus clade 1 (T30 representatives) ($F_{30} = 10.014$, $p < 0.001$).

SY and SP severity scores were significantly correlated with time periods and geographical location of the CTV isolates, respectively. Period 1 isolates induced significantly more severe SY ($H_1 = 6.165$, $p = 0.013$) and XC isolates induced significantly more severe SP ($H_2 = 7.636$, $p = 0.022$). Spatiotemporal analysis of the SY and SP reactions indicated significantly higher SY reactions for SC-1 isolates in comparison to SC-2 and CC-2 ($F_{30} = 5.033$, $p = 0.007$) and significantly higher SP reaction for XC-2 in comparison to SC-1 and -2 and CC-2 ($H_3 = 8.035$, $p = 0.045$). CTV isolates clustered in clade 5 (VT representative) had significantly more severe SY reactions in comparison to isolates from clade 1 (T30 representative) ($F_{27} = 8.297$, $p < 0.001$). CTV source types and genotypes had no significant effects in SY and SP reactions ($p > 0.05$).

DISCUSSION

The one-of-a-kind century-old *in planta* CCPP CTV collection has proven valuable for CTV research. From the early days of CTV discovery, detection, and biological characterization to today's molecular era, the collection has provided important information for the virus in California (Gumpf et al., 1987; Garnsey et al., 1991; Marco and Gumpf, 1991; Roistacher, 1991; Nikolaeva et al., 1998; Vidalakis et al., 2004; Wang et al., 2012). We understand that the data developed in this report is based on a relatively small sample size to reach any definitive or general conclusions for the genotype or evolutionary relationships of CTV isolates in California. However, each isolate tested was often a lone selection among many isolates detected as a representative of location, time, host combination, symptomology, etc. Furthermore, the

Table 4 | Biological characterization reactions of the *Citrus tristeza virus* (CTV) isolates in the Citrus Clonal Protection Program collection.

Name	ML	Seedling yellows				Stem pitting		
		SO	Le	GF	Sum	GF	SW	Sum
SY550	5	5	3	5	13	0	0	0
SY551	2	3	3	5	11	0	2	2
SY553	1	3.2	3	3.5	9.7	0	0	0
SY554	3	2.5	5	3.5	11	0	0	0
SY555	5	0	0	0	0	5	0	5
SY556	4	1	1.8	4.4	7.2	4.1	3.6	7.7
SY557	2	5	5	4.8	14.8	0	5	5
SY558	5	4.2	4.9	3.6	12.7	0	4.3	4.3
SY560	3	4.7	4.4	4.6	13.7	0	0	0
SY561	5	0	5	4	9	0	0	0
SY563	4	4.6	4.4	4.6	13.6	0	0	0
SY565	3	4	3.7	4.8	12.5	0	0	0
SY566	4	5	5	5	15	0	4.1	4.1
SY568	5	5	5	5	15	3.3	5	8.3
SY575	3	5	2	5	12	0	1	1
SY576	1	5	4	5	14	0	0	0
SY577	4.3	5	5	5	15	0	4	4
SY578	5	2	3	0	5	0	4	4
SY579	5	0	5	3.5	8.5	0	0	0
SY580	2	0	0	0	0	5	0	5
SY583	5	5	5	5	15	5	0	5
T500	2	0	1	1	2	0	0	0
T505	3	0	0	2	2	0	2	2
T506	2.5	0	0	0	0	0	0	0
T508	2	0	2	2	4	0	0	0
T510	1	2	2	0	4	2	0	2
T511	1	0	1	2	3	0	0	0
T514	3.5	0	1.5	1	2.5	0	0	0
T515	2.5	3	0	3	6	0	0	0
T525	3.5	0	0	0	0	0	0	0
T535	5	4.2	5	5	14.2	3.0	2.8	5.8

ML, Mexican lime (Citrus aurantifolia Christm. Swing.); SO, sour orange (C. aurantium L.), Le, Eureka or Lisbon lemon (C. limon L. Burm.f.); GF, Duncan grapefruit (C. paradisi Macf.), and SW, Madam Vinous or Pineapple sweet orange (C. sinensis)0.0–5: Symptoms evaluation scale. 0 = negative; 1 = very mild; 2 = mild; 3 = moderate; 4 = severe; and 5 = very severe.

statistical analysis served to normalize data from unequal or non-uniform CTV samples in the different categories tested.

The frequencies of CTV genotypes and biological characterization of the isolates from the past 100 years, different location, and source types in California were consistent with that expected due to selected citrus introductions [e.g., selection against stem pitting (SP) isolates], eradication and suppression efforts (e.g., reduced genotype diversity in period 2 and CPDA interceptions), and use of virus-tested stock (e.g., reduced genotype diversity in CC-2) (Roistacher et al., 1981; Yokomi and Deborde, 2005; Barnier et al., 2010). If citrus germplasm were introduced freely in California (i.e., without CTV testing and elimination) several CTV genotypes would be expected to be present in the state. Moreover, CTV association with aphid vectors over time would have created more genotype combinations in California. This study revealed only three of the five known CTV genotypes. Furthermore, 90% of the isolates had T30 genotypes in single and double infections with VT genotypes. Even though factors such as transmission efficiency of different genotypes or other molecular evolutionary events (mutations, recombination, etc.) could have affected our results, the data agrees with and provides support to previous reports identifying T30 and VT as the most common CTV genotypes in California (Yokomi and Deborde, 2005; Yokomi et al., 2011a).

Many CCPP CTV isolates were mixtures of multiple genotypes thus; it is difficult to ascertain which genotypes present directly induced the observed SP and SY symptoms. Interestingly, less than half of the CCPP CTV isolates induced SP with low symptom severity. The opposite was observed for SY. Almost all CTV isolates induced SY reactions with the majority being severe. SP is expressed in the field on various citrus scion species but not SY. SY is a greenhouse indicator reaction that is not expressed in field trees. This supports the hypothesis that early citrus researchers, nurserymen and growers selected citrus planting stock for California from non-SP symptomatic vigorous trees. In contrast, the SY phenotype would have been undetectable in the field and it would have required biological indexing for identification. Thus, selection of citrus stock from the 1800's to the 1950's would have no way to be tested for CTV SY and, as a result, SY isolates were likely unwittingly allowed to pass into California.

The diverse phylogenetic relationships and increased virulence of XC and SC CTV isolates should be expected as they represent the earliest arrival of CTV populations before any controls were imposed by certification and eradication programs. This was evident from the limited phylogenetic relationships and genotype diversity of the CC CTV isolates compared to SC isolates due to the benefits of the fore-mentioned control measures implemented in California (Roistacher, 1995; Gottwald et al., 2002; Barnier et al., 2010).

The CCPP CTV collection also provided a combination of biological and molecular isolates to evaluate performance of CTV detection/characterization tools. Mexican lime is well known and widely used CTV indicator host. The data presented here provided experimental evidence and statistical support that Mexican lime can be considered as the standard method for broad-spectrum CTV detection since it reacted with all CTV isolates tested regardless of genotype, origin, etc. In addition, the MMM protocol as described by Hilf et al. (2005) and Roy et al. (2010) readily identified older as well as recent isolates with single CTV genotypes T30 and VT. In contrast, identification of the T3 genotype and various other genotype mixtures were problematic. It is likely that recombination events, especially in mixed CTV infections, contributed to the observed irregular MMM results (Vives et al., 1999; Hilf et al., 2005; Vives et al., 2005; Moreno et al., 2008; Roy and Brlansky, 2010).

Our study also highlighted the need to use complimentary analysis by different methodologies of CTV characterization. For example, MMM analysis did not identify B165/T68 genotypes and the identification of T3 genotype was inconclusive for the CCPP CTV isolates. However, use of CP gene sequencing and

phylogeny suggested relationships of CCPP CTV isolates with T3 and B165/T68 genotypes. In addition, the MMM analysis identified T30 genotypes in CTV isolates that induced severe SY reaction in plant indicators. While T30 genotypes are not known to produce SY, it is possible, that other genotypes that were not identified by the utilized MMM protocols were present and responsible for the observed reactions. Specific genome regions, such as p23, of the CCPP CTV T30 isolates that induced SY reactions should also be further investigated (Albiach-Marti et al., 2010). Regardless of the mechanism behind the association of T30 genotypes with SY reactions, the present study indicated that in the absence of any biological information, isolates such as SY563 could have been considered benign based on genotype information alone. In our case, the combination of different methodologies provided an opportunity for careful interpretation of the molecular data as well as testable hypotheses for further experimentation with specific CTV isolates.

California has been fortunate so far in avoiding introduction of exotic CTV isolates such as A18, Taiwan-Pum/SP/T1 (JX266712), and NUagA and eradicating virulent CTV-SP isolates, such as SY568, SY553 (Meyer lemon) and T535 (Dekopon), before they could spread to commercial citrus (Calavan et al., 1980; Roistacher and Dodds, 1993; Vives et al., 1999; Gottwald et al., 2002; Moreno et al., 2008; Herrera-Isidrón et al., 2009; Ruiz-Ruiz et al., 2009; Roy et al., 2010; Saponari and Yokomi, 2010). Our genotype, phylogenetic, and biological analysis provided useful information for monitoring of CA-exotic CTV isolates, development of diagnostics and management strategies such as CTV-SP cross-protection (Roistacher et al., 1988; Roistacher and Dodds, 1993; Karasev, 2000; Folimonova et al., 2010; Folimonova, 2012; Matos et al., 2013).

ACKNOWLEDGMENTS

The authors are grateful to all past and current CCPP personnel for their dedicated work and especially for creating and maintaining the *in planta* CTV collection through time. We would like to thank Chester Roistacher, Mark Hilf, and Stephen Garnsey for their comments and assistance with the manuscript. We also thank Karen Xu (Statistical Consulting Collaboratory, UCR) for her advice with the statistical analysis. This work was partially supported by a specialty crop block grant (SCB 10005) from the California Department of Food and Agriculture and the Citrus Research Board (5300-147, 5100-147, and 6100). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 February 2013; accepted: 18 November 2013; published online: 10 December 2013.

Citation: Wang J, Bozan O, Kwon S-J, Dang T, Rucker T, Yokomi RK, Lee RF, Folimonova SY, Krueger RR, Bash J, Greer G, Diaz J, Serna R and Vidalakis G (2013) Past and future of a century old Citrus tristeza virus collection: a California citrus germplasm tale. Front. Microbiol. ***4****:366. doi: 10.3389/fmicb.2013.00366*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

METHODS ARTICLE
published: 02 July 2013
doi: 10.3389/fmicb.2013.00165

A genetic system for *Citrus Tristeza Virus* using the non-natural host *Nicotiana benthamiana*: an update

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In nature *Citrus tristeza virus* (CTV), genus *Closterovirus*, infects only the phloem cells of species of *Citrus* and related genera. Finding that the CTV T36 strain replicated in *Nicotiana benthamiana* (NB) protoplasts and produced normal virions allowed development of the first genetic system based on protoplast transfection with RNA transcribed from a full-genome cDNA clone, a laborious and uncertain system requiring several months for each experiment. We developed a more efficient system based on agroinfiltration of NB leaves with CTV-T36-based binary plasmids, which caused systemic infection in this non-natural host within a few weeks yielding in the upper leaves enough CTV virions to readily infect citrus by slash inoculation. Stem agroinoculation of citrus and NB plants with oncogenic strains of *Agrobacterium tumefaciens* carrying a CTV-T36 binary vector with a GUS marker, induced GUS positive galls in both species. However, while most NB tumors were CTV positive and many plants became systemically infected, no coat protein or viral RNA was detected in citrus tumors, even though CTV cDNA was readily detected by PCR in the same galls. This finding suggests (1) strong silencing or CTV RNA processing in transformed cells impairing infection progress, and (2) the need for using NB as an intermediate host in the genetic system. To maintain CTV-T36 in NB or assay other CTV genotypes in this host, we also tried to graft-transmit the virus from infected to healthy NB, or to mechanically inoculate NB leaves with virion extracts. While these trials were mostly unsuccessful on non-treated NB plants, agroinfiltration with silencing suppressors enabled for the first time infecting NB plants by side-grafting and by mechanical inoculation with virions, indicating that previous failure to infect NB was likely due to virus silencing in early infection steps. Using NB as a CTV host provides new possibilities to study virus-host interactions with a simple and reliable system.

Keywords: CTV, infectious cDNA clones, agroinoculation, *Agrobacterium tumefaciens*, oncogenic strains, graft transmission, *N. benthamiana* protoplasts, RNA silencing suppressor

INTRODUCTION

Citrus tristeza virus (CTV), a member of genus *Closterovirus*, is one of the more economically important plant viruses. Almost 100 million trees propagated on sour orange (*Citrus aurantium* L.) rootstocks died worldwide from different tristeza epidemics, and presently, many millions more propagated on decline-tolerant rootstocks are debilitated by severe CTV isolates inducing stem pitting in commercial citrus varieties regardless of the rootstock used (Bar-Joseph and Dawson, 2008; Moreno and Garnsey, 2010).

CTV virions (2000 × 10–12 nm) are composed of a single-stranded, positive-sense genomic RNA (gRNA) of about 20 kb and two coat proteins of 25 (CP) and 27 (CPm) kDa that encapsidate about 97 and 3% of the gRNA, respectively (Bar-Joseph and Lee, 1989; Satyanarayana et al., 2004; Gowda et al., 2009). The CTV gRNA has 12 open reading frames (ORF) and untranslated regions (UTR) of 107 and 273 nt at the 5′ and 3′ termini, respectively (Karasev et al., 1995). ORFs 1a and 1b, encompassing the 5′ half of the genome, encode replicase-related proteins that are translated from the gRNA and contain papain-like protease, methyltransferase-like, helicase-like and RNA-dependent RNA polymerase domains. The ten 3′- proximal ORFs encode proteins p33, p6, p65, p61, p27, p25, p18, p13, p20, and p23, which are expressed via 3′ coterminal subgenomic RNAs (sgRNAs) (Hilf et al., 1995), promoted by internal controller elements (Gowda et al., 2001). Proteins p6, p65, p61, p27, and p25 are part of a module conserved among closteroviruses that is involved in virion assembly and movement (Satyanarayana et al., 2000, 2004; Dolja et al., 2006; Gowda et al., 2009; Tatineni et al., 2010). The p33, p18, and p13 proteins are dispensable to systemically infect some citrus species (Tatineni et al., 2008), but they are required to invade others like grapefruit (*C. paradisi* Macf.) and sour orange (Tatineni et al., 2011). Moreover, p33 is required in CTV-infected plants to exclude superinfection by isolates of the same strain (Folimonova et al., 2010; Folimonova, 2012), and the expression ratio between p33 and p13 or p18 seems to determine the stem pitting symptom (Tatineni and Dawson, 2012). Proteins p25, p20, and p23 have been shown to act as silencing suppressors in *Nicotiana benthamiana* (NB) and *N. tabaccum* plants (Lu et al., 2004), with p23 being also a pathogenicity determinant

(Ghorbel et al., 2001; Fagoaga et al., 2005; Albiach-Martí et al., 2010).

In nature, the CTV host range is restricted to species of a few genera within the subfamily *Aurantioideae*, and within infected plants, the virus invades only phloem tissues. Although CTV was experimentally transmitted to *Passiflora gracilis* and *P. caerulea* (Müller et al., 1974; Roistacher and Bar-Joseph, 1987), two perennial vines, trials to mechanically transmit it to herbaceous and other non-rutaceous woody species, including NB and other *Nicotiana* species, were unsuccessful (Müller and Garnsey, 1984; our unpublished results). Moreover, although citrus can be mechanically inoculated by slashing citrus stems with CTV virions (Garnsey et al., 1977), all attempts to mechanically inoculate them with virion RNA or RNA transcripts from a cDNA clone were unsuccessful (Satyanarayana et al., 2001). These limitations and the large size of the CTV genome that hindered preparation of full-length cDNA clones and of intact RNA transcripts for inoculation greatly delayed development of a genetic system for this virus. After Navas-Castillo et al. (1997) showed that CTV virions were able to replicate in NB protoplasts, Satyanarayana and associates (1999) developed a full-length cDNA clone of the CTV isolate T36 (CTV-T36), from which they synthesized *in vitro* RNA transcripts that infected NB protoplasts and produced normal CTV virions. Due to the large size and fragility of the RNA transcripts and the difficulty to inoculate protoplasts with such large RNAs, the protoplast infection rate was only about 10^{-4} and the amount of virions produced was insufficient to infect citrus plants by mechanical inoculation. Virion amplification by successive cycles of protoplast inoculations yielded amounts of virions which were able to systemically infect citrus plants and incite the symptoms characteristic of the wild T36 isolate (Satyanarayana et al., 2001). However, the above limitations and frequent failures in virion amplification and transfer between protoplast batches made this genetic system very tenuous.

To overcome these problems we developed an improved genetic system based on agroinfiltration of NB leaves with binary plasmids carrying a cDNA of the CTV-T36 genome and different silencing suppressors and performed a time-course analysis of CTV accumulation in those leaves (Ambrós et al., 2011). Unexpectedly, we found that agroinoculated plants of this species, presumed to be "non-host," were systemically invaded by CTV-T36 with high viral titers, particularly in the upper leaves in which the virus eventually invaded some non-phloem tissues and incited typical disease symptoms. Citrus plants mechanically inoculated with virions produced in NB became systemically infected, displayed the symptoms characteristic of the wild CTV-T36 isolate, and restricted the virus to the phloem, suggesting that replication and movement in NB tissues does not alter CTV-T36 properties.

This new genetic system based on the use of NB as an intermediate host was simpler and more reliable than the former system based on protoplast transfection but it still had at least three potential limitations:

1. Direct agroinoculation of citrus plants would be an easier and faster procedure than using NB as an intermediate host for producing virions. Although previous efforts to agroinfect citrus with CTV using binary plasmids and different *A. tumefaciens* strains were unsuccessful (Gowda et al., 2005, and our unpublished data), virulence and transformation efficiency of *A. tumefaciens* strains are widely determined by experimental conditions and specific interactions with the plant that make some *Agrobacterium* strains more virulent than others in a particular host species, including members of the *Rutaceae* (Cervera et al., 1998). Transient and stable transformation of herbaceous and woody plants has been performed mainly with non-oncogenic (disarmed) strains in which the oncogenes of the wild-type T-DNAs were removed (Gelvin, 2005). In this work we tried agroinoculation of citrus plants using two oncogenic strains, the virulent strain C58 (pTiC58) and the supervirulent strain A281 (pTiBo542), transfected with binary vectors carrying a plant expression marker gene and a cDNA of CTV-T36.
2. The present genetic system relies on the ability of CTV-T36 to replicate in NB cells and to eventually move cell-to-cell and long distance, but the ability of other CTV genotypes to infect this host remains unknown. Therefore, testing if these virions can replicate in NB protoplasts is a preliminary step to develop a similar genetic system for other genotypes, a step that would be easier and faster if virions could be successfully introduced into NB cells. Here we tried to develop a procedure to mechanically inoculate NB plants with virions of different CTV isolates.
3. CTV-infected NB plants show dwarfing, necrosis and often die after a few months and further work with different CTV hybrid constructs or mutants, would require a procedure to maintain these constructs in NB without needing new agroinoculations. For this purpose here we developed a graft transmission procedure to transmit CTV from infected to healthy NB plants.

We found that the oncogenic *Agrobacterium* strains efficiently induced tumors expressing GUS in different plant species, including citrus, and that systemic CTV infection developed in some NB plants. However, CTV virions were not detected in tumors of citrus plants. Mechanical inoculation of CTV virions on NB plants agroinfiltrated previously with a silencing suppressor resulted in systemic infection with CTV-T36, but not with CTV T318A, even though this latter isolate replicated and accumulated in NB protoplasts to the same extent as CTV-T36. CTV-T36 was readily graft transmitted from infected to healthy NB plants after agroinfiltrating the receptor plants with a silencing suppressor. The possibilities and limitations of the new genetic system are further discussed.

MATERIALS AND METHODS

VIRUS ISOLATES AND PLANT GROWTH

The CTV isolate 947R is a clonal virus population obtained from the infectious CTV-T36 cDNA clone from Florida (Satyanarayana et al., 2001) that is maintained in Mexican lime [*C. aurantifolia* (Chritm.) Swing.] and alemow plants (*C. macrophylla* West.). Isolates T305 and T318A, inducing stem pitting on sweet orange [*C. sinesis* (L.) Osb.] and grapefruit (Ruiz-Ruiz et al., 2006; Sambade et al., 2007), and T385, a very mild isolate (Vives et al., 1999), are part of the IVIA collection of citrus viruses.

Citrus plants were grown in individual pots as previously reported (Arregui et al., 1982) and kept in a temperature-controlled (18/26°C night/day) greenhouse, whereas *N. benthamiana* plants were grown in a customized growth chamber kept at 23–24°C constant temperature and 50–60% relative humidity with a 16/8 h light/dark photoperiod.

BINARY VECTORS AND AGROBACTERIUM STRAINS

The BAC plasmid pCH20 (18 kb) contains two *Not*I sites flanking the unique *Bam*HI cloning site in the *sac*B gene of the T-DNA, a unique *Swa*I site and a selectable marker for resistance to kanamycin in bacteria (Hamilton, 1997) (**Figure 1A**). The *gus*-intron gene cassette from the pCAMBIA 2301 plasmid was PCR amplified using specific primers and the *Pfu* DNA polymerase (Stratagene). After phosphorylation, the ∼2.7 kb amplified fragment was cloned into the unique *Swa*I site of pCH20 to obtain the pCH20-GUSi plasmid (20.8 kb) (**Figure 1B**). The catalase intron of the GUS marker gene blocks its expression in transformed *A. tumefaciens* ensuring that glucuronidase activity is possible only in eukaryotic cells. The CTV9R expression cassettes (with or without an intron in the ORF 1a) were *Not*I excised from the original BAC vectors (Ambrós et al., 2011) and ligated vía *Not*I to the pCH20-GUSi plasmid to yield the pCH20-GUSi-CTV vector. *E. coli* DH10B transformants of these plasmids were selected on LB plates with kanamycin (50 mg/l) and sucrose (5%), and purified plasmid used to transform *A. tumefaciens* strains by electroporation. The C58 (pTiC58) *A. tumefaciens* is an oncogenic wild-type strain from the nopaline group, and A281 (pTiBo542) is a transconjugant of C58 belonging to the L,L-succinamopine group (both maintained in the IVIA Collection of Plant Pathogenic Bacteria). Transformed cells of both strains were selected on Luria-Bertani medium (LB) containing rifampicin (25 mg/l) and carbenicillin (20 mg/l) for C58 or nalidixic acid (20 mg/l) for A281 strain. Plasmids transfected in these strains were additionally selected with kanamycin (50 mg/l).

STEM AND LEAF AGROINOCULATIONS

A. tumefaciens colonies of C58 and A281 strains harboring the pCH20-GUSi or pCH20-GUSi-CTV vector were grown overnight at 28°C in LB supplemented with appropriate antibiotics. After centrifugation, 20 µl of bacterial suspensions at OD600 1.0 were inoculated on the stems of the different plant hosts by wounding with a sterile scalpel in 3 (herbaceous hosts) or 5 (citrus) sites, and then protecting inoculation sites from desiccation with plastic wraps. Control plants were inoculated with water or *Agrobacterium* strains without binary vector. Tumor formation was monitored visually and expression analyses were performed at 3–5 weeks post-inoculation.

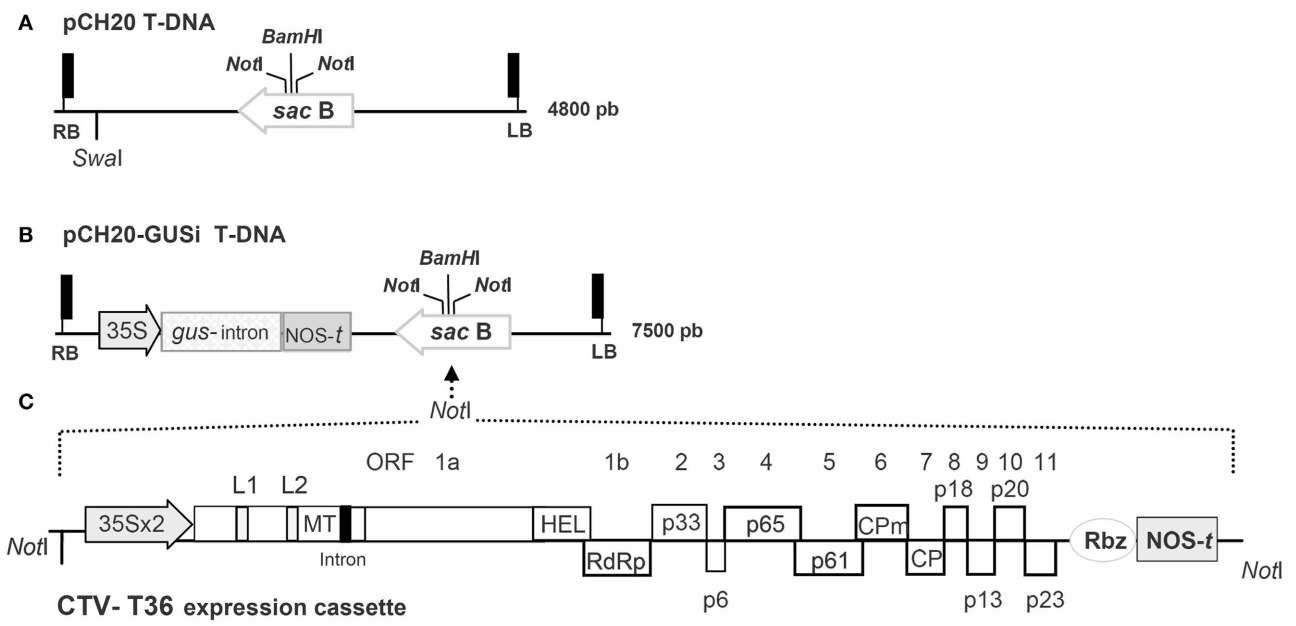

FIGURE 1 | Outline of the binary plasmids used in this study. (A) Detail of the T-DNA region in the pCH20 binary vector with its size indicated at the right. RB and LB are the right and left borders, the *Not*I restriction sites for subcloning, the unique *Swa*I site of the T-DNA and the unique *Bam*HI site present in the *sac*B gene are indicated. **(B)** Detail of the T-DNA region in the pCH20-GUSi binary vector. The cassette of the *gus*-intron selectable marker gene containing a catalase intron is shown. Shadowed boxes represent the 35S promoter of the *Cauliflower mosaic virus* (CaMV) and the nopaline synthase terminator (NOS-*t*) present in the cassette. Other details as in **(A)**. **(C)** Schematic representation and relative orientation of the CTV-T36 expression cassette containing the full-genome cDNA clone CTV9R from the agroinfectious BAC CTV-vector (Ambrós et al., 2011). Shadowed boxes represent the double enhanced 35S promoter (35S × 2), the *Hepatitis delta virus* ribozyme (Rbz) and the NOS-*t* present in the cassette. The genome organization of the CTV gRNA is denoted by boxes indicating the open reading frames (ORFs) and their corresponding translation products. L1 and L2 mean the two leader papain-like proteases; MT, methyltransferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase; CPm and CP, the minor and major coat proteins; intron, the *ST-LS1* intron incorporated in some CTV clones (Ambrós et al., 2011). The *Not*I sites flanking the construct are indicated and the strategy for subcloning into the T-DNA of pCH20-GUSi is marked with dotted arrow lines.

Agroinfiltration of NB or citrus leaves was performed as described (Ambrós et al., 2011), but in co-infiltration experiments, *A. tumefaciens* cultures harboring the candidate binary plasmids (empty or carrying the CTV genomic sequence) and those expressing the silencing suppressor p19 of *Tomato bushy stunt virus* (TBSV) (Voinnet et al., 2003) or p23 (CTV) (Lu et al., 2004) were mixed in a 2:1 ratio prior to infiltration.

GUS AND ELISA ASSAYS

Thin slices from the stem tumors induced by *Agrobacterium* C58 and A281 in tomato, *Nicotiana spp*. and Mexican lime plants were assayed individually for GUS activity at 4–6 (herbaceous hosts) or 6–8 (citrus) weeks after bacterial inoculations. GUS assays were performed by overnight incubation of tissue slices at 37°C with a 2 mM X-Gluc solution as described by Peña et al. (2004b).

CTV infection was monitored by a double-antibody sandwich-ELISA protocol using monoclonal antibodies 3DF1 and 3CA5 (Vela et al., 1986).

RNA AND DNA EXTRACT PREPARATION

Total RNA extracts (RNAt) were prepared from citrus, NB or *N. occidentalis* leaves or stem tumors, ground to powder with liquid nitrogen following a standard protocol (Ruiz-Ruiz et al., 2007) and then treated with RNase-free DNase (Ambion) before using it as template for RT-PCR techniques. Extracts enriched in double-stranded RNA (dsRNA) were obtained from CTV-infected citrus bark as reported previously (Moreno et al., 1990).

Genomic DNA (gDNA) extracts were prepared from a tissue pool including two slices from individual tumours in each plant according to Llop et al. (1999).

PCR, RT-PCR, AND QRT-PCR REACTIONS

Conventional PCR reactions to monitor transformation events were performed using gDNA extracts from tomato or citrus tumor tissues and four sets of primers: the primer set PM111 (5′-ATGACGCACAATCCCACTATCCTTCGC-3′)/PM250 (5′-GAG TGACCGCATCGAAACGCAGC-3′) amplifies a 581-bp fragment of the GUS-intron cassette; the set PM175 (5′-GCAGTCTCAG AACGAGGTGGC-3′)/BB2-5′ (5′-GGAAGGAGCTGACTGGGT TGAAGGC-3′) amplifies a 1063-bp fragment spanning the p23 ORF of CTV and the 5′ BAC polylinker; the set PM214b (5′-TTT CTGGGCGAACAGGTTGAAT-3′)/BB2-3′ (5′-GAAGACATACA TGACAAAAACGCTAGACGGC-3′) amplifies a 1,5-kb fragment including the 32 first nucleotides of the CTV 5′UTR and the 3′ BAC polylinker; and the set PM118/PM119 flanking the intron insertion point in the CTV ORF 1a amplifies a 622-bp fragment (intron-containing constructs) or a 433-bp fragment (intron-less templates). PCR conditions were essentially as reported (Ruiz-Ruiz et al., 2006).

Reverse transcription (RT) followed by PCR amplification to detect CTV gRNA was performed with primers PM118–PM119 and 1–2 μg DNase-treated RNAt as described (Ruiz-Ruiz et al., 2006; Ambrós et al., 2011). Control reactions included the absence of reverse transcriptase for each sample and negative reactions using water instead of RNAt, or RNAt from healthy plants or from plants agroinoculated with an empty binary vector. Positive controls were run by using RNAt or dsRNA-rich extracts from CTV-infected citrus plants, or PCR amplification of plasmid DNA containing CTV cDNA with or without the intron.

Quantitative assays (qRT-PCR) and estimations of the absolute number of T36 gRNA copies/ng of RNAt were performed as reported (Ruiz-Ruiz et al., 2007), including similar positive and negative controls as in conventional RT-PCR reactions.

TRANSFECTION OF NB MESOPHYLL PROTOPLASTS AND NORTHERN BLOT ANALYSIS

Isolation of mesophyll protoplasts from *N. benthamiana* leaves and transfection mediated by polyethylene glycol was as reported (Navas-Castillo et al., 1997; Satyanarayana et al., 1999). Virions from CTV isolates T36, T385, T305, and T318A were used for transfections. Protoplasts were harvested at 1–5 days post inoculation (dpi) and used to obtain RNAt extracts to analyze viral progeny (gRNA and sgRNAs) accumulation by Northern blot hybridization (Satyanarayana et al., 1999) with a digoxigenin-labeled riboprobe specific for the 3′ terminal region of the T318A gRNA.

MECHANICAL AND GRAFT INOCULATION OF NB PLANTS

Mechanical inoculation of young NB plants (∼1.5 months old) were performed by rubbing on the surface of three carborundum-dusted leaves 20–40 μl of virion extract from a sucrose gradient or crude sap extract from an infected plant. For graft transmission, symptomatic young shoots, or leaf petioles were excised from CTV-infected NB plants and used for V-shaped side-grafting on the stem of adult receptor plants. The grafts were protected with parafilm which was removed after 8–10 days. Before mechanical or side-graft inoculation, 3–4 fully expanded leaves of each receptor plant were agroinfiltrated with binary plasmids expressing a silencing suppressor protein as reported (Ambrós et al., 2011), with bacteria concentration being adjusted to 0.2 OD_{600}.

INDEXING IN CITRUS INDICATOR PLANTS

Infectivity bioassays of CTV virions from systemically infected leaves of agroinoculated NB plants were performed by slash-inoculation on four alemow plants (Garnsey et al., 1977). Inoculum consisted of virions purified in a sucrose gradient (Satyanarayana et al., 2001) or crude sap extracts, as indicated. Controls consisted of a similar number of indicator plants inoculated with virion extracts from citrus plants infected with the 947R CTV isolate. CTV infection of new leaves was detected at 1–2 months post inoculation (mpi) by ELISA and by symptom observation.

RESULTS

AGROINOCULATION OF CTV WITH ONCOGENIC *A. tumefaciens* STRAINS PRODUCES VIRUS-INFECTED TUMOURS AND SYSTEMIC INFECTION IN NB BUT NOT IN MEXICAN LIME

In an attempt to simplify the newly developed genetic system for CTV (Ambrós et al., 2011) we tried direct agroinoculation of citrus with C58 and A281, two oncogenic strains of *A. tumefaciens* that produce good tumours in citrus plants, transfected with a suitable binary vector carrying the cDNA of CTV-T36 isolate (Satyanarayana et al., 2001; Ambrós et al., 2011) and an appropriate plant selectable marker to monitor cell transformation.

For this purpose we developed the plasmid pCH20-GUSi, with a *gus*-intron marker gene (**Figures 1A,B**) that ensures that positive GUS expression was derived from transformed plant cells and not from residual bacteria, and then the vector pCH20-GUSi-CTV containing the expression cassette of CTV-T36 (**Figure 1C**). The functionality of this latter vector was first tested in *N. benthamiana* using the non-oncogenic *A. tumefaciens* strain COR308 and the ratio of CTV systemically infected plants obtained was similar to that reported previously (Ambrós et al., 2011 and data not shown). Suspensions of *A. tumefaciens* C58 and A281 harboring the pCH20-GUSi-CTV or pCH20-GUSi were then agroinoculated on the stems of several plant species, some susceptible to CTV infection (citrus and NB), and others (*N. occidentalis* and tomato) that are natural hosts for *A. tumefaciens* but not CTV hosts. Tomato plants were a positive control for the ability of both oncogenic strains to induce tumours (essentially 100% of the inoculation points) and a virulence phenotype, with A281 inciting more necrosis than C58 (data not shown). Similar results were observed in NB and *N. occidentalis* (**Figure 2A**, left panel). In Mexican lime, tumor formation frequency with both strains was also about 85%, but A281 elicited larger tumours than C58 and these appeared earlier (**Figure 2A**, right panel).

Analysis of GUS activity in tumor tissues revealed significant differences between A281 and C58 strains in tomato (∼90% vs. 60% of GUS expressing tumours), whereas these percentages were similar for both strains in Mexican lime (∼80%) (**Table 1**, **Figure 2B**). However, C58 induced more than twice the number of high-GUS-activity cells incited by A281 in lime tumours (**Figure 2B**, right panel), indicating higher frequency of independent transformation events. In NB C58 also induced over 90% of tumours with a high number of GUS-positive cells (**Table 1**).

The efficiency of T-DNA integration in tumor cells was assessed by PCR detection of different target regions of the T-DNA insert using gDNA from tumours as template. In tomato, PCR assays detected three different T-DNA regions including the *gus*-gene and the CTV cDNA cassette in about 40% of the GUS positive tumours developed by both *Agrobacterium* strains. In Mexican lime, PCR amplified at least two of these T-DNA targets from 80 % of the tumor samples, and the four T-DNA regions including three of the CTV cDNA (**Figure 2C**), from 50% of the tumours. These results suggest that integration of the full T-DNA in the nucleus of lime cells by both oncogenic strains is relatively frequent.

Transient expression of the CTV cDNA was monitored by detecting the coat protein (ELISA) or the viral RNA (RT-PCR and qRT-PCR) in tumor tissues. While no positive CTV detection was ever observed in tomato tumours and only a faint amplification by qRT-PCR (with about 10^2 CTV RNA copies/ng RNAt) was detected in some *N. occidentalis* galls, in NB most tumours became CTV infected as confirmed by positive ELISA readings, and the ratio of plants containing some CTV-infected tumor at 2 mpi was about 80% (**Table 1**). Accumulation of CTV gRNA in tumor tissues was very variable among plants of the same experiment and between assays, ranging from $\sim 10^2$ to 10^5 copies of CTV gRNA/ng RNAt (**Figures 3BI,II**). CTV expression was never observed in Mexican lime tumours, even when plants were co-inoculated with a binary vector expressing the p19 silencing suppressor of TBSV (Voinnet et al., 2003), suggesting either a strong plant silencing reaction against CTV or a failure to produce functional RNA transcripts in the lime tumor cells.

About 23% of the NB plants agroinoculated with the C58 strain transfected with pCH20-GUSi-CTV (with or without an intron in the CTV cDNA), became systemically infected by CTV at 2–3 mpi (**Table 1**), as confirmed by ELISA, qRT-PCR in upper leaves and by expression of specific symptoms. The number of transformation events obtained with C58 in NB seemed to increase when co-inoculated with a binary vector expressing the p19 silencing suppressor, as revealed by the slightly higher GUS activity and CTV systemic infection rate (**Table 1**). However, no association was observed between the presence of tumours with high CTV accumulation as detected by ELISA or qRT-PCR and systemic infection of the corresponding plant (**Figures 3BI,II**). Moreover, some plants with CTV-infected primary tumours developed new leaves beside the agroinoculation sites (**Figure 3A**) and these secondary tumorigenic tissues showed high CTV accumulation as confirmed by ELISA and q-RT-PCR (**Figure 3B**I, right panel), but systemic CTV infection did not occur.

CTV distribution as monitored by tissue-print-ELISA and symptom expression in systemically infected NB plants (**Figures 4A,B**) were similar to those reported using agroinfiltration with disarmed *Agrobacterium* strains (Ambrós et al., 2011). Viral titer in upper leaves was also variable among plants and experiments and generally ranged from $\sim 10^4$ to 10^5 CTV gRNA copies/ng RNAt (**Figure 4C**), albeit in some plants we observed a 2–60-fold excess of CTV gRNA in comparison with values obtained in plants agroinfiltrated with disarmed strains (**Figures 4C,D**, and Ambrós et al., 2011). Indeed when crude sap extracts from those plants were used to mechanically inoculate alemow plants, 42% of them (3/7) became infected. As expected, inoculation with purified virion preparations from the same NB plants resulted in 100% (4/4) of citrus plants infected at 1 mpi and they displayed the symptoms characteristic of the wild T36 isolate.

Systemic CTV infection was never observed in other agroinoculated hosts, as expected from low or no CTV expression in their tumours. Moreover, agroinfiltration of Etrog citron (*C. medica* L.), alemow or Mexican lime leaves with the C58 and A281 oncogenic strains, albeit inducing functional leaf tumours, did not result either in systemic infection and only a faint qRT-PCR amplification of CTV targets was occasionally observed at 14–28 dpi, suggesting restricted infections that never progressed.

PRE-TREATMENT WITH A SILENCING SUPPRESSOR ENABLED SYSTEMIC INFECTION OF NB PLANTS AFTER MECHANICAL INOCULATION WITH CTV VIRIONS

Previous attempts to mechanically transmit CTV from citrus to herbaceous or non-rutaceous woody species, including NB, were unsuccessful (Müller and Garnsey, 1984 and our unpublished results). Since experimental aphid transmission of CTV to some *Passiflora* species has been reported (Müller et al., 1974; Roistacher and Bar-Joseph, 1987), we tried to aphid transmit CTV-T36 to healthy NB plants using *A. gosypii* (>100 individuals)

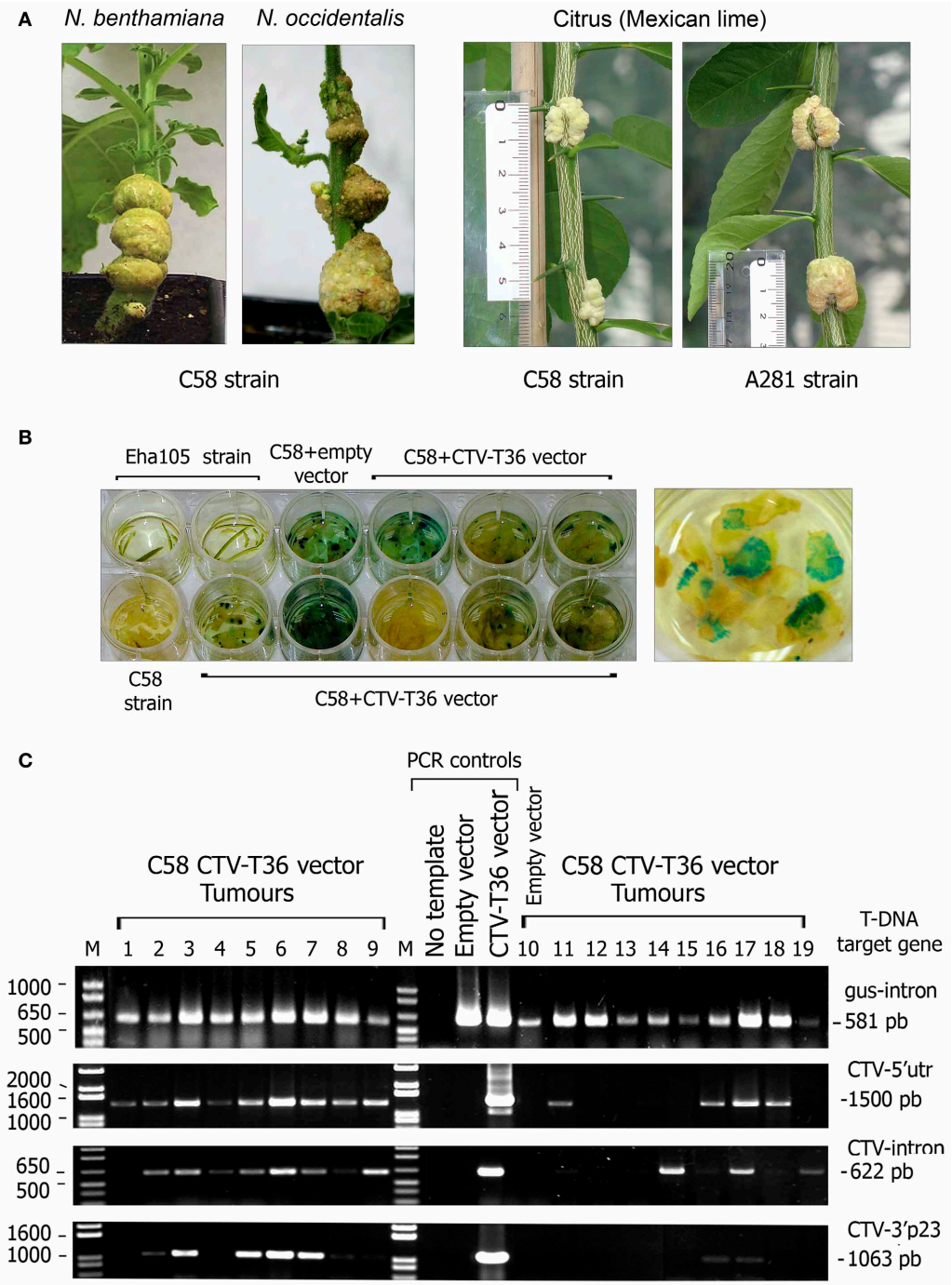

FIGURE 2 | Tumours incited by oncogenic *A. tumefaciens* strains in herbaceous and citrus hosts. (A) Left panel, tumours induced by *A. tumefaciens* C58 strain in *Nicotiana* species at 4 weeks post inoculation (wpi) (NB) or 5 wpi (*N. occidentalis*). Right panel, tumours induced by *A. tumefaciens* C58 and A281 strains in Mexican lime at 7 wpi. The rule on the left indicates the size of individual tumours. **(B)** GUS activity assay of stem tumours from individual lime plants agroinoculated with *A. tumefaciens* C58. Each dish contains slices from individual tumours from plants agroinoculated with C58 harboring the pCH20-GUSi (empty vector) or the pCH20-GUSi-CTV (CTV-T36 vector) plasmids and incubated with X-Gluc solution at 8 wpi. Controls consisted of lime bark tissue agroinoculated with the non-oncogenic *A. tumefaciens* Eha105 strain or with the C58 strain without plasmid. A detail of tumor slices showing many blue spots of high-GUS activity is presented on the right. **(C)** Confirmation of partial T-DNA integrations in the cell nucleus of agroinfected lime plants. PCR amplification products obtained from gDNA extracts of tumours incited by *A. tumefaciens* C58 harboring the pCH20-GUSi-CTV or the pCH20-GUSi vectors (line 10). Controls: PCR amplification products from pCH20-GUSi-CTV (containing an intron in the CTV ORF 1a) and pCH20-GUSi plasmids, or from distilled water. The T-DNA target regions amplified (from the right to the left border as indicated in **Figure 1**) and the size of the DNA fragments are indicated at the right. Each lane corresponds to an individual tumor sample from different plants in the same experiment and DNA bands under the same number in different gels correspond to the amplification products obtained using different primers and the same gDNA (obtained from two slices of the same tumor). M, 1 Kb Plus DNA marker (Invitrogen, Fisher), with relevant sizes of DNA fragments indicated at the left.

Table 1 | Efficiency of tumor and systemic CTV infections in *N. benthamiana, N. occidentalis,* tomato cv. Roma (*L. sculentum L.*) and Mexican lime plants stem agroinoculated with oncogenic *A. tumefaciens* strains harboring the pCH20-GUSi empty vector or the pCH20-GUSi-CTV vector carrying the CTV expression cassette (CTV9R).

			Source		
			Tumors		Plants
Plant host	**Vector**	**Strain[a]**	**GUS[b] activity**	**Infectivity[c]**	**Infectivity[d]**
N. benthamiana	CTV9R	C58	35/37	25/37	4/37
	Empty	C58	9/10	0/10	0/10
	CTV9R+p19[e]	C58	7/7	6/7	3/7
	Empty+p19[e]	C58	2/2	0/2	0/2
N. occidentalis	CTV9R	C58	6/7	0/7	0/7
	Empty	C58	2/2	0/2	0/2
Tomato	CTV9R	C58	19/36	0/19	0/19
		A281	14/15	0/7	0/7
	Empty	C58	6/8	0/5	0/5
		A281	9/9	0/3	0/3
	–	C58	0/3	0/3	0/3
	–	A281	0/3	0/3	0/3
Citrus	CTV9R	C58	118/138	0/44	0/44
	CTV9R	A281	72/90	0/31	0/31
	Empty	C58	2/3	0/3	0/4
		A281	3/4	0/4	0/4
	–	C58	0/2	0/2	0/2
	–	A281	0/2	0/2	0/2
	–	Eha105	–	–	0/2

[a] *Oncogenic (C58 and A281) and disarmed (Eha105) A. tumefaciens strains used for stem agroinoculation.*

[b] *No. of N. benthamiana (NB) or N. occidentalis plants with GUS positive tumours/No. of plants with tumours. In tomato and citrus plants the values represent the No. of individual tumours with GUS activity/No. of tumours assayed (obtained from 3–4 independent bioassays).*

[c] *No. of plants with CTV infected tumours detected by ELISA at 2 mpi or later/No. of plants with tumours.*

[d] *No. of CTV systemically infected plants/No. of stem agroinoculated plants. Infection in upper leaves was detected by ELISA at 2–3 mpi or later.*

[e] *Co-infiltration with a vector expressing the p19 silencing suppressor protein of TBSV.*

–Indicates agroinoculations with bacteria strains carrying no vectors, absence of GUS activity or absence of infectivity.

fed for 24 h on infected citrus or NB leaves. Although CTV was detected in some aphids by qRT-PCR no receptor plant was infected.

We then examined other inoculation procedures that could enable testing infectivity of different CTV genotypes in NB without using protoplasts or aphids. For this purpose leaves of young NB plants were mechanically inoculated with crude sap from systemically infected (T36 strain) NB leaves or with purified CTV virions from these plants or from CTV-infected citrus bark (**Table 2**). Since all transmission trials were unsuccessful, bioassays were repeated agroinfiltrating the receptor plants with a vector expressing the p19 silencing suppressor (TBSV) 3 days before mechanical inoculation. Unexpectedly, using this pretreatment we were able to mechanically transmit for the first time CTV virions to NB (**Table 2**). Although transmission efficiency was variable among bioassays, an average of 27–39% infected plants was obtained, with vein clearing and stunting symptoms being indistinguishable from those previously observed in this host (Ambrós et al., 2011), albeit they appeared at 3–4 mpi (a 2–3 months delay in comparison with the leaf agroinfiltration procedure).

Since not all CTV genotypes are able to successfully replicate in NB (Navas-Castillo et al., 1997; Satyanarayana et al., 2000) we first assayed virion infectivity of T318A and T305, two Spanish isolates inducing stem pitting in grapefruit and sweet orange, in NB protoplasts in comparison with T36 and T385 virions, used as positive and negative controls, respectively. Northern blot analysis of protoplasts transfected with T318A virions showed viral progeny accumulation at 2 dpi with the viral gRNA and sgRNAs being readily visible at 3 dpi (**Figure 5**, left panel). Generally, viral RNA accumulation of this isolate was similar to that of the CTV-T36 control, and higher than that displayed by T305. Moreover, accumulation of T318A RNAs increased up to 5 dpi in surviving protoplasts and their hybridization signal remained as intense as

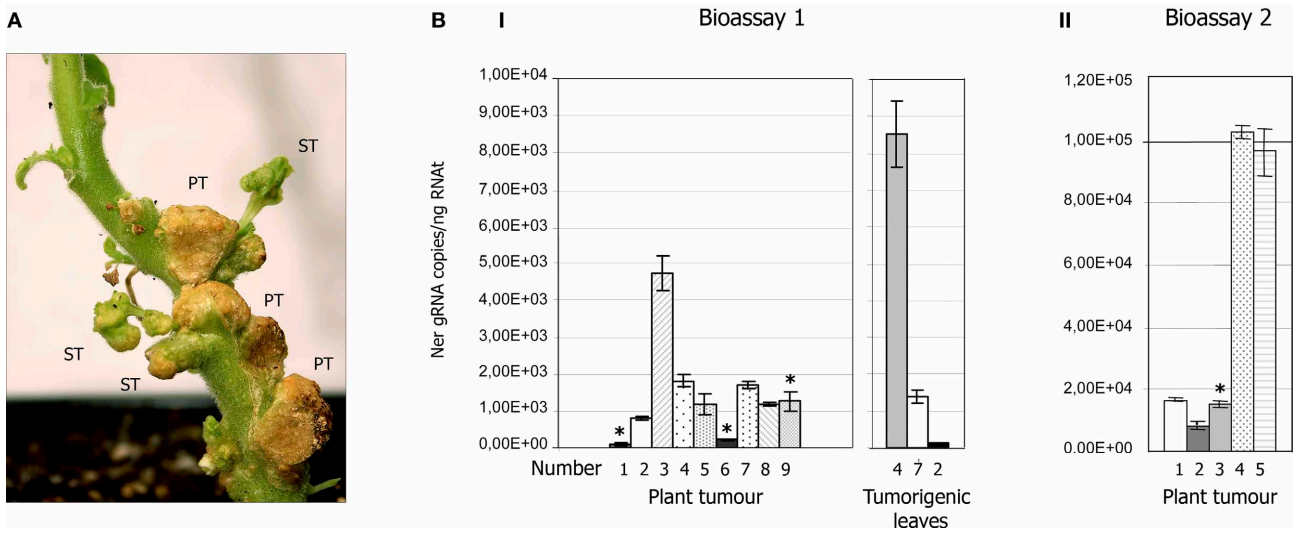

FIGURE 3 | CTV accumulation in tumours of NB agroinoculated with *A. tumefaciens* C58 harboring the pCH20-GUSi-CTV vector. (A) Detail of tumor tissues induced by C58 in the stem of NB at 7 wpi showing three primary tumours (PT) and some tumorigenic leaves (ST, secondary tumours) that developed later beside the agroinoculation sites. **(B)** CTV accumulation in tumor tissues (or tumorigenic leaves) of NB plants agroinoculated with the C58 strain estimated by qRT-PCR (Ruiz-Ruiz et al., 2007) and expressed as number of CTV gRNA copies per ng of RNAt. Means and standard deviations were obtained from four technical replicates and two independent bioassays. Panels I and II show CTV accumulation in tumours of individual plants in bioassays 1 and 2, respectively. Asterisks above some bars indicate plants of these bioassays that became systemically infected by CTV. Right graphic in panel I shows the viral titer in tumorigenic leaves produced in three plants of bioassay 1.

that of CTV-T36, whereas this signal was very weak for T305, and no signal was observed for T385 (**Figure 5**, right panel). Although T318A virions successfully replicated in NB protoplasts, mechanical inoculation of these virions on NB plants, with or without an agroinfiltration pre-treatment with the p19 silencing suppressor, failed to cause systemic infection of those plants (**Table 2**).

Overall, these results indicate that the interaction between CTV-T36 and NB must be genotype-specific.

CTV CAN BE GRAFT-TRANSMITTED FROM INFECTED TO HEALTHY NB PLANTS

To maintain wild or mutant CTV genotypes in NB without starting periodically a new agroinfection process we tried to develop a system to graft-transmit CTV from systemically infected to healthy NB plants. For this purpose we assayed different types of tissues from the donor plant putting two side-grafts per receptor plant and protecting them to avoid desiccation. Suculent petioles or short young shoots (**Figure 6**) from CTV-infected NB plants were the best inoculum source since larger shoots or stems showed reduced survival. Around 40% graft survival was observed at the end of the first month, with surviving grafts showing CTV symptoms or fluorescence when a *gfp*-tagged virus (Tatineni et al., 2008) was used (**Figure 6**). In spite of inoculum survival, essentially no CTV transmission was observed in receptor NB plants without pre-treatment with a silencing suppressor, except for a single plant out of the 70 inoculated in several experiments (**Table 3**). In contrast, agroinfiltration with binary plasmids expressing the p19 (TBSV) or the p23 (CTV) silencing suppressors 3 or 6 days prior to graft inoculation resulted in 50 or 28% average transmission rates, respectively, at 2 mpi. The rate of infected plants still increased at 4 mpi for plants pre-treated with p19.

Symptoms of infected plants were similar to those reported before, but they appeared earlier than in plants inoculated mechanically, since vascular connections between the graft and the receptor plant likely allowed direct loading of CTV virions into the phloem tubes.

DISCUSSION

A new genetic system based on agroinfiltration of NB leaves with disarmed *Agrobacterium* cultures transfected with binary vectors, carrying the CTV-T36 cDNA, was recently developed (Ambrós et al., 2011). Although this system was easier, faster and more reliable than the former protoplast system (Satyanarayana et al., 1999, 2001), it still showed limitations that we tried to overcome in this work. The most obvious was, why can we not directly agroinoculate citrus plants? *A. tumefaciens* has been widely used for efficient delivery of viral genomes into different plants including a citrus-infecting virus and several phloem-limited viruses some of which belong to the *Closteroviridae* family (Grimsley et al., 1986; Prokhnevsky et al., 2002; Chiba et al., 2006; Vives et al., 2008; Liu et al., 2009; Wang et al., 2009). Although transient or stable transformation of citrus species has also been demonstrated (Cervera et al., 1998; Tzfira et al., 2004; reviewed in Peña et al., 2004a), previous trials to agroinfect citrus plants with CTV using different binary plasmids, silencing suppressors and disarmed *Agrobacterium* strains were unsuccessful (Gowda et al., 2005, and our unpublished data). Infiltration of citrus leaves with *Agrobacterium* is usually very inefficient for bacterial penetration and usually leads to low level of T-DNA expression, as

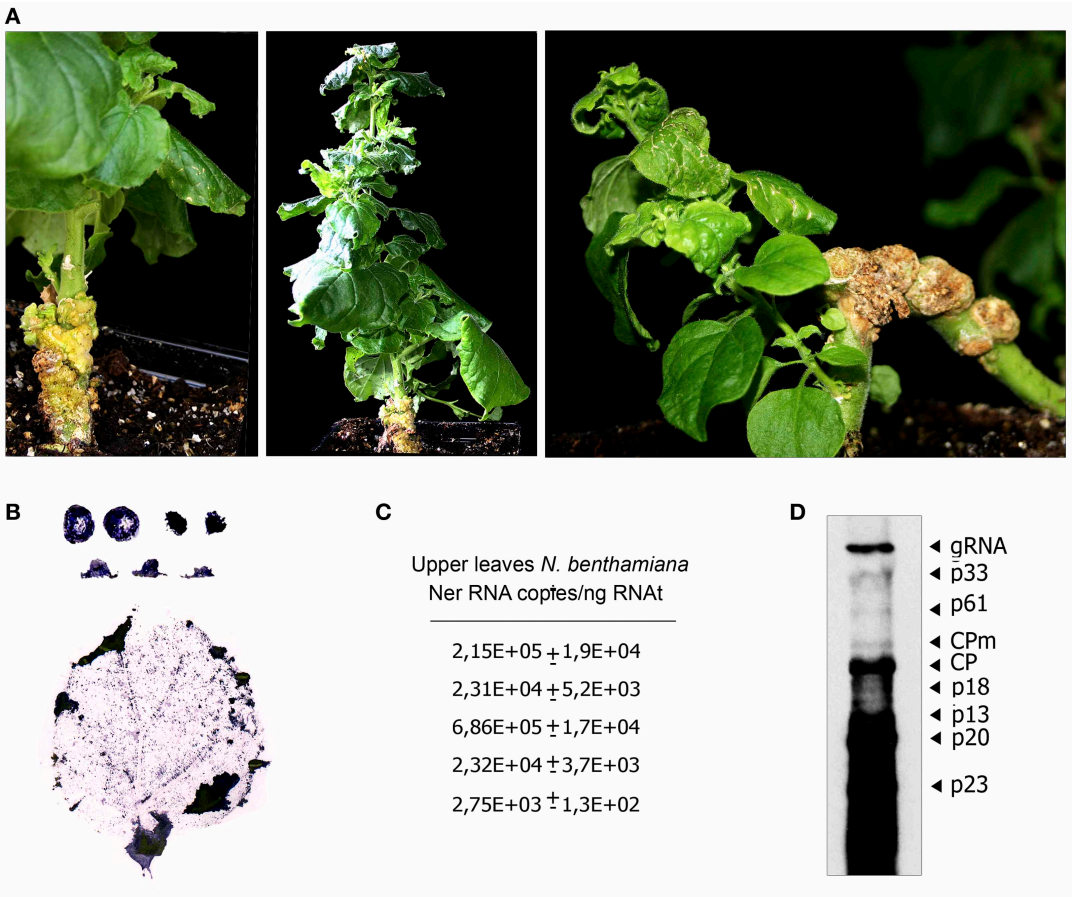

FIGURE 4 | Symptom expression and CTV distribution in NB plants systemically infected after agroinoculation with *A. tumefaciens* C58 harboring the pCH20-GUSi-CTV plasmid. (A) Detail of symptoms induced by CTV in agroinfected *N. benthamiana*. Left, stem tumours and vein clearing symptoms in a new young lateral shoot (right) at 7 wpi. Middle, epinasty in NB at 8 wpi. Right, new shoot in an old systemically infected NB (10 wpi) showing stunting, vein clearing and crinkly leaf. **(B)** Relative accumulation and distribution of CTV in shoots (first line), petioles (second line) and upper leaves of systemically infected NB. **(C)** Absolute accumulation of CTV in upper leaves from different NB plants estimated by qRT-PCR (Ruiz-Ruiz et al., 2007) as in **Figure 3**. **(D)** Northern blot analysis of RNAt (4 μg) extracted at 10 wpi from the upper leaves of a CTV-infected NB plant. Positions of the CTV genomic (gRNA) and subgenomic RNAs (sgRNAs) are indicated by arrows on the right. The blot was hybridized with a digoxigenin-labeled riboprobe specific for the 3′-terminal region of the CTV-T36 gRNA.

Table 2 | Transmission of CTV by mechanical inoculation of NB healthy plants with or without pre-treatment with a silencing suppressor.

CTV isolate	Inoculum source	Type of inoculum[a]	Suppressor treatment[b]	Infectivity[c]
T36	Infected NB upper leaves[d]	Crude sap	-	0/6, 0/6
	Infected NB upper leaves	Virions	-	0/6, 0/6
	Infected citrus bark	Virions	-	0/6
	Infected NB upper leaves	Crude sap	p19	0/6, 0/6
	Infected NB upper leaves	Virions	p19	2/3, 0/3, 2/4
	Infected citrus bark	Virions	p19	2/4, 1/3, 0/3
T318A	Infected citrus bark	Virions	-	0/6
	Infected citrus bark	Virions	p19	0/3, 0/3

[a] *Crude sap extracts or gradient purified virions.*

[b] *Pre-infiltration with a vector expressing the p19 silencing suppressor protein of TBSV.*

[c] *No. of infected NB plants/No. of inoculated plants at 2–3 mpi or later in independent bioassays.*

[d] *Upper leaves from systemically infected NB plants.*

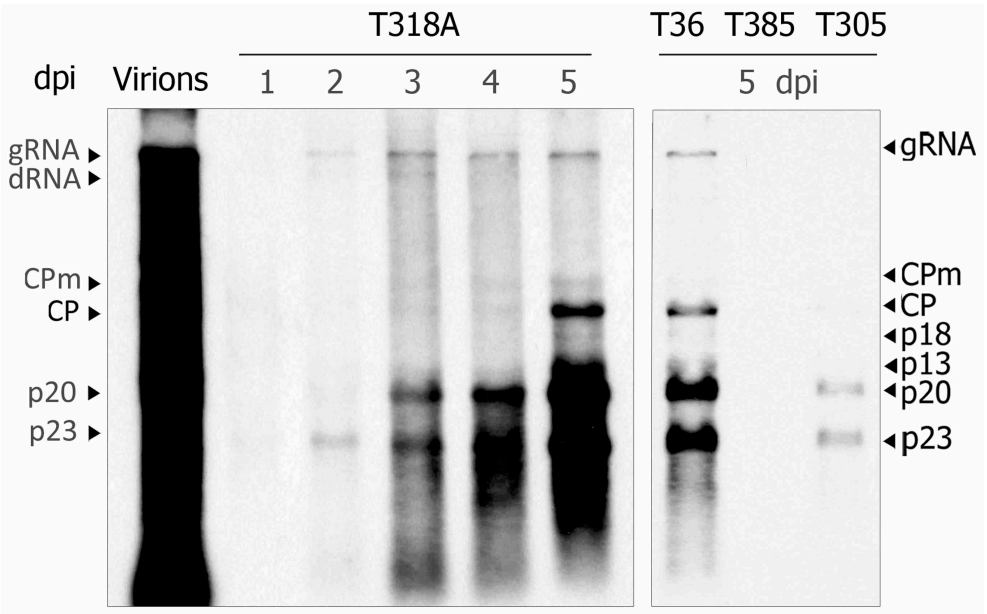

FIGURE 5 | Northern blot analysis of RNAt from NB mesophyll protoplasts transfected with purified virions from citrus plants infected with the CTV isolate T318A at 1 through 5 days post-inoculation (dpi) (left panel), and the T36, T385, and T305 isolates at 5 dpi (right panel). Left lane, virion extract used to transfect protoplasts. Positions of the CTV gRNA and sgRNAs are indicated by arrows at both sides. A large dRNA present in T318A and its relative position is shown on the left. The blot was hybridized with a digoxigenin-labeled riboprobe specific for the 3′-terminal region of the CTV-T318A gRNA.

FIGURE 6 | CTV systemic infection of NB plants after side-graft inoculation. Left, detail of side-graft inoculation of NB plants using CTV infected petioles (upper panel) or young shoots with vein clearing symptoms (lower panel). Right, severe stunting and crinkly leaf in a NB plant systemically infected (7 wpi); one surviving graft shows CTV symptoms.

Table 3 | Side-graft transmission of CTV from infected to healthy NB plants with or without pre-treatment with a silencing suppressor.

Silencing suppressor[a]	Pre-treatment day[b]	Infectivity[c]	%[d]
-	-	0/17, 0/13, 0/21, 1/19	<2%
p23	D3	2/9	22%
p23	D6	3/9	33%
p19	D3	8/9, 3/5, 3/5	70%
p19	D6	5/9, 8/17	52%

[a] *Pre-infiltration with a vector expressing the p19 silencing suppressor protein of TBSV or the p23 silencing suppressor protein of CTV.*

[b] *Pre-infiltration of leaves of the receptor plants was performed 3 or 6 days prior to graft inoculations.*

[c] *No. of infected NB plants/No. of inoculated plants in independent bioassays at 4–6 mpi.*

[d] *Average transmission rate.*

observed with other recalcitrant species (Wroblewski et al., 2005). However, the ability of wild oncogenic strains to incite tumours on some citrus species proved very efficient for citrus transformation (Cervera et al., 1998). Here the virulent strain C58 was as efficient as the supervirulent strain A281 inducing tumor formation in Mexican lime plants, albeit tumours induced by the latter grew earlier, were larger in size and showed a necrotic phenotype, in agreement with the supervirulence reported by Cervera et al. (1998). Both oncogenic strains, carrying the empty or the CTV expressing vector, produced good vascularized tumours and

high GUS transient expression in NB and lime, with C58 giving better results than A281 in the latter host species. Using a *gus*-intron marker gene engineered in the T-DNA of the binary vector guaranteed that GUS expression occurred only *in planta* and, therefore, it was indicative of cell transformation. Most tumours incited by C58 in NB became CTV infected at 1–2 mpi, as detected by ELISA and qRT-PCR assays, and 23% of the plants became systemically infected by CTV at 2–3 mpi, as expected from the high vascularization of these tumours. Remarkably, systemic infection of these plants was not associated with highest CTV titers in tumours. Moreover, some plants developed secondary tumorigenic tissues with high CTV titer, but systemic infection of these plants never occurred, further supporting our previous suggestion that systemic infection of NB results from CTV invasion of the vascular system and not from *Agrobacterium* migration within agroinfiltrated plants (Cubero et al., 2006; Ambrós et al., 2011). Contrasting with NB, essentially no CTV coat protein or viral RNA was detected in *N. occidentalis* tumours in spite of the high GUS expression observed, suggesting limited virus replication if any in cells of this host, in agreement with the lack of CTV replication observed previously in protoplasts of this species (Navas-Castillo et al., 1997; Satyanarayana et al., 2000). Neither CTV coat protein nor viral RNA was detected in Mexican lime tumours in spite of the high proportion of cells showing intense GUS activity, indicative of total or partial T-DNA integration. Moreover, PCR detection of different T-DNA regions in individual tumours suggested that integration of the full CTV cDNA in the Mexican lime cells occurs at an effective rate. Therefore, failure to detect CTV infection in lime tumours does not seem to depend on the oncogenic strain, tumor formation or cell transformation efficiency. Indeed co-agroinoculation with a binary plasmid expressing the p19 silencing suppressor (Voinnet et al., 2003) slightly increased GUS activity and the CTV systemic infection rate in NB tumours, but it had no effect in lime tumours. Since Mexican lime is known to be highly susceptible to CTV infection, our results suggest a strong silencing response against CTV at the very early steps of infection, or more likely, failure to get enough functional RNA transcripts reaching the cytoplasm of transformed lime cells. Moreover, alemow and Mexican lime leaves pre-infiltrated with a silencing suppressor and then with the vector carrying CTV-T36 developed tumours, but only trace amounts of CTV gRNA could be detected by qRT-PCR in some of them at 14–28 dpi, suggesting occasional restricted infections that never progressed. These results indicate that a CTV genetic system based on direct agroinoculation of citrus hosts presently is unworkable and that, at least in the near future, the use of NB as an intermediate host to produce CTV virions will be necessary.

Since the present genetic system relies on the ability of CTV-36 to replicate in NB cells and to eventually move cell-to-cell and long distance, developing a similar system with new CTV genotypes requires testing previously the ability of their virions to replicate in NB cells. Previous observations indicated that not all CTV genotypes can replicate in NB protoplasts. Here we confirmed that while isolate T318A (Ruiz-Ruiz et al., 2006) replicated and accumulated in protoplasts to the same extent as CTV-T36, the isolate T305 replicated at low level and the isolate T385 did not replicate at all. Phylogenetic comparison of the full-genome sequence of different CTV isolates has revealed that these belong to at least six different strains, with most genetic differences being located in the 5′ moiety of the gRNA (Harper, 2013). Differences in the replicase components may affect their interactions with host factors and thus determine the ability of each strain to replicate in NB protoplasts. To avoid the need for protoplast preparation we tried to mechanically inoculate NB plants by rubbing leaves with CTV-T36 and T318A virion extracts from citrus. While no infection was observed in plants without pre-treatment with a silencing suppressor, up to 39% infection rate was achieved in pre-treated plants mechanically inoculated with CTV-T36 virions. This is the first time that CTV is mechanically transmitted to a new host by leaf rubbing. Although co-infiltration of NB plants with a silencing suppressor was not essential for CTV agroinfection with disarmed *A. tumefaciens* strains, in line with results reported for the crinivirus *Lettuce infectious yellowing* (LIYV) (Wang et al., 2009), silencing suppressors expedited systemic infection and often increased infectivity (Ambrós et al., 2011). This finding and the need for a silencing-suppressed receptor plant to achieve infection in mechanically inoculated plants underlines the importance of the antiviral silencing reaction in the early stages of CTV infection. Unfortunately no infection was obtained in pre-treated plants inoculated with T318A virions, even though this genotype replicated like CTV-T36 in NB protoplasts, suggesting that viral factors other than the replicase interact differently with host factors in T36 and in T318 genotypes. Previous studies have shown that resistance to CTV infection is often strain dependent (Yoshida, 1985, 1993, 1996; Garnsey et al., 1987; Gmitter et al., 1996; Mestre et al., 1997a,b,c). Furthermore, this resistance was often due to inability of the virus to move cell-to-cell or long distance, since CTV replicated and accumulated in protoplasts of resistant citrus varieties to the same extent as in susceptible varieties, and normal infectious virions were produced (Albiach-Martí et al., 2004). It is possible that, although the CTV T318A gRNA efficiently replicates in *N. benthamiana* protoplasts and likely in cells, virions may not be correctly assembled, or virion proteins may not interact properly with host factors, thus impairing virus movement. These results are a major concern for the actual possibilities to use the present genetic system with CTV genotypes other than CTV-T36. Additional CTV isolates replicating in NB protoplasts should be tested for infectivity on NB plants to ascertain if systemic infection of this species is T36-specific or it can be achieved by other virus genotypes. In any case, availability of CTV genotypes capable to produce systemic infection of this host species, others able to replicate but not to spread systemically, and still others unable to replicate, may be helpful to dissect the CTV-NB interactions at the genetic level.

A final limitation of the new genetic system based on agroinoculation of NB plants was the need for new plant agroinoculation cycles to maintain in this host the CTV-T36 or other interesting hybrid constructs that could be produced. This limitation was overcome by developing an efficient graft inoculation system to transmit CTV from infected to healthy NB plants. Pre-treatment of the receptor plants by agroinfiltrating a silencing suppressor, proper selection of the inoculum for long-term survival and

incubation conditions to avoid inoculum desiccation were critical factors that increased the transmission rates. Although side grafts allow direct contact of the donor and the receptor phloem tissues, pre-treatment with a silencing suppressor raised transmission rate from less than 2% to about 50% at 3 mpi, underlining again the importance of antiviral silencing defense of the plant in the early stages of CTV infection. Several reports have documented that virus loading into the vascular system is a complex process involving a strong bottleneck for the virus population (Ding et al., 1995; Gilbertson and Lucas, 1996; Wintermantel et al., 1997; Cruz, 1999; Li and Roossinck, 2004; Ali and Roossinck, 2010). Reduction in the effective population size and virus silencing by the new host might explain in part the long delay necessary for systemic infection. Finding that higher infectivity was obtained pre-treating with p19, a silencing suppressor acting at cellular and systemic levels (Voinnet et al., 2003), than with p23, a CTV-encoded suppressor acting only at cellular level (Lu et al., 2004), support the idea that virus loading in the vascular system is an important step for systemic infection and that suppressing long-distance silencing of the receptor plant helps to overcome this obstacle. Moreover, plants pre-treated with p19 not only showed a higher rate of infection at 2 mpi but, contrasting with those pre-treated with p23, this rate further increased at 4 mpi.

CTV distribution and symptom expression in NB plants systemically infected after agroinoculation with oncogenic strains, mechanical inoculation of virions or graft transmission, mimic those reported previously on this host (Ambrós et al., 2011), supporting the notion that essential host-viral interactions leading to viral movement and symptom development remain unaltered. The main difference between agroinoculation and mechanical or graft inoculation was the significant delay in systemic CTV infection observed with the two latter methods, particularly with mechanical inoculation that sometimes had a lag period of up to 4–6 months, probably due to the low number of CTV virions initiating infection. Biological characteristics of CTV virions from NB systemically infected by either procedure remain unaltered and upon slash inoculation to alemow plants these displayed the symptoms characteristic of the CTV-T36 isolate.

Summarizing, agroinoculation of NB with CTV-T36-based vectors by either agroinfiltration or stem agroinoculation are presently the best procedure to assay new CTV hybrid constructs in citrus plants. These new constructs can now be easily maintained in NB plants for future transmissions to citrus or for eventual studies on CTV stability or evolutionary adaption to this non-natural host after successive passages. Infection of NB by leaf rubbing with virion extracts is to our knowledge the first report on how a non-natural host species may become susceptible to mechanical inoculation with a virus that is naturally phloem-restricted, after pre-treatment with a silencing suppressor. This procedure may potentially help developing future infectious CTV clones if virus genotypes other than CTV-T36 replicating in protoplasts are found capable of invading NB plants.

ACKNOWLEDGMENTS

This research was supported by grants AGL2007-61885/AGR and AGL2010-20221 co-financed by FEDER funds and by the Ministerio de Ciencia e Innovación (MICINN), and project 5953 from the IVIA. Silvia Ambrós was recipient of contracts from the Ramón y Cajal program of the Ministerio de Educación y Ciencia, the Agroalimed Foundation and the IVIA (Generalitat Valenciana). Susana Ruiz was recipient of a predoctoral fellowship from the Ministerio de Educación y Ciencia. We thank Manuel Martínez for his valuable and excellent technical assistance, plant care and help with viral inoculations.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 February 2013; accepted: 03 June 2013; published online: 02 July 2013.

*Citation: Ambrós S, Ruiz-Ruiz S, Peña L and Moreno P (2013) A genetic system for Citrus Tristeza Virus using the non-natural host Nicotiana benthamiana: an update. Front. Microbiol. **4**:165. doi: 10.3389/fmicb.2013.00165*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 23 April 2013
doi: 10.3389/fmicb.2013.00093

Citrus tristeza virus: evolution of complex and varied genotypic groups

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Amongst the Closteroviridae, *Citrus tristeza virus* (CTV) is almost unique in possessing a number of distinct and characterized strains, isolates of which produce a wide range of phenotype combinations among its different hosts. There is little understanding to connect genotypes to phenotypes, and to complicate matters more, these genotypes are found throughout the world as members of mixed populations within a single host plant. There is essentially no understanding of how combinations of genotypes affect symptom expression and disease severity. We know little about the evolution of the genotypes that have been characterized to date, little about the biological role of their diversity and particularly, about the effects of recombination. Additionally, genotype grouping has not been standardized. In this study we utilized an extensive array of CTV genomic information to classify the major genotypes, and to determine the major evolutionary processes that led to their formation and subsequent retention. Our analyses suggest that three major processes act on these genotypes: (1) ancestral diversification of the major CTV lineages, followed by (2) conservation and co-evolution of the major functional domains within, though not between CTV genotypes, and (3) extensive recombination between lineages that have given rise to new genotypes that have subsequently been retained within the global population. The effects of genotype diversity and host-interaction are discussed, as is a proposal for standardizing the classification of existing and novel CTV genotypes.

Keywords: *Citrus tristeza virus*, evolution, strain, genotype, divergence, recombination

INTRODUCTION

All organisms carry, in their genome, traces of their evolutionary history: past selective events, diversification, and recombination, all of which provide an insight into the adaptive landscape over which these organisms evolved. The small, simple genomes of viruses are ideal for study, as even a single non-synonymous mutation can alter the phenotype. Viral evolution and epidemiology are interdependent; the continued spread of a virus via vector species into new hosts relies on its ability to adapt (Pybus and Rambaut, 2009), although both processes are subject to drift. One aspect of viral adaptation, of any given species, is the bifurcation of an ancestral sequence or population into two or more lineages that over time develop novel phenotypic characteristics, utilize novel vectors, and infect new host species. Members of a distinct phylogenetic lineage that possess a shared evolutionary history are, to all intents and purposes, strains.

The existence of multiple strains exhibiting differences in infectivity, host range, transmission, or virulence is common amongst animal viruses, such as *Hepatitis C virus* (HCV) (Gray et al., 2011), *Influenza A virus* (Smith et al., 2009), and *Simian immunodeficiency virus* (Etienne et al., 2011), and in plant viruses, such as *Cucumber mosaic virus* (CMV) (Roossinck, 2001) and *Plum pox virus* (PPV) (Candresse and Cambra, 2006). Amongst the Closteroviridae, the existence of multiple strains is a rarity, due in part to the limited host range of most species, phloem-specific tissue tropism, or lack of opportunity for spread due to absence of polyphagous vectors (Karasev, 2000), as well as a distinct lack of research on species infecting less economically important crops. With the possible exception of *Grapevine leafroll virus-3* (Bester et al., 2012), *Citrus tristeza virus* (CTV) is the only closterovirus species to possess multiple, phylogenetically distinct strains (Moreno et al., 2008).

Citrus tristeza virus is one of the most significant pathogens to afflict citrus, and has been responsible for the loss of over 100 million trees either killed or rendered unproductive over the past century (Moreno et al., 2008). CTV is a member of the *Closterovirus* genus in the family *Closteroviridae*, with a 19.3-kb ssRNA genome encoding 12 open reading frames. ORF1 expresses one large polyprotein (ORF1a) containing helicase, methyltransferase, and duplicated protease domains, as well as the RNA-dependent RNA-polymerase (ORF1b) via a +1 frameshift (Karasev et al., 1995). The 10 other ORFs, expressed through subgenomic RNAs, encode the major and minor coat proteins (p25 and p27), three suppressors of RNA silencing (p25, p20, and p23) (Lu et al., 2004), two genes expressing a heat shock protein homolog (p65) and a protein with a diverged coat protein motif, both required for virion assembly (Satyanarayana et al., 2000), and three proposed host range genes (p33, p13, and p18) (Tatineni et al., 2011). CTV causes three major host reactions or syndromes: seedling yellows, stem pitting, and quick decline, of which the last two are significant problems for citrus cultivation. Symptom expression and severity is dependent on three factors: the species or cultivar infected, the

species of the rootstock on which the scion is grafted, and finally, the particular infecting strain or mixture of CTV isolates (Moreno et al., 2008).

Citrus tristeza virus diseases, in all their forms, are the result of concentrated agricultural production; a setting quite unlike the natural environment in which both citrus and CTV evolved. Citrus have been used for trade, as a source of medicinal compounds, and as an item of religious significance for over 2000 years and have been extensively propagated throughout much of the world (Webber et al., 1967). Throughout much of their history importation of citrus plants occurred only as seed, avoiding CTV spread as the virus is not transmissible by pollen or seed (Moreno et al., 2008); it is only with the rise of rapid shipping in the mid-to-late nineteenth century that the movement of whole plants and later, live cuttings, became possible, leading to the global distribution of CTV (Moreno et al., 2008). This coincided with the rise of large-scale commercial citrus production in the late nineteenth century and adoption of monocultures; a departure from earlier production for local consumption in which a variety of species and/or cultivars were grown in one locale. Monoculture production promotes the occurrence of tristeza epidemics, which have punctuated the last century in South America in the 1930s and early 1940s, as well as Florida in 1951, Spain in 1957, Israel in 1970, and Venezuela in 1980 (Bar-Joseph et al., 1989; Moreno et al., 2008), by providing a genetically and phenotypically uniform host range susceptible to the introduction or evolution of a pathogenic strain, or combination of strains.

With the sequencing of the first CTV genomes, T36 from Florida (Karasev et al., 1995), VT from Israel (Mawassi et al., 1996), followed by T385 from Spain (Vives et al., 1999) and it's near identical homolog T30 from Florida (Albiach-Marti et al., 2000), it became apparent that these three strains diverged markedly from one another, with two different trajectories: the VT-like and T30-like isolates on one hand, and the T36-like on the other (Hilf et al., 1999). Additional sequencing of novel isolates over the past decade suggests that the global CTV diversity is far higher than previously thought, and that new genotypes have diverged from the ancestral population, or have arisen through recombination with previously described strains (Ruiz-Ruiz et al., 2006; Harper et al., 2009, 2010; Melzer et al., 2010; Roy and Brlansky, 2010). Identification of new genotypes is complicated by asymmetry between the 5′ and 3′ halves of the genome, for most of the divergence between the groups is most apparent in the 5′ end of the genome and the ORF1a/1b genes (Hilf et al., 1999; Albiach-Marti et al., 2000) which contain the replication associated proteins. It is in the 5′ end of the genome that the more recently described T3 and NZ-B18/B165 isolates can be distinguished from one another and from VT, as they are all otherwise homologous in the 3′ subgenomic RNA coding genes (Hilf et al., 2005; Harper et al., 2009; Roy and Brlansky, 2010). Classification of CTV genotypes is further confused by the existence of recombinant isolates such SY568 (Vives et al., 2005) and HA16-5 (Melzer et al., 2010). Yet, both divergence and recombination are an important component of CTV evolution (Martin et al., 2009), and it may be proposed that the existence of multiple strains is responsible for the wide range of phenotypes observed within and between different citrus cultivars and species, particularly when multiple strains are in mixture (Scott et al., 2013).

Therefore, in this study an array of complete genomic sequences of CTV from around the world was examined to elucidate their complex and interwoven evolutionary histories, and to establish how the strains we see today came to be. Such knowledge is a necessary first step to understanding the interaction between specific virus isolates or strains and host cultivars, and hence, understanding pathogenicity. A standardized system of classification for identifying and grouping the strains present around the world, as well as a framework for incorporating novel strains, on a genotypic basis is also proposed.

MATERIALS AND METHODS

CTV ISOLATES

The CTV isolates examined in this study were obtained from two major sources: a collection of isolates from the state of Florida, maintained at the Citrus Research and Education Center, University of Florida, and from sequences from around the world deposited in the NCBI database (**Table 1**). An infectious clone based on the T36 isolate that was maintained under glasshouse conditions for 7 years in a single host was also examined (Satyanarayana et al., 1999, 2001).

SMALL RNA SEQUENCING OF CTV ISOLATES

A total of 2 g of young green bark tissue from samples obtained either from field or glasshouse collections were ground to a fine powder in liquid nitrogen, and total RNA extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), with modifications to the protocol to account for scale. Briefly, the powdered tissue was homogenized in 10 mL of Trizol reagent and 2 mL of chloroform and incubated on ice for 10 min. Samples were then separated by centrifugation at $12000 \times g$ for 20 min, and the upper aqueous phase mixed with an equal volume of isopropanol before precipitation at −20°C for at least 2 h. Total RNA was pelleted by a further round of centrifugation, and washed with 70% ethanol before air-drying at room temperature. The pellets were re-suspended in 100 μL of dH_2O, and the small RNA fraction, fragments of less than 200 bp, recovered by processing through an Ambion *mir*Vana miRNA isolation kit (Ambion, Austin, TX, USA) as per the manufacturer's protocol. Small RNA presence and quality was checked on an Agilent 2100 Bioanalyzer platform (Agilent Technologies, Palo Alto, CA, USA).

Small RNA libraries were constructed using the ABI SOLiD small RNA expression kit (Applied Biosystems Inc., Foster City, CA, USA) as per the manufacturer's protocol and sequenced using a SOLiD 5500xl platform at the Interdisciplinary Center for Biotechnology Research, University of Florida. The resulting reads for each sample were trimmed to remove adapters, and reads with a length of less than 19 nt and greater than 25 nt were discarded, giving a total of between 3.8×10^6 and 1.2×10^7 reads per sample. The reads for each sample were depleted by removal of sequences present in mirBase19 (Kozomara and Griffiths-Jones, 2011) and the plant snoRNA databases (Brown et al., 2003), the *Citrus sinensis* chloroplast sequence (Bausher et al., 2006), *C. sinensis* genome scaffolds, and the *Arabidopsis thaliana* mitochondrion sequence (Unseld et al., 1997). Reads for each sample were

Table 1 | Provenance of CTV isolates used in this study.

Sequence name	Accession no.	Genotype	Isolation host	Country of origin	Sequencing method	Reference
FL202	KC517493	VT	*Citrus sinensis*	FL, USA	SOLiD 5500xl	This study
FS674	KC517485	T36	*C. sinensis*	FL, USA	SOLiD 5500xl	This study
FS701	KC517494	VT	*C. sinensis*	FL, USA	SOLiD 5500xl	This study
	KC517489	T30				
	KC517486	T36				
FS703	KC517492	VT	*C. sinensis*	FL, USA	SOLiD 5500xl	This study
	KC517491	T30				
	KC517487	T36				
FL278	KC517490	T30	*C. macrophylla*	FL, USA	SOLiD 5500xl	This study
FS577	KC517488	T36	*C. macrophylla*	FL, USA	SOLiD 5500xl	This study
FS02-2	EU937519	VT	*C. sinensis*	FL, USA	Affymetrix microarray	Weng et al. (2007)
	EU937520	T30				
	EU937521	T36				
T3	KC525952	T3	*C. sinensis*	FL, USA	Sanger	Hilf et al. (unpublished)
NZ-M16	EU857538	T3	*C. aurantifolia*	New Zealand	Sanger	Harper et al. (2009)
T68-1	JQ965169	T68	*C. sinensis*	FL, USA	Sanger	This study
HA16-5	GQ454870	Unknown	Unknown	Hawaii, USA	Sanger	Melzer et al. (2010)
NZ-B18	FJ525436	T68	*C. sinensis*	New Zealand	Sanger	Harper et al. (2009)
CT14A	JQ911663	T68	*C. sinensis*	China	Sanger	Unpublished
B165	EU076703	T68	*C. reticulata*	India	Sanger	Roy and Brlansky (2010)
NZRB-TH28	FJ525433	RB	*Poncirus trifoliata*	New Zealand	Sanger	Harper et al. (2010)
NZRB-TH30	FJ525434	RB	*Poncirus trifoliata*	New Zealand	Sanger	Harper et al. (2010)
NZRB-M17	FJ525435	RB	*C. aurantifolia*	New Zealand	Sanger	Harper et al. (2010)
NZRB-M12	FJ525431	RB	*Poncirus trifoliata*	New Zealand	Sanger	Harper et al. (2010)
NZRB-G90	FJ525432	RB	*Poncirus trifoliata*	New Zealand	Sanger	Harper et al. (2010)
B301	JF957169	RB	*C. sinensis*	Puerto Rico	Sanger	Roy et al. (unpublished)
HA18-9	GQ454869	RB	Unknown	Hawaii, USA	Sanger	Melzer et al. (2010)
T30	AY260651	T30	Unknown	FL, USA	Sanger	Albiach-Marti et al. (2000)
T385	Y18420	T30	Unknown	Spain	Sanger	Vives et al. (1999)
VT	U56902	VT	Unknown	Israel	Sanger	Mawassi et al. (1996)
T318A	DQ151548	VT	Unknown	Spain	Sanger	Ruiz-Ruiz et al. (2006)
Nuaga	AB046398	VT	Unknown	Japan	Sanger	Suastika et al. (2001)
CT11A	JQ911664	VT	*C. sinensis*	China	Sanger	Unpublished
AT-1	JQ061137	VT	*C. sinensis*	China	Sanger	Unpublished
KPG3	HM573451	VT	*C. reticulata*	India	Sanger	Biswas et al. (2012)
T36	U16304	T36	Unknown	FL, USA	Sanger	Karasev et al. (1995)
T36 (Clone)	AY170468	T36	N/A	FL, USA	Sanger	Satyanarayana et al. (2001)
538 (Clone)	N/A	T36	*C. macrophylla*	FL, USA	SOLiD 5500xl	This study

then mapped against extant genome sequences, and assembled using a combination of SHRiMP v2.0 (David et al., 2010) and CLC Genomics Workbench v5.5.1 (CLC Bio, Aarhus, Denmark), producing matches of between 9.5×10^5 and 3.5×10^6 reads per sequence. *De novo* assembly was also attempted using a word size of 100, length fraction of 0.5, and similarity of 0.8. Completed sequences were deposited in the NCBI database (**Table 1**).

PHYLOGENETIC AND EVOLUTIONARY ANALYSES

Complete genome sequences were aligned using Muscle 3.8 (Edgar, 2004a,b) and manipulated in BioEdit 5.0.9 (Hall, 1999). Annotations were applied using CTV reference isolates T36 (Karasev et al., 1995), T30 (Albiach-Marti et al., 2000), and T318A (Ruiz-Ruiz et al., 2006) as references.

As CTV is known to frequently recombine (Vives et al., 2005; Harper et al., 2010) which creates phylogenetic ambiguity two methods, maximum parsimony (MP) and neighbor network (NN), were applied as it has been shown that these are less error prone in inferring topology in the presence of recombination (Woolley et al., 2008). MP was applied to the complete genome alignment using MEGA 5.10 (Tamura et al., 2011) with the subtree-Pruning-Regrafting algorithm with a search level of 1 in which the initial trees were obtained by the random addition of 10 sequences, branch lengths were calculated using the average pathway method. NN construction was performed using SplitsTree 4.12.3 (Huson and Bryant, 2006) with LogDet distance correction, exclusion of gap, and parsimony-uninformative sites and splits filtered using a weakly greedy algorithm.

Tests for selection, and episodic diversifying selection within sites of CTV ORFs were performed using the Fixed Effects Likelihood (FEL) (Kosakovsky Pond and Frost, 2005a) and Mixed Effects Model of Evolution (MEME) (Murrell et al., 2012) algorithms respectively, using the Datamonkey webserver (Kosakovsky Pond and Frost, 2005b). All alignments were screened for recombination, and where necessary partitioned, using the GARD algorithm (Kosakovsky Pond et al., 2006). Branch-Site Random Effects Likelihood (Branch-Site REL) analysis (Kosakovsky Pond et al., 2011) was also performed on the aforementioned alignments to search for episodic diversifying selection within branches, and for comparison with MEME results.

The presence of co-evolution between domains of ORF1a and ORF1b was detected and analyzed using the MirrorTree webserver (Ochoa and Pazos, 2010); Pearson correlation coefficient values greater than 0.8 were considered to be indicative of co-evolution (Clark et al., 2011). MatrixMatchMaker v2 (Rodinov et al., 2011) was also used to confirm co-evolutionary events within strains.

Recombination analysis was performed using RDP v3.34 (Martin et al., 2010) using the RDP (Martin and Rybicki, 2000), BootScan (Martin et al., 2005), SiScan (Gibbs et al., 2000), Chimera (Posada and Crandall, 2001), Geneconv (Padidam et al., 1999), MaxChi (Maynard Smith, 1992), and 3Seq (Boni et al., 2007) methods to generate a consensus of regions that may be recombinant in origin. Recombination events that were not identified by at least three of the seven models used were discarded, as were events for which the parental sequences could not be identified. Isolate HA16-5 was excluded from this analysis as its divergent sequence generated a large number of unconfirmed recombinant events.

RESULTS

COMPLETE GENOME ANALYSIS

Examination of the complete genome phylogenies of 36 extant CTV sequences developed using MP (**Figure 1**) and NN (**Figure 2**) methods indicated the presence of five major previously described CTV strains: VT, T30, T3, RB, and T36. Interestingly, MP was able to resolve a further clade containing four isolates, T68-1 from Florida, CT14A from China, NZ-B18 from New Zealand, and B165 from India, that we have termed the T68 strain; NN analysis also identified this clade, though also indicated significant and repeated recombination events between this and the VT clade. This phylogeny also correctly placed the RB-T36 recombinant isolates NZRB-TH30 and NZRB-M17 (Harper et al., 2010) as part of the RB lineage rather than the T36 lineage suggested by maximum likelihood analysis (data not shown). Finally, the Hawaiian isolate HA16-5 could not be placed into one of the extant clades using MP or NN, suggesting that this is a very novel isolate and/or a recombinant as suggested by the NN analysis, and potentially a novel strain.

Bifurcation was observed within the VT lineage (**Figures 1** and **2**), which segregated the Israeli and US VT-like isolates, henceforth termed "Western," from the Asian (AT-1, CT11A, and Nuaga) and Spanish (T318A) isolates, henceforth termed "Asian," which we suggest represents the spread of two distinct sequence variants around the world, and likely reflects the historical movement of plant material. The Indian isolate KPG3, a suggested recombinant (Biswas et al., 2012), remained separate from both subtypes in the phylogeny.

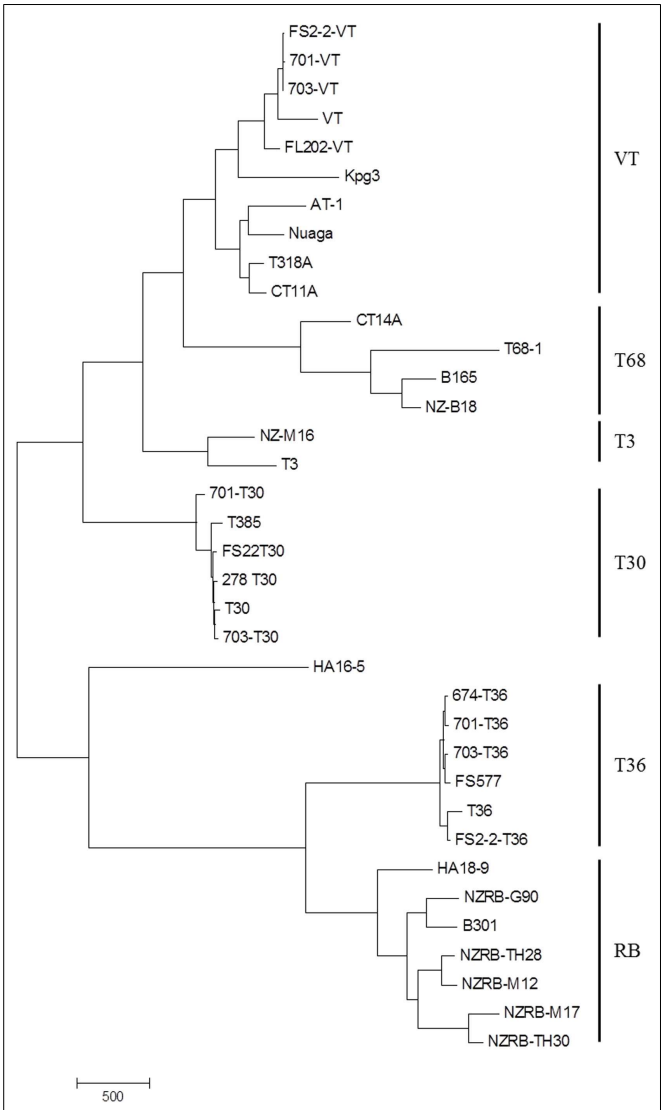

FIGURE 1 | Maximum parsimony phylogeny of the complete genomes of *Citrus tristeza virus* isolates examined in this study. Major strains are indicated.

Each of the major strains share an average of 85.1% nucleotide identity across the length of the genome, with a range of 92.4% nucleotide identity between VT and T3 lineages, to 80.5% between the T36 and T68 lineages (**Table 2**). This average identity is not evenly distributed throughout the length of the genome, for example ORF1a of the RB strain shares an average of 73.1% identity with the T30 strains, whilst the p61, p27, and p6 ORFs of these two strains possess much higher identities of 94.5, 95.5, and 95.7% respectively (**Table 2** and data not shown). Amino acid identities follow a similar pattern to the nucleotide sequences, ranging between 73.4 and 92.1% for ORF1a to a high of 94.1–98.6% for p27 (**Table 2**). Within-strain nucleotide identities suggest conservation (**Table 3**), with a range of between 94.9 and 97.4% per ORF for VT and 99.2 to 99.9 for T36; T3 has lower 3′ gene identities as one member, NZ-M16, is recombinant.

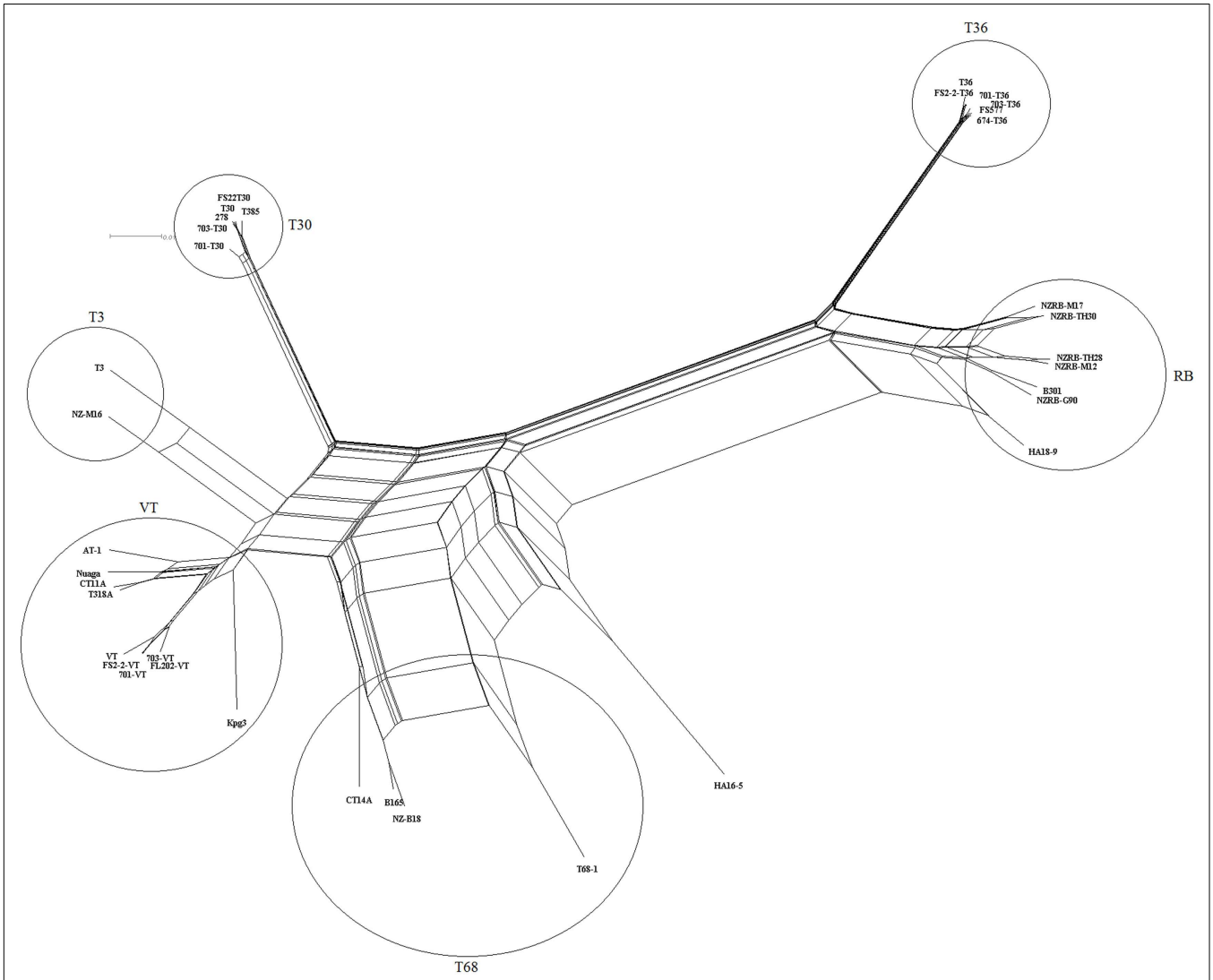

FIGURE 2 | Neighbor network reconstruction of the complete genomes of *Citrus tristeza virus* examined in this study. Major strains groupings are indicated.

Tests for selective pressures on each of the CTV ORFs (**Table 4**) revealed basic patterns. First, the 3′ ORFs (p33 through p23) each have a similar proportion of codons under negative or purifying selection, ranging from 19.6 to 30.4%, while the 5′ ORFs required for replication (Karasev et al., 1995) have a much higher number of codons under negative selection with a range of 47.0–54.8%. In contrast FEL analysis, across all CTV strains, indicated that a very small proportion of codons, less than 2% in all cases, of both 5′ and 3′ ORFs show evidence of positive selection (**Table 4**). MEME analysis, which operates under similar assumptions, though has greater resolving power than FEL (Murrell et al., 2012), found more positively selected codons for each ORF (**Table 4**), of which many were selective events basal to one or more of the extant strains; the location of positively selected codons specific to single isolates rather than strains were not recorded. Even though more positively selected codons were identified by MEME, these represent less than 5% of the total which, when added to the total of negatively selected codons, suggests that the majority of the coding sequence of each ORF operates under neutral selection. In contrast to Branch-Site REL analysis which identified episodic diversifying selection only in terminal branches of VT-like isolates and indicated selection was similar between lineages (data not shown), MEME analysis did indicate significant episodic diversifying selection in sites that could be mapped to specific lineages (**Table 4**). This was particularly prevalent in ORFs 1a and 1b as well as p23, p33, and p61. The latter two genes possessed positively selected sites in branches leading to the RB, T30, and T36 genomes suggesting that they had, over evolutionary time, diversified from the VT, T3, and T68-like strains in these genes, while diversification in ORF1b was common to all strains except VT, with further diversification of T36; MEME did not resolve the 18 amino acid insertion unique to the T36 strain in ORF1b. Mapping

Table 2 | Average nucleotide and amino acid between strain identities for the (A) complete genome, (B) ORF1a, (C) p25, and (D) p27 genes.

	RB	T36	T3	T68	T30	VT	HA16-5		RB	T36	T3	T68	T30	VT	HA16-5
(A) GENOME								**(B) ORF1a**							
RB								RB		*91.4*	*86.9*	*92.1*	*74.8*	*74.6*	*79.6*
T36	90.9							T36	90.6		*85.7*	*91.7*	*74.6*	*74.3*	*80.2*
T3	81.0	80.5						T3	85	82.9		*85.8*	*74.4*	*74.5*	*86.1*
T68	80.9	80.5	88.0					T68	90.8	90.4	83		*74.3*	*74.3*	*79.9*
T30	82.0	81.9	90.0	86.1				T30	73.1	72.9	72.6	72.6		*91.6*	*73.5*
VT	81.2	80.8	92.4	89.9	90.3			VT	73.2	72.9	72.7	72.9	91.2		*73.4*
HA16-5	81.8	80.3	84.0	86.2	83.5	83.8		HA16-5	78.0	78.1	82.8	77.9	72.1	72.2	
	RB	**T36**	**T3**	**T68**	**T30**	**VT**	**HA16-5**		**RB**	**T36**	**T3**	**T68**	**T30**	**VT**	**HA16-5**
(C) p25								**(D) p27**							
RB		*95.7*	*96.5*	*96.8*	*95.3*	*96.4*	*97.2*	RB		*95.1*	*95.4*	*96.0*	*96.5*	*95.8*	*96.1*
T36	93.9		*95.8*	*95.6*	*96.2*	*95.2*	*96.2*	T36	92.9		*94.5*	*95.5*	*96.0*	*95.4*	*94.9*
T3	92.6	92.3		*97.8*	*96.4*	*97.4*	*98.2*	T3	87.9	87.8		*97.1*	*94.1*	*97.2*	*94.4*
T68	92.6	93.0	95.1		*95.7*	*97.9*	*97.3*	T68	89.3	89.1	92.8		*95.8*	*98.6*	*94.5*
T30	92.4	93.1	93.1	92.6		*95.5*	*96.4*	T30	95.5	93.9	88.1	89.4		*95.6*	*95.6*
VT	93.1	92.8	95.4	96.4	92.7		*97.1*	VT	89.2	89	93.8	96.2	89.5		*94.3*
HA16-5	93.4	92.2	91.9	91.1	91.5	92.4		HA16-5	92.5	92.0	87.5	88.5	92.1	88.4	

Amino acid identities are italicized.

the number of events onto a neighbor-joining phylogeny of ORF1a (**Figure 3**) revealed that there has been significant episodic diversification in first the T36, RB, and T68 lineages from T3, VT, and T30 (9 events), followed by separation of RB and T36 from the T68 lineage (37 and 12 events), and RB from T36 (5 and 3 events respectively). There are also a large number of positively selected sites, 11 and 8 respectively, in the bifurcation of the T3 and T30 genotypes, and three sites under selection in the branch leading to the Asian VT isolates, separating them from the Western VT isolates. These data therefore suggest significant, concerted separation of the major CTV lineages, and it should be noted the analysis likely underestimates the total number of diversifying events as negative selection in extant isolates to maintain sequence can obscure ancestral positive selection (Murrell et al., 2012), as suggested by Branch-Site REL analysis in this study (data not shown).

ORF1a is an example of the varying selective pressures within a single gene. It contains four domains: the L1 and L2 papain-like proteases, methyltransferase, and helicase domains (Karasev et al., 1995), separated by regions that if not non-coding, are of unknown function at time of writing. The four domains all show conservation of sequence; FEL analysis identified between 43.2 and 66.2% of residues under negative selection, and between 0 and 1.4% of residues under positive selection, higher than the surrounding regions which ranged between 33.1 and 63.9% and 1.9 and 12.5% for negative and positively selected residues respectively (**Table 5**). MEME analysis detected more positively selected codons, although several of these pertained to single isolates rather than historical evolutionary events during strain evolution (data not shown). This strong negative selective pressure is reflected in the overall level of amino acid identity in all four of the functional domains, ranging between 86.2 and 93.6% for the L2 protease and methyltransferase respectively, while being notably lower between domains, with an amino acid identity range of 74.7–89.2%.

Co-evolution was detected using MirrorTree between the ORF1a and ORF1b (RdRp) domains L1-L2, L1-MET, L1-HEL, L1-RdRp, L2-MET, L2-HEL, L2-RdRp, MET-HEL, MET-RdRp, and HEL-RdRp with Pearson's correlation coefficient values of between 0.847 and 0.972 (**Table 6**). Higher coefficient values were obtained within strains for each of these events, for example within L1-MET the coefficient values were 0.919, 0.994, and 0.942 between isolates of the T30, VT, RB, and T36, strains respectively; the latter two strains share the same motifs and were considered together. This was not consistent across all domains examined, however, as some pairings only one strain had a coefficient value above the threshold, such as L2-HEL and MET-HEL in which the RB/T36 strain had values of 0.971 and 0.976 respectively (**Table 6**). In contrast, the MatrixMatchMaker algorithm found only weak evidence of co-evolution in most domains with weighted scores of less than 1, with the exception of VT isolates between L1 and L2, and VT and T3 isolates between the L2-HEL domains (data not shown). This is to be expected as MMM is not optimized for resolving co-evolution between closely related domains (Clark et al., 2011). Overall, these results correlate with the translated amino acid sequence of four domains of ORF1a, in which the major genotypes maintain a unique motif of amino acid substitutions, suggesting that co-evolution has occurred not only between domains, but have co-evolved within strains.

RECOMBINATION ANALYSIS

Recombination is a major factor in the evolution of the recognized CTV strains as indicated by the NN analysis (**Figure 2**). Analysis of the extant genome sequences in this study using RDP found that nearly every isolate contained trace evidence of

Table 3 | Average nucleotide and amino acid identities within the six major CTV strains, divided by ORF.

	Genome		p13		p18		p20		p23		p25		p27		p61		p65		p33		p6		ORF1a		ORF1b	
	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
VT	96.4	–	94.9	96	96.1	95.4	97.4	98.6	96.3	96.8	96.3	97.5	96.1	98.5	96.2	97.2	97.3	98.3	95.3	95.6	96.3	95.8	96.2	95.9	97.4	98.5
T3	95.9	–	93.3	95	93.8	94.6	94.9	96.2	91.9	90.4	94.3	98.2	90.4	97.1	92	94.7	93.6	98.3	97.5	96	92.8	98	97.8	97.4	97.8	98.6
T68	94.2	–	96.2	95.4	97.3	98.2	98.9	99.7	98.6	97.8	98.6	99.1	98.8	99	98.1	97.7	98.2	99.2	97.8	97.3	100	100	90.8	91.8	92.2	95.3
T30	99.4	–	99.6	99.7	99.3	99.4	99.4	100	99.1	99	99.7	99.7	99.3	99.5	99.6	99.5	99.2	99	99.5	99.2	99.6	100	99.4	99.4	99.4	99.6
RB	96.2	–	94.8	93.5	95.1	94.8	95.6	97.6	95.1	94.5	96.3	97.5	95.4	97.4	96.3	95.5	97.9	98.4	96.8	95.5	97.4	96.7	95.8	95.3	97.4	98.2
T36	99.4	–	99.2	99.3	99.9	99.8	99.8	99.7	99.5	99.2	99.6	99.6	99.5	99.8	99.5	98.9	99.7	99.4	99.5	99.6	98.8	98.4	99.4	98.4	99.8	99.8

recombination either within or between strains, although these events were weakly supported and identified by less than four models, or the parental sequences could not be identified. Recombination events supported by four or more models, with acceptable p-values ($p < 0.01$), were identified in members of four strains, RB, VT, T3, and T68 as well as the potentially novel strain HA16-5 (**Table 7**), and can be classified into two major groupings: the insertion of fragments within an ORF, or the complete replacement of the 3′ or 5′ half the genome at a point within or between the ORF1b and p33 ORFs (**Figure 4**). The former includes both inter- and intra-strain recombination, for example members of the RB all retain an ancestral recombination event, the partial replacement of the p65 ORF from a VT-like isolate, while three isolates also have undergone subsequent recombination events, with NZRB-M17 and TH30 of the RB strain acquiring T36-like segments at the beginning of ORF1a, while HA18-9 has acquired a VT-like segment between the partial p27 through partial p13 ORFs (**Table 7**; **Figure 4**). Three of the four T68 isolates have acquired VT-like fragments in ORF1a, although interestingly while isolates B165 and NZ-B18 possess the western VT-like insertions, isolate CT14A maintains a longer ∼5 kb fragment that shares higher identity to Asian VT-like isolates of 97.3 versus 94.7% to the western VT isolates. The VT-like isolates by contrast show only two events of inter-strain recombination, with a T30-like insertion in ORF1a between bases 4368 and 5695, and repeated T3-like insertions in the 3′ half of isolate Kpg3 (**Figure 4**). Finally, isolate AT-1, an Asian VT-like isolate maintains an insertion of approximately 3.1 kb that shares higher identity with western VT isolates; it cannot be discounted that this is the result of conservation of an ancestral proto-VT sequence rather than recombination.

The replacement of the 5′ or 3′ half of the genome also occurs, most notably in the unclassified isolate HA16-5 which possesses a complete 3′ replacement, introducing an RB, or more specifically HA18-9 fragment, on to the end of a T68-like ORF1, while NZ-M16, a T3-like isolate has a VT-like complete 3′ replacement. All members of the T68 strain possess a complete 3′ replacement with a VT-like isolate that is likely the result of a single ancestral event, as it is largely conserved between T68-like isolates with an average 98.3% nucleotide identity.

EVOLUTION WITHIN LINEAGES

It has already been observed that there is a high degree of similarity within but not between strains; in this study two lineages, VT and T36, were examined in detail for evidence of within-strain evolution to determine how and where closely related isolates diverge from one another. The VT strain is, at present, the most diverse of the recognized strains, with members sharing an average of 96.4% nucleotide identity (**Table 2**). As mentioned earlier, the VT strain can be separated into two sub-strains, encompassing the Israeli and US VTs, and the Spanish and Asian VTs; the VT-like Kpg3 isolate is a recombinant and does not group with the two major clades. The two subgroups differ by 3.7% at the nucleotide level, with the majority of the diversification located in ORF1a. Although it should be noted that comparatively few result in positively selected non-synonymous substitutions, with only six in ORF1a, one in p20 and two in p61 (data not shown). Most show no evidence of positive or negative selection and simply may be neutral for strain

Table 4 | Positively and negatively selected codons present in CTV ORFs identified by FEL and MEME analysis.

CTV gene		Fixed effects likelihood model		Mixed effects model of evolution	
ORF	**No. codons**	**No. negatively selected sites**	**No. positively selected sites**	**Sites with episodic diversifying selection**	**Lineage specific codon diversification**
p6	51	15 (29.4%)	0	0	
p13	119	36 (29.4%)	1 (0.8%)	1 (0.8%)	6 (RB, T36)
p18	167	41 (24.5%)	2 (1.7%)	2 (1.7%)	
p20	182	42 (23.1%)	0	4 (2.2%)	105 (Florida VT); 115 (T3)
p23	209	41 (19.6%)	4 (1.9%)	8 (3.8%)	3 (T36); 27 (T36) (RB, T30); 29 (RB, T30); 78 (RB, T30); 79 (T36) (RB, T30);177 (RB)
p25	223	57 (25.5%)	0	4 (1.8%)	
p27	240	73 (30.4%)	1 (0.4%)	2 (0.8%)	102 (T36)
p33	303	84 (27.7%)	2 (0.7%)	8 (2.4%)	117 (VT, T68, and T3); 219 (T68); 224 (Florida VT)
p61	536	137 (25.6%)	8 (1.5%)	17 (3.2%)	203 (T30); 333 (RB, T30, and T36); 372 (RB, T30, and T36)
p65	594	178 (29.9%)	3 (0.5%)	13 (2.2%)	412 (T30)
ORF1b	500	274 (54.8%)	5 (1.0%)	11 (2.2%)	237 (RB and T36)
ORF1a	3124	1469 (47.0%)	59 (1.8%)	138 (4.4%)	7 (T3, RB, T30, T36, T68); 27 (T36, RB); 30 (T68); 39 (T68, T36, RB); 47 (T36, RB); 49 (T36, RB); 52 (T36, RB); 53 (T36, RB) (Asian VT); 62 (T36, RB); 75 (T36, RB, T68); 92 (RB); 95 (T68) (T3, T30); 109 (T30) (T36, RB); 163 (T68, RB, T36); 179 (T3, T30) (T68, RB, T36); 234 (T3, T30) (RB); 238 (T3); 255 (T68, RB, T36); 299 (T3) (T68, RB, T36); 302 (RB) (T68); 310 (RB, T36); 336 (RB, T36); 378 (T30); 442 (T68); 471 (T68); 505 (RB, T36); 548 (T68); 571 (T68); 595 (RB, T36); 660 (T68, RB, T36);661 (T36) (T68); 671 (RB, T36); 694 (RB, T36, T68) (T3); 698 (RB, T36) (T68); 699 (RB, T36) (T3) (T68); 718 (T36, RB); 762 (T36, RB); 767 (T68); 875 (RB, T36); 881 (T68, RB, T36); 884 (T68, T3); 924 (RB); 1003 (T30) (RB, T36); 1075 (T68, RB, T36); 1527 (T3) (T36, RB); 1596 (T36); 1607 (T3, Asian VT); 1660 (T3); 1671 (T3), (T36, RB); 1845 (RB, T36); 1850 (T36); 1995 (RB, T36); 2004 (T30); 2016 (RB, T36); 2026 (T3), (T36, RB); 2030 (Asian VT) (T36, RB); 2039 (RB, T36); 2043 (RB); 2073 (Asian VT) (T3) (T36, RB); 2093 (RB, T36); 2103 (RB, T36); 2125 (RB, T36); 2129 (RB, T36); 2143 (T30) (RB, T36); 2168 (T3); 2173 (T30, T36); 2329 (T30); 2381 (T30); 2384 (T3, T30); 2397 (T30) (RB, T36); 2428 (RB, T36); 2464 (T30); 2526 (RB, T36); 2656 (T36, RB); 2683 (RB, T36); 2785 (RB, T36); 2852 (T68)

Location of strain-specific codon diversification given, per ORF.

evolution. Despite diversification, it should also be noted that both subgroups contain the conserved VT-strain motifs in the ORF1a L1, L2, MET, and HEL domains; and as mentioned earlier, show evidence of within-strain co-evolution between these domains.

The T36 strain shows significantly less diversity, based on extant sequences, than the VT strain with an average nucleotide identity of 99.4% (**Table 2**), which may be due in part to the sequencing of isolates from one geographic locale, and no obvious segregation into sub-strains. There is a small divergence of 0.46–0.57% at the nucleotide level between the type isolate of the strain, T36, which has been propagated in glasshouse conditions for approximately 40 years (S. Garnsey, personal communication), and isolates FS577, FS674, and FS701 extracted recently from field samples. These minor changes are significant in that the T36 isolate is phenotypically different from the extant field isolates, with a decrease in aphid transmission efficiency from approximately 40–50% down to 1% by *Toxoptera citricida*, and a decrease in virulence, producing only mild stem pitting on susceptible *C. macrophylla* hosts (S. Garnsey, personal communication; Harper, unpublished). The substitutions are distributed throughout the genome and have produced a total of 17 non-synonymous mutations in ORF1a, nine in p61, three in p65, and one change each in p6, p18, p20, p23, p25, and p33.

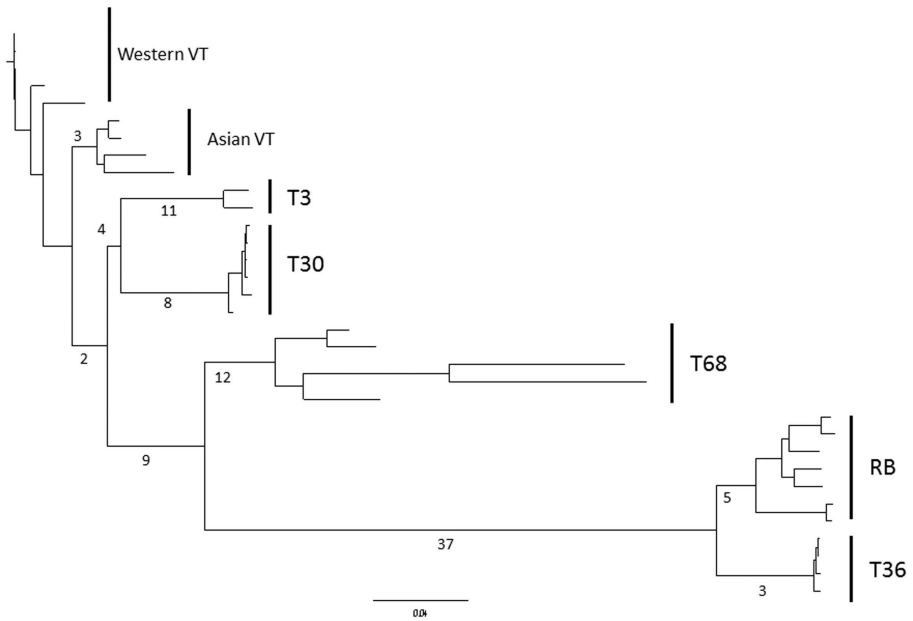

FIGURE 3 | Frequency of episodic diversifying selection events in ORF1a within CTV lineages as determined by MEME analysis, mapped on to a neighbor-joining nucleotide phylogeny. CTV strains and subtypes indicated.

Table 5 | Positively and negatively selected codons present within recognized domains of ORF1a identified by FEL and MEME analysis.

Domain	Sites	Amino acid identity (%)	No. residues	No. negatively selected sites	No. positively selected sites	
					FEL	MEME
L1	1–338	82.5	338	112 (33.1%)	14 (4.1%)	36 (10.7%)
	339–485	91.3	146	63 (43.2%)	2 (1.4%)	6 (4.1%)
	486–831	74.7	345	132 (38.3%)	11 (3.2%)	30 (8.7%)
L2	832–977	86.2	145	79 (54.5%)	0	7 (4.8%)
	978–1039	89.2	61	39 (63.9%)	0	1 (1.6%)
MET	1040–1349	93.6	309	174 (56.3%)	1 (0.3%)	4 (1.3%)
	1350–2701	79.8	1351	609 (45.1%)	26 (1.9%)	71 (5.3%)
HEL	2702–3099	92.2	397	263 (66.2%)	3 (0.8%)	7 (1.8%)
	3100–3124	83.3	24	0	3 (12.5%)	4 (16.7%)

Finally, the possession of a T36 based clone allowed us to explore the evolutionary rate of a single isolate. Isolate 538, introduced by bark-flap inoculation of a virion preparation into a *C. macrophylla* host 7 years earlier, was reconstructed by sequencing of the siRNA population present. Comparison of the reconstructed isolate 538 sequence with the clone reference sequence AY170468 indicated that only nine nucleotide substitutions had become fixed in the consensus sequence, an evolutionary rate of 6.67×10^{-5} per site, per year; these substitutions included five non-synonymous substitutions, located in ORF1a (positions 606 T-A and 2228 T-I), p61 (324 G-D), and p18 (59 I-V, and 129 K-M). Two of the substitutions (p61 324 G-D, and p18 129 K-M) restored the residue to that found in the T36-like field samples, while the others introduced amino acids of similar properties, with the exception of the substitution at site 2228 of ORF1a, which may be the result of drift or neutral evolution. This indicates a very slow rate of evolution in a single CTV isolate, under stable conditions, over time.

DISCUSSION

Before considering how the extant strains of CTV evolved, we should ask a more basic question: what is a strain in this context? Throughout much of their history, CTV isolates were classified by the presence or absence, and severity of, symptoms on citrus indicator species, and later by serological classification using monoclonal antibodies, such as MCA13, that distinguished between

Table 6 | Strain-specific co-evolution events between recognized domains of ORF1a identified by MirrorTree.

	L1		L2		MET		HEL	
L2	Overall	0.862						
	RB and T36	**0.974**						
	T30	**0.901**						
	VT	0.184						
MET	Overall	0.884	Overall	0.89				
	RB and T36	**0.942**	**RB and T36**	**0.977**				
	T30	**0.919**	T30	0.111				
	VT	**0.994**	**VT**	**0.941**				
HEL	Overall	0.847	Overall	0.972	Overall	0.927		
	RB and T36	**0.936**	**RB and T36**	**0.971**	**RB and T36**	**0.976**		
	T30	0.732	T30	0.722	T30	0.45		
	VT	**0.986**	VT	−0.196	VT	0.751		
RDRP	Overall	0.865	Overall	0.765	Overall	0.92	Overall	0.832
	RB and T36	0	RB and T36	0	RB and T36	0	RB and T36	0
	T30	**0.962**	T30	−0.397	T30	0.668	**T30**	**0.896**
	VT	**0.992**	VT	−0.556	**VT**	**0.967**	**VT**	**0.992**

Events with a Pearson's correlation coefficient of 0.8 or higher were considered to be significant (highlighted in bold).

Table 7 | Location and provenance of recombination events present in CTV isolates examined in this study.

Recombinant isolate	Start	End	Parental strain 1	Parental strain 2	RDP	Geneconv	BootScan	MaxChi	Chimera	SiSscan	3Seq
M17/TH30	111	3281	T36	RB	5.64E-47	6.46E-24		6.92E-29	1.09E-31	7.88E-29	1.47E-81
B165, NZ-B18	3949	8305	VTs	T68	1.25E-05			3.18E-04	8.65E-06		2.72E-40
CT14A	5229	9390	Asian VTs	T68	1.98E-210	7.77E-197	1.29E-102	1.63E-68	2.91E-68	2.63E-90	1.55E-316
B165	607	1012	VTs	T68	2.33E-56	7.77E-54		1.40E-15	5.33E-15	6.40E-15	3.66E-27
AT-1	393	3489	VTs	Asian VTs	3.22E-25	1.70E-30	1.33E-16	9.30E-24	3.59E-17	3.38E-14	6.22E-49
RB	12175	13809	VTs	T30	1.69E-14		9.47E-14	2.07E-14	4.07E-16	6.78E-17	9.21E-26
NZ-B18	13154	13605	RB	T68	6.46E-18	5.55E-16	9.67E-12	6.39E-05	6.17E-05	3.21E-04	
CT14A	16667	17410	VT	T3	7.75E-12	1.21E-07	4.13E-07	1.20E-04	2.65E-05	2.56E-04	
NZ-M16	36	11532	VT	T3	2.82E-98	1.99E-85	7.43E-88	4.78E-40	8.65E-05	2.10E-43	5.70E-158
HA16-1	21	11281	HA18-9	T68-1	1.84E-317	3.84E-303		3.43E-42	1.12E-54	9.18E-74	5.78E-218
Asian VTs	4368	5695	T30	VTs	6.60E-04		1.25E-02	9.71E-08	4.29E-08	2.81E-08	2.20E-02
T68-1	7	10848	Unknown (itself?)	VTs	7.44E-160	7.08E-90	2.17E-66	1.39E-46	4.01E-46	1.00E-34	6.53E-168
Kpg3	10804	14424	T3	VTs	4.31E-52	6.31E-37	1.17E-37	5.04E-27	6.46E-29	6.67E-29	4.56E-75
Kpg3	14858	16000	T3	VTs	8.65E-24	2.50E-16	3.28E-26	1.10E-09	1.47E-09	2.35E-11	3.30E-27
Kpg3	17438	18040	T3	VTs	1.61E-08	2.03E-03	7.09E-09			6.19E-03	
HA18-9	15781	17434	VT	NZRB-TH28	1.63E-85	7.03E-64	2.66E-86	1.87E-13	8.29E-08	4.25E-18	4.61E-26

mild and severe strains (Moreno et al., 2008). It is only with the advent of sequencing, over the past quarter century, that strain classification was applied on a genetic basis.

In this study we apply the label "strain" to describe a single phylogenetic lineage, which implies a high level of sequence identity and a shared evolutionary history. It is important to reiterate here that one cannot apply a phenotypic label, such as a "seedling yellows" or a "stem pitting" isolate on a genetic basis alone. Phylogenetic analysis indicated the existence of at least six extant strains, named T36, VT, T3, RB, T68, and T30; the recombinant isolate HA16-5 (Melzer et al., 2010) represents a potential seventh strain, although until homologs are found this remains speculative. An examination of genomes of these six strains indicates that their evolutionary history is a complex mixture of diversification, with differential selective pressures operating within and between genes, as well as between strains, of extensive recombination, and adaptation to an ever changing environment. How this process occurred is described in the following discussion.

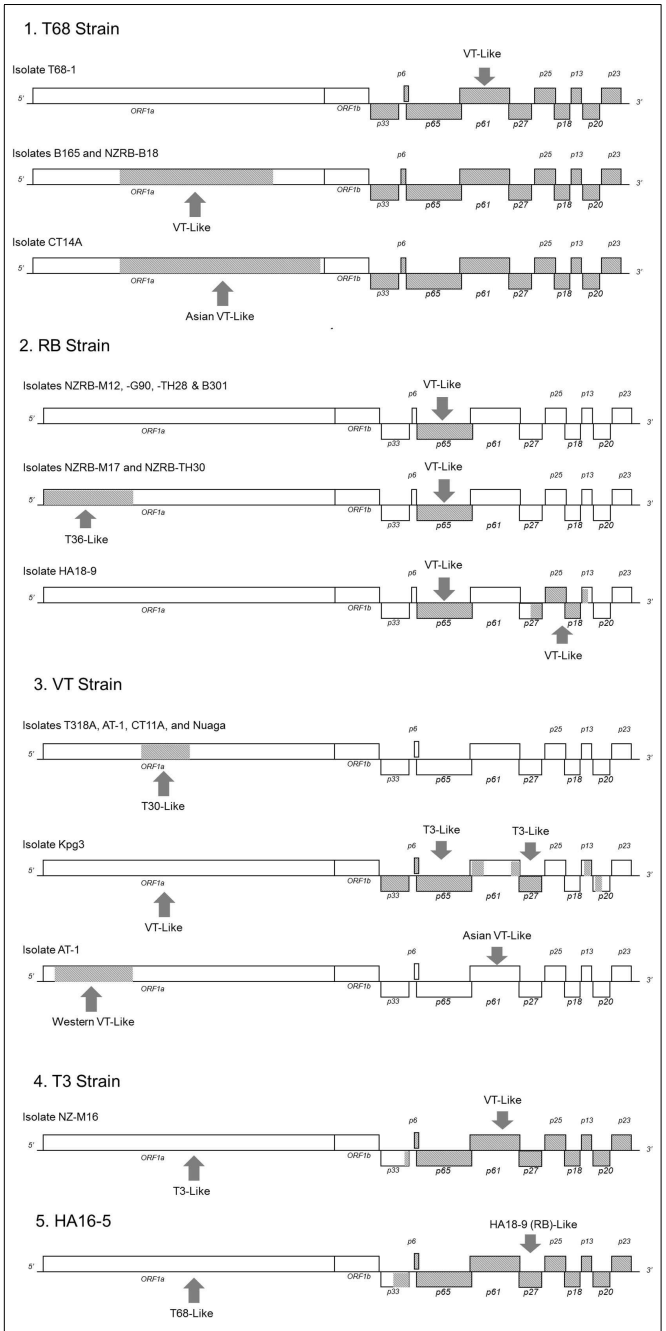

FIGURE 4 | Location and origin of the recombination events present in CTV isolates examined in this study, divided by strain.

THE EVOLUTION OF *CITRUS TRISTEZA VIRUS* STRAINS

The adaptive landscape, first proposed by Wright (1932) is a means of projecting all possible mutations and gene combinations of a species or population onto a topography on which selective pressures from the environment create fitness peaks and valleys (Wright, 1988; Pigliucci, 2008). The combined processes of mutation, selection, and drift drive a species or population across this landscape. In essence, to explore the landscape is to evolve. If we apply this metaphor to CTV, can we reconstruct the evolutionary history, the processes and selective pressures that have produced the six extant CTV lineages?

First, we must consider whether there was either a single, common ancestral proto-CTV sequence that has diversified, or whether there were multiple introductions of a proto-closterovirus into citrus. Evidence for the latter is subject to conjecture as only the 5′ half of the genome supports this hypothesis, due to the conservation of sequence in the 3′ half of the genome (Mawassi et al., 1996; Hilf et al., 1999). It has been proposed that this asymmetry results from the recombination between a proto-CTV isolate and an unknown closterovirus (Karasev, 2000), which is plausible as recombination between different virus species or families has been observed in both animal (Maori et al., 2007; Davidson and Silva, 2008) and plant viruses (Fernandez-Cuartero et al., 1994; Tan et al., 2004; Tiendrebeogo et al., 2012), and is particularly common amongst luteoviridis (Gibbs and Cooper, 1995; Smith et al., 2000). Recombination, particularly between species, allows a distinct shift in evolutionary trajectory (Sztuba-Solinska et al., 2011), moving the sequence across the adaptive landscape. Such shifts cannot occur by stepwise mutation alone, unless the selective constraints are relaxed, for stabilizing selection will tend to keep a population grouped around an adaptive peak, where any non-neutral mutant is likely to have lower fitness, and to shift between peaks will require multiple mutations to pass through a "valley" of lower fitness (Wright, 1988; Pigliucci, 2008), a cost avoided by recombination.

The extant recombinant CTV sequences HA16-5 (Melzer et al., 2010) and NZ-M16, as well as the T68 and RB strains (Harper et al., 2010), indicate that 5′-3′ recombination events are common, and the ORF1b-p33 junction may represent a selectively favored site for recombination as has been observed in other virus species (Smith et al., 2000; Ohshima et al., 2007). If we consider VT, T3, and T30, which share 90.6% nucleotide identity in ORF1a to be descendants of one proto-CTV, this suggests that there were two additional proto-CTVs or unknown closteroviruses introduced into citrus, whose descendants are T36 and RB, and T68 and HA16-5 respectively. It is also possible that the strain-specific divergence of ORF1a may be the recombination of the proto-CTV with a CTV-derived defective RNA (dRNA), as dRNAs have been proposed to act as "spare parts" to repair, via recombination, mutated, or non-functional genomic sequences (Batuman et al., 2010). dRNAs are frequently found in mixture with intact CTV isolates (Mawassi et al., 1995; Ayllon et al., 1999) and as they are non-coding and replicated by a helper genome, have the potential to diverge from the parental sequence under neutral selective conditions. Yet evidence for divergence, or eventual mutational meltdown and elimination via Muller's ratchet, is lacking as most CTV dRNA sequences show little change from the parental sequence, suggesting that either the dRNAs were recently generated, or that selection does act upon the dRNAs (Batuman et al., 2010). Indeed, the conservation (Knorr et al., 1991; Graves et al., 1996) and repair of mutant dRNAs (Kim et al., 1993) has been observed, indicating the latter situation is most probable. In the absence of a non-CTV descendant of the hypothetical novel closterovirus, or discovery of strongly divergent dRNAs in citrus, the recombinant origin of the asymmetrical 5′ and 3′ halves cannot be conclusively proven.

The alternative is that there was a single proto-CTV strain whose genes have evolved under differential selective pressure (Mawassi et al., 1996; Karasev, 2000), both within the genome, and between strains, over evolutionary time. That selective pressures are not equal across the genome can be inferred from the FEL and MEME analysis in this study, in which it was found that the percentage of negatively selected sites varied from 19.6 to 54.8% per ORF, and positively selected sites from 0 to 1.9%. Interestingly, the most diverse region of CTV, ORF1a is also under very strong negative selection, with 47.0% of residues under negative selection; similar results were reported by Martin et al. (2009), although current purifying selection can mask episodes of ancestral positive selection (Murrell et al., 2012). The strength of selection is also not consistent within a single gene, for an examination of ORF1a found that, each of the functional domains showed a higher percentage of negatively selected residues and conversely fewer positively selected residues than the inter-domain regions. These selective pressures correlated with an average of 90.8% sequence identity within domains, as opposed to 81.9% in the inter-domain regions.

It has been remarked upon previously (Albiach-Marti et al., 2000; Silva et al., 2012) that CTV is an inherently stable virus with a very low rate of nucleotide substitutions, or rate of evolution, estimated to be 1.73×10^{-5} nucleotide changes, per site, per year based on coat protein sequences (Silva et al., 2012). In this study we observed a rate of 6.67×10^{-5} changes, per site, per year, although only changes fixed in the population across the entire genome were considered, leading to a possible underestimation of the rate. The low nucleotide substitution rate of CTV may be due to linear rather than exponential replication (Silva et al., 2012), loss of fitness in mutants, or due to population size, in which small populations evolve faster than larger populations (Sanjuan, 2012). However, estimating substitution rates assumes a constant rate of evolution, whereas a population may evolve rapidly when confronted with a changing landscape of selective conditions, such as movement into new areas, hosts, or vector systems (Nichol et al., 1993; Holland and Domingo, 1998; Moya et al., 2000).

Higher rates of evolution within specific regions of viral genomes have been observed in the E1/E2 genome region of HCV (Gray et al., 2011), the HA1 domain of *Influenza A virus* (Bhatt et al., 2011), and the coat and HAM1h proteins of *Ugandan cassava brown streak virus* (Mbanzibwa et al., 2011); critically, the rapidly evolving regions are involved in host-pathogen interaction or defense responses, indicating the importance of external factors on evolutionary rates, which will be discussed in the following section.

Did ORF1a and, to a lesser extent, other regions of CTV rapidly diversify in the past, taking separate paths across the adaptive landscape? Evidence from MEME and FEL analysis suggests that this was the case, although it is likely that there was a significant difference in rate between lineages. Differences in evolutionary rate between genotypes of the same species has been observed in the E1/E2 and NS5a genes of HCV subtypes 1a and 1b (Gray et al., 2011), within a beta-barrel epitope of the envelope of *Japanese encephalitis virus* (Murrell et al., 2012) and the coat protein of subgroups 2a, 3a, and 3b of CMV (Moury, 2004). For CTV, MEME analysis found episodic diversifying selection in most ORFs, with the exception of p6. Four genes, p23, p61, ORF1a, and ORF1b had multiple positive selection events in lineages leading to the extant CTV strains. The first two genes are respectively responsible for suppression of silencing, as well as controlling negative strand accumulation (Satyanarayana et al., 2002; Lu et al., 2004), and virion assembly (Satyanarayana et al., 2000) respectively, while the latter two are necessary for replication (Satyanarayana et al., 1999). The diversification of p23 is to be expected, as both host antiviral RNAi genes and viral suppressors of silencing are known to rapidly evolve (Obbard et al., 2009), and strain-specific mutations may be the result of adaptation to specific hosts. The p61 protein is a HSP90-type molecular chaperone, involved in CTV virion assembly (Satyanarayana et al., 2000) and, in other viruses, RNA recruitment and assembly of the viral replication complex (Huang et al., 2012). Plant homologs of p61 have also been implicated in assembling RNA-induced silencing complexes with AGO1 (Iki et al., 2010), therefore strain-specific diversification of CTV p61 may be involved in host-interaction or as a pathogenicity factor. The replication components, ORF1a and 1b have evolved under strong host-specific selection, as they interact with co-opted host RNA-binding proteins and molecular chaperones to form a viral replication complex (Huang et al., 2012; Mine and Okuno, 2012). In addition, the helicase domain of the *Tobacco mosaic virus* (TMV) replicase protein has been found to bind to the host NAC-domain transcription factor, suppressing host defense responses (Wang et al., 2009), suggesting that replication associated proteins have multiple functions, hence multiple selective pressures acting upon them, and this shifting balance will move a sequence across the adaptive landscape.

To summarize, it is possible that CTV evolved through multiple introductions of one or several proto-closteroviruses in citrus and subsequently recombined. Unfortunately this remains hypothetical in the absence of a non-CTV closterovirus descended from one of these proto-closteroviruses. Recombination with a dRNA is also possible, although little is known about how much variation a dRNA can develop whilst still retaining the major functional domains. It is more likely that the divergence observed in ORF1a is the result of an adaptive radiation in different proto-citrus hosts, with a variable evolutionary rate within and between strains. The extent of the divergence differs between the 5′ and 3′ halves of the genome, which is due in part to extensive recombination, discussed later, and to competing selective pressures of adaptation to new host species and new selective peaks, whilst retaining multiple biological functions within and between domains.

PROMISCUOUS RECOMBINATION

Recombination is a significant factor in CTV evolution (Martin et al., 2009), producing variants with potentially different properties to the parental isolates (Sztuba-Solinska et al., 2011), and as mentioned earlier, allowing a shift of evolutionary trajectory. To continue the adaptive landscape metaphor, recombination allows a population to leap from fitness peak to peak if selectively favored, but if not it can be akin to jumping off a cliff, leading to extinction of that genotype. Recombinants have long been known to be a factor in the emergence of new CTV strains; one of the earliest genomes to be sequenced, SY568 from California (Yang et al., 1999) is a known recombinant, from a mixed population (Vives

et al., 1999, 2005), as are B165 (Roy and Brlansky, 2010), Kpg3 (Biswas et al., 2012), all members of the RB (Harper et al., 2010; this study), and T68 genotypes (this study). Recombinants readily occur in mixed infections of CTV strains (Rubio et al., 2001; Scott et al., 2013), which raises the question of why, if recombination can repair defective sequences (Rao and Hall, 1993; Borja et al., 1999), and allow a rapid change in fitness (Sztuba-Solinska et al., 2011) and evolutionary trajectory, recombinants are not found between all CTV strains, and in all regions?

The probability of generating a viable recombinant depends on both viral and host factors. First, it requires that both parental strains be present in the same host, and infect the same cell (Sztuba-Solinska et al., 2011). The recombinant must then be able to replicate and establish a systemic infection. Evidence in this study indicates that there is strain-specific co-evolution in functional domains of ORF1a/1b and, although not investigated, potentially other parts of the genome. Furthermore, the majority of CTV recombinants identified are between isolates of more closely related strains, for example in Kpg3 and NZ-M16 between VT and T3, in NZ-B18 and B165 between T68 and VT, and in isolates NZRB-M17 and TH30 between RB and T36 (**Figure 4**). Recombination events between more diverse strains were rare, the insertion of a VT-like p65 ORF into RB is one example although, as the rest of the 3′ half of that strain is T30-like, it may not be as drastic a change. One may suggest that co-evolution of functional domains within strains is a limitation on which genotypes may form viable recombinants *in vivo* that if not lethal, may at least reduce fitness and prevent the recombinant from becoming fixed in the population. The exception is the complete replacement of the 5′ or 3′ half of the genome, an event that as noted earlier, has produced the RB and T68 strains, as well as isolates NZ-M16, HA16-5, and SY568 (Vives et al., 1999). It may be proposed that complete replacement of the 5′ half avoids a reduction in fitness as all components necessary for replication are replaced en bloc.

The sites at which recombination can occur may be limited to specific hotspots, sites where recombination frequently occurs (Sztuba-Solinska et al., 2011). Such sites have been observed in PPV (Glasa et al., 2002), *Watermelon bud necrosis virus* (Kumar et al., 2010), and *Brome mosaic virus* (Olsthoorn et al., 2002; Shapka and Nagy, 2004); it may be proposed that there is such a site within the CTV region containing ORF1b-p33 (Vives et al., 1999, 2005; Hilf, 2010), although unlike in the aforementioned examples no features that would promote recombination, either stem-and-loop secondary structures (Glasa et al., 2002; Kumar et al., 2010) or AU-rich regions (Shapka and Nagy, 2004) have been identified in CTV at this site (Vives et al., 1999, 2005; Harper unpublished), or surrounding the p65 recombination site present in the RB strain (Harper et al., 2010).

Finally, if viable, the recombinant faces competition with, and selection against, other CTV isolates in the population; this is of particular importance as CTV isolates have been shown to exclude super-infection by closely related sequences (Folimonova et al., 2010). At time of writing one region involved in this response has been identified: the absence of homologous p33 sequence is necessary for super-infection of one isolate by another (Folimonova, 2012). If super-infection exclusion of a newly generated recombinant does occur, this reduces the probability it will become fixed in the population, or be acquired by an aphid vector and transmitted to a new host, thus in all likelihood leading to extinction.

THE SELECTIVE LANDSCAPE

Having established that each gene is evolving under differential selection pressure, and at a different rate, what factors may be at play in determining the topography of the adaptive landscape over which the CTV genotypes have evolved and diversified? We have already mentioned the powerful effect of host-adaptation on the evolution of specific CTV proteins to permit replication and systemic infection, yet little has been said about citrus itself, for diversification in the host is paralleled by diversification in the pathogen. Indeed, host range diversification may be proposed to be a necessary precondition for strain divergence, for two other plant viruses with recognized strain diversification, PPV and CMV, also exhibit significant host diversification, the former infecting many *Prunus* species (Candresse and Cambra, 2006), whilst the latter infects over 1000 herbaceous, shrub, and tree species (Roossinck, 2001). This is not true of all viruses however, for TMV infects species from 30 different families, yet shows little segregation into strains (Kearney et al., 1999); it is possible that the evolution and diversification of viruses into strains differs markedly between those infecting annual hosts that are removed or die at the end of a growing season, and perennial species in which an infection can persist for decades.

The host range of CTV is limited to members of the *Rutaceae,* with the exception of few non-Rutaceous *Passiflora* species (Moreno et al., 2008). All species of the genus *Citrus*, including the commercially important sweet and sour oranges, limes, grapefruit, lemons, and mandarins are susceptible to CTV to some degree (Muller and Garnsey, 1984; Moreno et al., 2008), as are members of the related genera *Microcitrus*, *Clausena*, *Eremocitrus*, *Aegle*, *Aeglopsis*, *Afraegle*, *Citropsis*, *Severinia*, *Swinglea*, and *Atalantia* (Muller and Garnsey, 1984; Yoshida, 1996), although the last three demonstrate some degree of resistance to the virus (Muller and Garnsey, 1984; Garnsey et al., 1987; Mestre et al., 1997), as do *Fortunella crassifolia* and *Poncirus trifoliata* (Mestre et al., 1997). Such a range of host species creates a bewildering array of potential selective factors, peaks, and valleys across the landscape. Each species will differ to some degree in physiology, gene expression, metabolism, and antiviral defenses, and an isolate at an adaptive peak in one host may be less fit in another. For example, CTV isolate T36 has been shown, through use of a GFP-expressing clone, to readily infect *C. macrophylla*, yet have a decreasing gradient of cells infected in *C. sinensis* and *C. paradisi*, to a few scattered cells in *C. aurantii* (Folimonova et al., 2008), which would suggest that T36 has a minimum capacity for replication and movement in *C. aurantii*. Curiously, it has also been found that different combinations of three genes, p33, p13, and p18, are dispensable for infection of *C. macrophylla*, *C. aurantifolia*, *C. sinensis*, *C. paradisi*, *C. micrantha*, *C. latifolia*, and *C. medica* by a T36 clone (Tatineni et al., 2011), while *C. aurantii* requires the genome to be intact, suggesting that each host species exhibits variable selective pressure on different regions of the CTV genome.

There are also differences in virulence between strains, for example T36 isolates can readily infect *C. maxima* cv. "Red Shaddock" pummelo, yet members of the VT and T30 strains take

much longer to produce a detectable infection; suggesting a form of resistance in this cultivar (Hilf, 2005). Differential reactions to CTV strains have also been observed in *P. trifoliata* (Harper et al., 2010) and *C. maxima* (Garnsey et al., 1996) suggesting that host-specificity has contributed to the diversity of strains observed today. Furthermore, it may be proposed that resistance genes have contributed to the emergence of the resistance-breaking or "RB" strain of CTV that can systemically infect *P. trifoliata* (Harper et al., 2010), where the resistant host provides a refuge free of competition from other strains, and a potential reservoir of inoculum to spread to other trees. This is most clearly illustrated with soybean in which three resistance loci against *Soybean mosaic virus* (SMV) exist in different cultivars (Chowda-Reddy et al., 2011), which has led to the evolution of specific strains capable of overcoming a single loci, yet no "super strain" has emerged capable of overcoming all loci at once, as this requires multiple concerted mutation of the CI, HcPro, and P3 genes (Chowda-Reddy et al., 2011). Similar limitations in citrus hosts likely account for no characterized strain being capable of infecting all potential hosts equally.

It has been mentioned earlier that host defenses play a significant role in determining the topography of the adaptive landscape, for example resistance genes in *P. trifoliata* restricting virus movement, whilst selecting for mutants better able to replicate and systemically infect host species, such as the CTV resistance-breaking strain "RB" which can systemically infect *P. trifoliata* (Harper et al., 2010). One host defense mechanism, RNA interference, targets the viral genome for degradation via both host- and pathogen-derived small interfering RNAs (Dunoyer and Voinnet, 2005). Differences in host cellular siRNAs have been proposed to determine whether specific tissues are permissive of viral infection (Dunoyer and Voinnet, 2005). The strength of selection exerted by RNAi on viruses is illustrated by the prevalence of virally encoded suppressors of silencing, which are found in potexviruses, potyviruses, cucumoviruses, and closteroviruses (Dolja et al., 2006). CTV encodes three suppressors of silencing: p25, p20, and p23 (Lu et al., 2004), of which the latter two were observed in this study to show significant variation between strains, and frequent episodic diversifying selection, suggesting that there is constant adaptation to changes in the siRNA complex within and between hosts, in effect, an "arms race" (Obbard et al., 2009). Silencing itself can affect the evolution of viral genomes in two ways, by selecting for "escape" mutations that alter the target sequence and prevent recognition and degradation (Leonard et al., 2008), or by selecting for nucleotide compositional changes in the viral genome to match host mRNAs (Dunoyer and Voinnet, 2005); the latter has been observed in CTV (Cheng et al., 2012) and may be a significant genetic barrier to the divergence of CTV strains, and also a potential explanation for the absence of intermediate sequences between the major lineages, as an isolate cannot occupy all possible permutations of sequence space (Roossinck and Schneider, 2006; Domingo et al., 2012).

Finally, in the absence of human intervention, the only means by which CTV is transmitted is by aphid species (Roistacher and Moreno, 1990). The aphid vector species exerts selective pressure on CTV isolates by selectively transmitting some isolates or strains rather than others, for example T3 was transmitted by rates of between 19 and 30%, using *Aphis gossypii* (Bar-Joseph et al., 1977), while NZ-M16, a member of the same genotype was unable to be transmitted by *T. citricida* (Harper et al., 2009). Similarly, *A. gossypii* was capable of transmitting isolates of the VT strain whereas *Toxoptera aurantii* and *A. spiraecola* could not (Raccah et al., 1976). These data suggest co-evolution with specific vector species, likely those prevalent in the region, and those that feed on the host species, in which the strain originated. The same vector species will also transmit strains or isolates at different rates, suggestive of strain-specific co-evolution, for example Raccah et al. (1980) reported rates of transmission for a series of Israeli isolates of between 5.6 and 37.5% with *A. gossypii*, while Broadbent et al. (1996) reported transmission rates of between 5 and 55% with *T. citricida* in Australia. Aphid transmission is particularly important for the evolution of new or novel variants of a strain, for as mentioned earlier, weakly negative, neutral, or even positively selected variants or recombinants may not reach fixation, reducing the probability of transmission, without which it will become extinct with the death of the host. Aphid transmission also acts as a bottleneck, removing a proportion of the quasispecies from the source plant, to a new host where it may evolve in a different direction from the original population (Domingo et al., 2012). The T36 isolate, originally extracted by aphid transmission from a severely declining field tree in Florida in 1975 (S. Garnsey, personal communication) is an example of this phenomenon for it is less pathogenic than most T36 strain field isolates, which could be considered a neutral or positively selected trait, yet it is also very poorly transmitted by aphid species compared to other isolates of the strain (<1 versus 40–50% success rate) (Harper, unpublished). The separation of this otherwise negatively selected mutant from the original population eliminated much of parental quasispecies and allowed a different evolutionary path to be taken, such that today there is little probability that the original phenotype would be restored, as T36 and highly transmitted field isolates FS577, 701 and 703 differ by 35 non-synonymous substitutions spread across the genome, a significant genetic barrier.

In summary, the adaptive landscape over which CTV strains have evolved and diversified is comprised of host factors, including species, resistance genes, and active host defenses such as RNAi. Vector species also exert significant selection on specific strains, and are important for the persistence and spread of novel variation.

STRAIN CLASSIFICATION AND DIAGNOSIS

As mentioned earlier, CTV isolates have been classified and grouped by their phenotype, virulence, host range, serology, and more recently, using sequence homology of one or more genes (Moreno et al., 2008). Unfortunately, there has been no concerted effort to classify what constitutes a strain, leading to a proliferation of newly sequenced isolates being referred to as new strains with little justification. In addition, the link between genotype and phenotype is also unclear, and while the role of several genes in phenotypic expression has been indicated (Fagoaga et al., 2006; Albiach-Marti et al., 2010; Tatineni and Dawson, 2012), how often minor differences in sequence alter pathogenicity has not, therefore classification based on phenotype such as "stem pitting" or "seedling yellows" strains, or "severe" and "mild" strains is ill-advised.

Complicating matters is a lack of consistency in choosing a region or regions to analyze, with CP, ORF1a/b, and sundry 3′ genes all being targeted in different assays (Hilf et al., 2005; Nolasco et al., 2009; Roy et al., 2010). Diagnosis with the coat protein alone is a historical legacy, as it is the most highly conserved and least variable of the 3′ genes with 93.4% nucleotide identity and 96.3% amino acid identity between isolates examined in this study. Despite a suggestion that its conservation renders any mutation significant (Nolasco et al., 2009), the CP is not reflective of the complete genome and can in no way explain the divergent 5′ and 3′ halves of CTV strains, nor extensive recombination. For example the CP phylogeny of isolates examined in this study groups some T68-like isolates with VT, and splits the Asian VT subgroup, whilst grouping Kpg3, HA16-5, and HA18-1 together (data not shown). The other 3′ genes show differing levels of conservation and variation between strains, and while they may be appropriate for distinguishing one strain from the rest, we have identified no ORFs from which all six extant strains may be distinguished. In contrast, ORF1a/1b is the most suitable region for phylogenetic reconstruction, as divergence between strains, such as between VT and T3, is most apparent in the 5′ end of the genome (Hilf et al., 1999; Albiach-Marti et al., 2000), and contains conserved functional domains (L1, L2, HEL, and MET) that show strain-specific motifs and hence are suitable sites for primer design. Ideally, one would require the complete genome to be amplified to make an accurate diagnosis of strain type and to identify any potential recombinant regions, such as by using small RNA sequencing as in this study, although given the prevalence of mixtures in the field this is neither practical nor cost-effective for large-scale surveys. We do however suggest that future diagnostic assays be designed to (a) amplify multiple sites within both ORF1a/1b, given the frequent recombination, and (b) design specific primers or probes for each strain at the same site to correctly identify potential recombinants.

In this study we have described six strains of CTV named T36, VT, T3, RB, T68, and T30, defined by separation of their complete genome phylogenies, and distance between groups. We further propose, based on genome phylogeny and recombination analysis, that the type isolates for each strain be assigned as follows: T36: isolate T36 (U16304), T3: isolate T3 (KC525952), T30: isolate T30 (AY260651), RB: isolate NZRB-TH28 (FJ525433), and T68: isolate T68-1 (JQ965169). Due to the bifurcation of the VT genotype, we propose that while not divergent enough to separate into novel genotypes, the Asian and Western subtypes of VT be recognized with the type isolates T318A (DQ151548) and FS701 (KC517494) respectively. Isolate HA16-5 (GQ454870) on the basis of sequence appears to be a novel strain, through recombinant in origin; until a similar sequence is found, the classification remains tentative.

It is likely, as with many crop species (Roossinck and Schneider, 2006), that the ancestral population of CTV is far more diverse than what is currently known and only a subset are present in commercially produced citrus, therefore we must establish criteria to determine whether a new sequence is either novel, or a member of one of the presently described genotypes. Firstly, we must discourage the assigning of new or novel strains on the basis of partial or fragmentary sequence; the complete genome is required for accurate placement. To be a novel strain, the complete genome sequence should differ by >7.5% at the nucleotide level, the minimum distance between VT and T3, and by >8% at both the nucleotide and amino acid levels in ORF1a, the minimum distance between VT and T30. Finally, a novel strain must be examined for recombination with the type members of the extant strains listed above, whilst being recombinant in origin does not disqualify a sequence from being novel, it should show the nucleotide or amino acid divergence shown above to be classified.

CONCLUDING REMARKS

The existence of strains, if defined as distinct phylogenetic groups with a shared ancestry as found in PPV (Candresse and Cambra, 2006) and CMV (Roossinck, 2001), is a rare phenomenon amongst plant viruses and is almost unknown amongst the Closteroviridae. CTV is the exception, with at least six extant strains that exhibit a wide range phenotypic characteristics. These strains may have evolved through either a single introduction into citrus and subsequent radiation, or through multiple introductions followed by recombination; which scenario is more likely is obscured through subsequent evolution over time and the absence of extant protoclosterovirus sequences. Regardless of their origin, CTV strains have evolved and diversified across the adaptive landscape, a topology comprised of many host and vector species, that have exerted variable selective pressure on different parts of the genome, and indeed, between strains leading to diversity within non-functional domains regions, such those within ORF1a for example, while the 3′ genes, which include structural and replication associated proteins, are much more conserved. Functional constraints, together with co-evolution of the replication domains and host-selection pressures on codon choice have acted to decrease the likelihood of moving between adaptive peaks by mutation alone. Recombination, rather than mutation, has been shown to be the major factor in CTV strain evolution, producing three of the six extant strains, although evidence suggests that co-evolution reduces the likelihood of recombination between any two strains. Why then, is an understanding of strain evolution important? Knowledge of the selective pressures and constraints acting upon CTV strains is crucial to the development of cross-protection programs, for the development of infectious clones for field release, and for the breeding of new, resistant citrus cultivars. It is hoped that further research into the link between genotype and phenotype will yield significant advances in citrus production.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2013; accepted: 03 April 2013; published online: 23 April 2013.

Citation: Harper SJ (2013) Citrus tristeza virus: evolution of complex and varied genotypic groups. Front. Microbiol. ***4****:93. doi: 10.3389/fmicb.2013.00093*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 26 June 2013
doi: 10.3389/fmicb.2013.00151

Genetic variability and evolutionary dynamics of viruses of the family *Closteroviridae*

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RNA viruses have a great potential for genetic variation, rapid evolution and adaptation. Characterization of the genetic variation of viral populations provides relevant information on the processes involved in virus evolution and epidemiology and it is crucial for designing reliable diagnostic tools and developing efficient and durable disease control strategies. Here we performed an updated analysis of sequences available in Genbank and reviewed present knowledge on the genetic variability and evolutionary processes of viruses of the family *Closteroviridae*. Several factors have shaped the genetic structure and diversity of closteroviruses. (I) A strong negative selection seems to be responsible for the high genetic stability in space and time for some viruses. (2) Long distance migration, probably by human transport of infected propagative plant material, have caused that genetically similar virus isolates are found in distant geographical regions. (3) Recombination between divergent sequence variants have generated new genotypes and plays an important role for the evolution of some viruses of the family *Closteroviridae*. (4) Interaction between virus strains or between different viruses in mixed infections may alter accumulation of certain strains. (5) Host change or virus transmission by insect vectors induced changes in the viral population structure due to positive selection of sequence variants with higher fitness for host-virus or vector-virus interaction (adaptation) or by genetic drift due to random selection of sequence variants during the population bottleneck associated to the transmission process.

Keywords: *Closterovirus*, *Crinivirus*, *Ampelovirus*, recombination, selection, phylogeny, gene flow

INTRODUCTION

There are five basic mechanisms determining the genetic structure and evolution of biological populations: mutation, recombination, natural selection, genetic drift, and migration (Moya et al., 2004). RNA viruses have a great potential for high genetic variability, rapid evolution and adaptation to new conditions and environments due to their rapid replication, generation of very large populations, and high mutation rates (at least 10^5 times higher than those of their hosts) as a consequence of the lack of proofreading activity of RNA polymerases (Holland et al., 1982; García-Arenal and Fraile, 2011). In many of these viruses, genome recombination and/or reassortment of genomic segments (pseudorecombination) between divergent virus strains or virus species increase genetic variability and accelerate evolution (Chare and Holmes, 2004; Nagy, 2008). The genetic variation generated by mutation and recombination is limited and structured by the other three evolutionary forces: natural selection, genetic drift, and gene flow (Roossinck, 2003; Moya et al., 2004). Natural selection is a directional process by which variants that are fittest in a certain environment will increase their frequency in the population (positive or adaptive selection), whereas variants less fit will decrease their frequency (negative or purifying selection), this process being determined by numerous specific interactions of viruses with their plant hosts (Schneider and Roossinck, 2001), vectors (Power, 2000; Chare and Holmes, 2004) and even with other viruses co-infecting the same plant. Genetic drift consists of stochastic changes in allele frequencies in a finite population due to the random sampling of genes at reproduction (Moya et al., 2004). This supposes a reduction of the genetic variability and fixation of selectively neutral variants, and it has an important effect during severe and rapid reduction in population size produced by population bottlenecks or founder events (Ali and Roossinck, 2008). Genetic drift can occur in different events of the virus life cycle such as virus movement between plant cells (Sacristan et al., 2003; Li and Roossinck, 2004), transmission between plants by vectors (Ali et al., 2006; Betancourt et al., 2008) and interaction between coinfecting viruses (Fraile et al., 1997). Finally, migration (gene flow) among distinct geographical areas, plants or different parts of the same plant is an important factor shaping the genetic structure of viral populations. High migration favors genetic uniformity between populations and thus decreases the global genetic diversity (Moya et al., 2004). While mutation and recombination are intrinsic of the virus genome and its replication and expression systems, natural selection, genetic drift and gene flow are affected by virus biology (e.g., host type and range, means and extent of dispersal), environmental conditions, and population parameters (e.g., population size and history of population bottlenecks).

The study of genetic variability and the evolutionary mechanisms related to the different aspects of the virus biology is crucial

to understand virus epidemiology and emergence (Grenfell et al., 2004), designing specific diagnostic tools, and developing efficient and durable strategies of disease control (García-Arenal and McDonald, 2003; Acosta-Leal et al., 2011). Several reviews on evolution of the family *Closteroviridae* have been published, which were focused on macroevolution and taxonomy (Dolja et al., 1994; Agranovsky, 1996; Karasev, 2000; Dolja et al., 2006).

The family *Closteroviridae* is composed of viruses characterized by their long (up to 2000 nm) and flexuous, non-enveloped, polar, virions with two coat proteins, the major (CP), covering most of the genomic RNA, and the minor (CPm) located to one of the virion ends (Agranovsky et al., 1995; Febres et al., 1996; Tian et al., 1999). Its members have the largest genomes of all positive sense RNA plant viruses (up to 20 kb). Although the number and relative position of open reading frames (ORFs) vary between species, there is a common genome organization. ORFs 1a and 1b encode replication-related proteins, with protease, methyl-transferase, helicase, and RNA-dependent RNA polymerase conserved domains. Downstream ORFS include a conserved five-gene module encoding a small hydrophobic protein with affinity to cell membranes, a homolog of the plant heat shock proteins HSP70 (HSP70h), a ∼60 kDa protein with a diverged coat protein motif, the CP and the CPm. The functions postulated for the HSP70h are: cell-to-cell movement, involvement in the assembly of multisubunits complexes for genome replication and/or subgenomic RNA synthesis and assembly of virus particles, whereas the ∼60 kDa protein is required for incorporation of both HSP70h and CPm to the particle tail (Tian et al., 1999; Satyanarayana et al., 2000; Alzhanova et al., 2001). The genome expression strategy is based on: (I) proteolytic processing of the polyprotein encoded by ORF 1a, (II) +1 ribosomal frameshift for the expression of ORF1b, and (III) expression of the downstream ORFs via the formation of 3′ co-terminal subgenomic RNAs. Presently, there are three genera in the family (Martelli et al., 2011): *Ampelovirus*, *Crinivirus* and *Closterovirus*, although a new genus named *Velarivirus* has been proposed (Al Rwahnih et al., 2012). The characteristics of the accepted genera are:

- *Ampelovirus*. Mealybug-transmitted, monopartite genome, and the CPm gene is located downstream of CP gene or lacking in some species. Viruses studied here are: *Grapevine leafroll-associated virus* 1 (GLRaV-1), GLRaV-3, GLRaV-5, and GLRaV-11 (tentative member), and *Pineapple mealybug wilt-associated virus* 1 (PMWaV-1).
- *Crinivirus*. Whitefly-transmitted, bipartite or tripartite genome, and the CP gene is located upstream of the CPm gene. Viruses analyzed here are: *Blackberry yellow vein-associated virus* (BYVaV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Potato yellow vein virus* (PYVV), *Sweet potato chlorotic stunt virus* (SPCSV), *Tomato chlorosis virus* (ToCV), and *Tomato infectious chlorosis virus* (TICV).
- *Closterovirus*. Mostly aphid-transmitted, monopartite genome, and the CP gene located downstream of the CPm gene. Viruses studied here are: *Citrus tristeza virus* (CTV) and *Grapevine leafroll-associated virus* 2 (GLRaV-2).

In this work we performed an updated analysis of the genetic variation of viruses in the family *Closteroviridae* by analysis of the coat protein genes using nucleotide sequences retrieved from Genbank and present an updated review on the genetic variability and evolutionary processes of the viral populations of members of the family *Closteroviridae*.

MATERIALS AND METHODS

ALIGNEMENT OF NUCLEOTIDE SEQUENCES

Nucleotide sequences from worldwide isolates of members of the family *Closteroviridae* were retrieved from GenBank (http://www.ncbi.nlm.nih.gov and http://www.dpvweb.net). The coat protein genes (CP or CPm) were selected because they are present in all viruses and because it is the genomic region for which more sequences are available. Only those viruses with sequences of five or more different isolates were analyzed (**Table 1**). Multiple alignment was performed with the algorithm CLUSTAL W (Larkin et al., 2007) implemented in the program MEGA 5.05 (Tamura et al., 2011).

ANALYSIS OF NUCLEOTIDE SEQUENCES

The program MEGA 5.05 was used for: (1) inference of phylogenetic relationships between isolates of each viral species by the neighbor-joining method (Saitou and Nei, 1987), (II) estimation of nucleotide distances between sequence pairs and diversities (mean nucleotide distances) using Kimura-two-parameter as the nucleotide substitution model (Kimura, 1980), and (III) estimation of the ratio between non-synonymous and synonymous substitution (N/S) by the Pamilo-Bianchi-Li method (Pamilo and Bianchi, 1993) to study the role of natural selection at the protein level. $N/S \approx 0$ indicates neutral evolution, $N/S < 1$ negative or purifying selection and $N/S > 1$ positive or adaptive selection.

The program DnaSP 5.10 (Librado and Rozas, 2009) was used to assess genetic differentiation and the gene flow level between different countries or geographic areas with the statistic F_{st} (Weir and Cockerham, 1984). F_{st} can take values from 0, no genetic differentiation and complete gene flow, to 1, complete genetic differentiation as a consequence of null gene flow. Only countries or geographical areas with more than four isolates of the virus analyzed were taken into account.

Recombination between isolates of the same virus was analyzed with the program RDP3 (Martin et al., 2010) that incorporates the recombination-detecting algorithms GENECONV (Padidam et al., 1999), BOOTSCAN (Salminen et al., 1995; Martin et al., 2005), MAXCHI (Smith, 1992; Posada and Crandall, 2001), CHIMAERA (Posada and Crandall, 2001), SISCAN (Gibbs et al., 2000), 3SEQ (Boni et al., 2007), and RDP (Martin and Rybicki, 2000), using their default parameter values. Only those events recognized by at least four different algorithms were accepted as evidence for recombination. The effect of recombination was taken into account during analysis of selection.

RESULTS

GENETIC VARIATION BETWEEN VIRUS ISOLATES

Figure 1 shows the phylogenetic relationships between isolates of each virus species with branch length indicating genetic distances.

Table 1 | Genetic diversity and population genetic parameters of viruses of the family *Closteroviridae*.

Genus	Virus	Genome[a]	NI[b]	NC[c]	World Areas[d]							Population parameters[e]				Diversity within genetic groups[f]					
					N	*S*	*A*	*E*	*M*	*F*	*O*	*M*	*D*	*N/S*	F_{st}	I	II	III	IV	V	VI
Ampelovirus	GLRaV-1	CP	7	5	✓	–	✓	✓	✓	✓	–	0.175	0.093	0.069	N/A[g]	N/A	N/A	0.047	–	–	–
	GLRaV-3	CP	191	9	✓	✓	✓	✓	✓	✓	–	0.256	0.058	0.073	0.092	0.009	0.018	0.037	–	–	–
	GLRaV-4	CP	5	4	✓	–	–	–	✓	✓	–	0.391	0.254	0.096	N/A	0.075	N/A	N/A	–	–	–
	GLRaV-5	CP	79	5	–	✓	–	✓	–	–	–	0.089	0.058	0.171	0.172	–	–	–	–	–	–
	GLRaV-11	CP	15	2	–	✓	–	✓	–	–	–	0.171	0.109	0.127	N/A	0.008	0.006	0.030	N/A	0.073	–
	PMWaV-1	CP	6	5	✓	–	–	–	–	✓	–	0.016	0.008	0.353	N/A	–	–	–	–	–	–
Crinivirus	CYSDV	CP	41	13	✓	–	–	✓	✓	✓	–	0.108	0.030	0.008	0.955	0.003	0.002	–	–	–	–
	PYVV	CP	9	2	–	✓	–	–	–	–	–	0.021	0.012	0.214	N/A	–	–	–	–	–	–
	SPCSV	CP	39	8	–	✓	✓	–	–	✓	–	0.379	0.032	0.151	0.327	0.014	N/A	–	–	–	–
	ToCV	CP	23	12	✓	✓	✓	✓	✓	✓	–	0.021	0.011	0.111	0.560	–	–	–	–	–	–
	TICV	CPm	7	5	✓	–	–	✓	–	✓	–	0.007	0.004	0.250	N/A	–	–	–	–	–	–
	BYVaV	CP	34	1	✓	–	–	–	–	–	–	0.058	0.031	0.009	0.225	–	–	–	–	–	–
Closterovirus	GLRaV-2	CP	55	7	✓	–	✓	✓	–	✓	–	0.375	0.128	0.161	0.150	0.022	0.030	0.011	0.008	0.009	N/A
	CTV	CP	577	47	✓	✓	✓	✓	✓	✓	✓	0.124	0.073	0.158	0.373	–	–	–	–	–	–

[a] *Genomic region analyzed: CP, coat protein; CPm, minor coat protein.*

[b] *NI, number of isolates analyzed for each virus.*

[c] *NC, number of countries where the virus isolates were collected.*

[d] *World Areas: N, North America; S, South America; A, Sub-Saharian Africa; E, Europe + Mediterranean Africa; M, Middle east; F, Far East; O, Oceania.*

[e] *Population parameters: M, maximum nucleotide distance between isolate pairs; D, diversity (mean nucleotide distance between isolate pairs), F_{st} = measure of gene flow.*

[f] *Diversity within genetic groups. Groups included isolates that had nucleotide distances higher than 0.1 with respect to all isolates of the other groups.*

[g] *N/A, not applicable.*

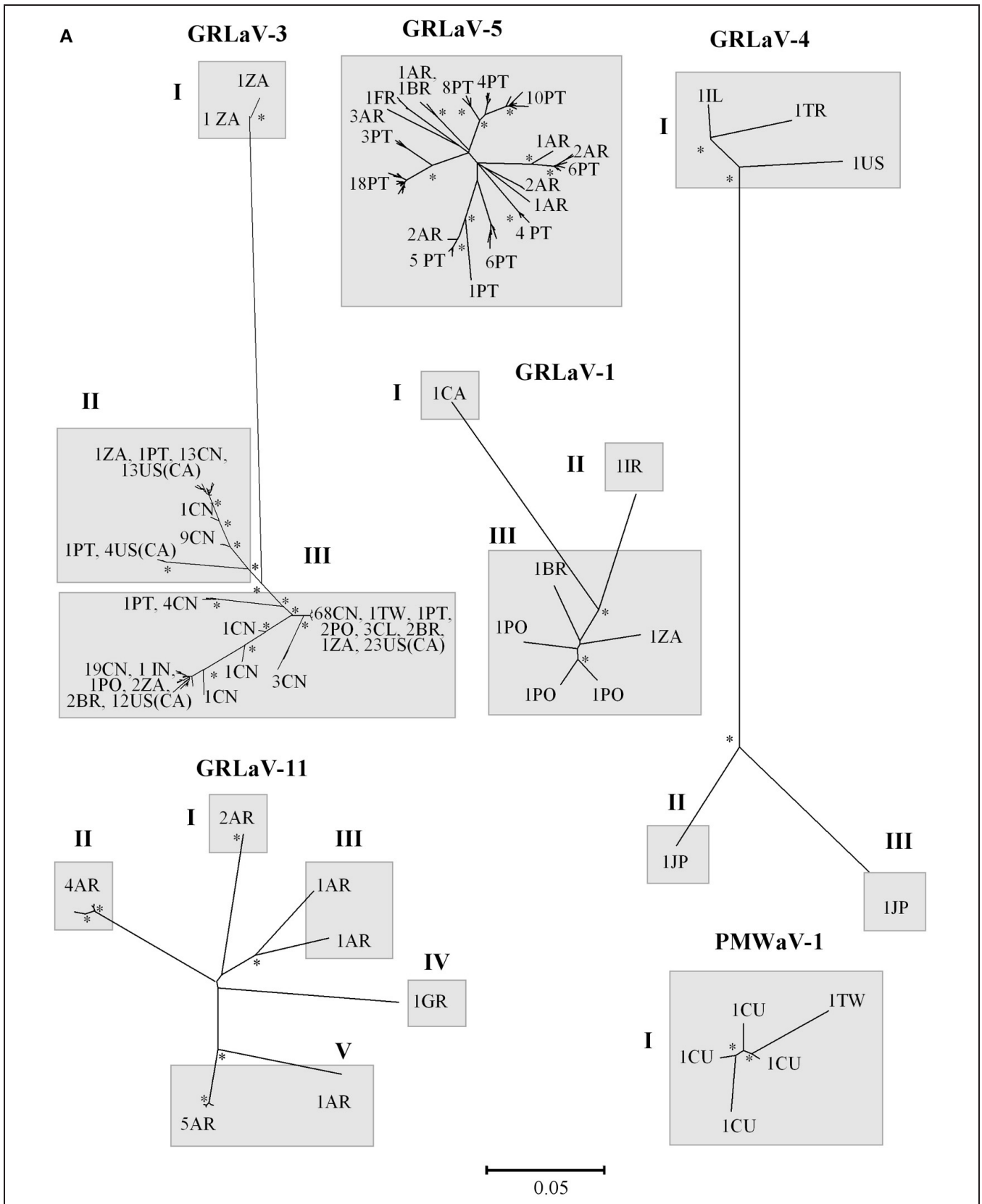

FIGURE 1 | Continued

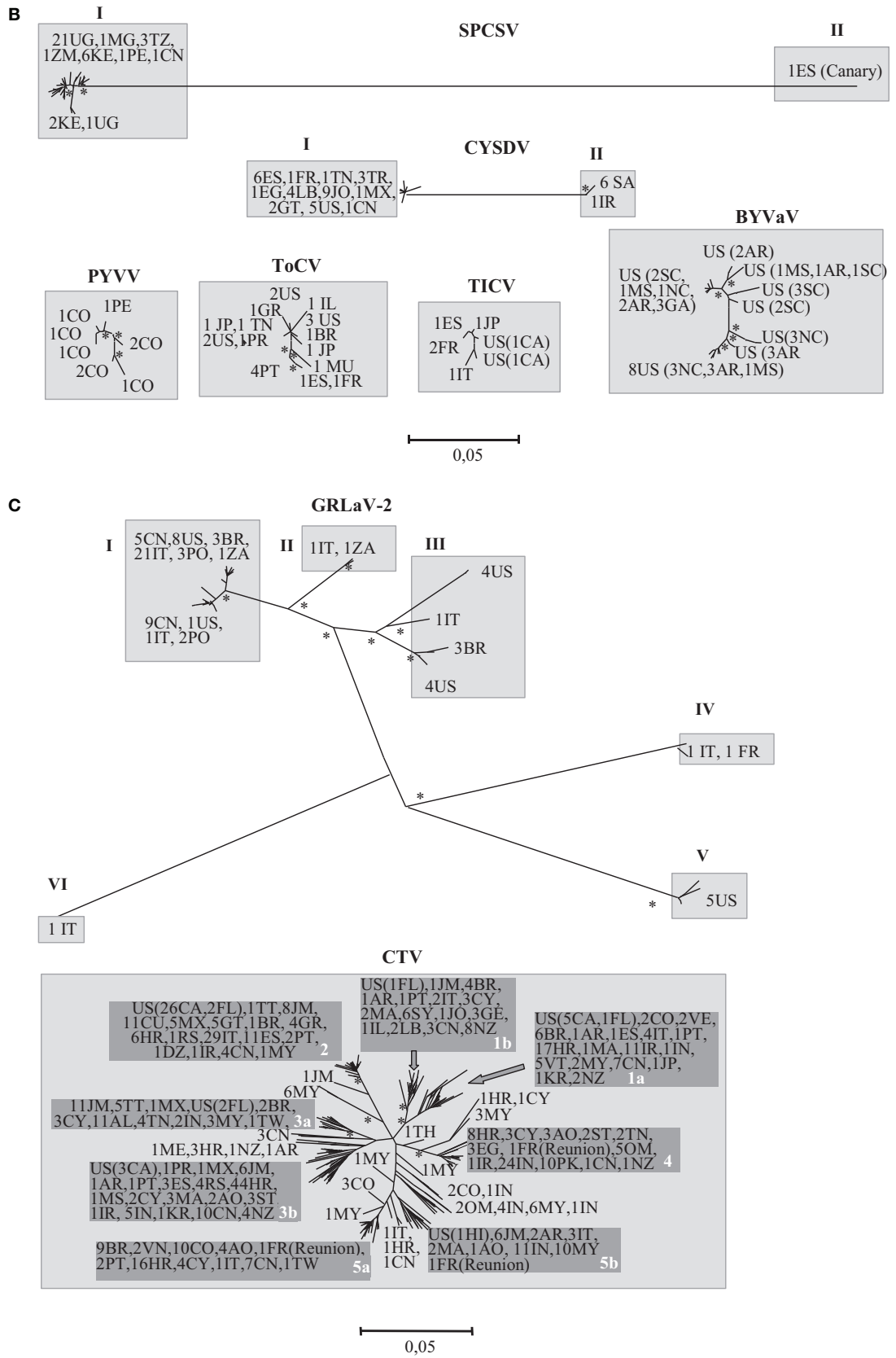

FIGURE 1 | Continued

FIGURE 1 | Neighbor-joining phylogenetic trees of the coat protein genes of different viruses of the family *Closteroviridae*. Bootstrap values higher than 0.75 are indicated with asterisks. Two-letter codes indicate countries (defined in ISO 3166-1) and/or US states. Numbers preceding the codes indicate the number of isolates analyzed from each country. For each virus, genetic groups are indicated in gray boxes with Roman numerals and include virus isolates having nucleotide distances higher than 0.1 with all isolates from other clades. Subgroups or clades in CTV are indicated in darker boxes with Arabic numbers. **(A)** Genus *Ampelovirus*, **(B)** Genus *Crinivirus*, and **(C)** genus *Closterovirus*.

For each virus, isolates were classified into genetic groups considered as those clades with all isolates having nucleotide distances higher than 0.1 with respect to all isolates of the other clades. These groups are indicated in gray boxes. **Table 1** shows the nucleotide diversities and other population genetic parameters.

In the genus *Ampelovirus*, four out of the six viruses studied comprised three to five genetic groups with distances between isolates of up to 0.391 (**Figure 1A** and **Table 1**). GLRaV-1 sequences (Alabi et al., 2011) formed three genetic groups with isolates from: (I) Canada, (II) Iran, and (III) Brazil, Poland and South Africa. GLRaV-3 sequences (Turturo et al., 2005; Fajardo et al., 2007; Chooi et al., 2009; Fuchs et al., 2009; Jooste et al., 2010; Gouveia et al., 2011; Sharma et al., 2011; Wang et al., 2011; Bester et al., 2012; Farooq et al., 2012) also fell into three groups including isolates from: (I) South Africa, (II) California, Portugal, South Africa and China, and (III) California, Brazil, Chile, Portugal, Poland, South Africa, China and Taiwan, with group I diverging much more than groups II and III, and with a maximum nucleotide distance between isolates of 0.256 (**Table 1**). GLRaV-4, in spite of having CP sequences of only five isolates (Saldarelli et al., 2006) was composed of three genetic groups with isolates from: (I) Israel, Turkey and USA, (II) Japan, and (III) Japan, with group I diverging more than groups II and III and with a maximum nucleotide distance of 0.391. This was the most variable member of the family with a nucleotide diversity value of 0.254 (**Table 1**). GLRaV-5, with most isolates from Portugal and Argentina, showed moderate genetic variability and all isolates clustered in a single genetic group. GLRaV-11, in spite of having sequences of only seven isolates (six from Argentina and one from Greece), was composed of five genetic groups: four from Argentina and one from Greece, with a nucleotide diversity value of 0.109 (**Table 1**). Finally, for the pineapple-infecting PMWaV-1, the CP sequences available include four isolates from Cuba and one from Taiwan which were similar and formed a unique genetic group.

The genus *Crinivirus* had the lowest genetic variability of the three genera, with nucleotide diversity values lower than 0.033 (**Table 1**). Four viruses were composed of isolates clustered in a unique genetic group and two viruses had isolates clustered in two divergent genetic groups (**Figure 1B**), with isolates within each group having very low variability (diversity below 0.015). The blackberry-infecting BYVaV isolates, all from USA, presented very low genetic variation. The cucurbit-infecting CYSDV was composed of two genetic groups located in: (I) Middle East (Iran and Arabia) and (II) Mediterranean Basin (South Europe, North Africa and Near East), North America and China. Each group had a very low nucleotide diversity (**Table 1**; Rubio et al., 1999, 2001a; Marco and Aranda, 2005; Sweiss et al., 2007). The potato-infecting PVYV was restricted to South America and presented very low genetic variation. The sweet potato-infecting SPCSV had two very divergent genetic groups with a nucleotide distance of up to 0.379 (**Table 1**): (I) located predominantly in Eastern Africa, but also with isolates in Peru and China; and (II) located in Western Africa. In our analysis the Western Africa group was represented by only one isolate from the Canary Islands, but analysis of the HSP70h gene of several isolates from Nigeria showed that they were also part of the Western Africa group which diverged from the East Africa group (Fenby et al., 2002). Finally, the tomato-infecting TICV and ToCV showed very low genetic variation in spite of their wide distribution. TICV isolates were from USA, Europe and Japan; and ToCV isolates from Europe, North America, Mediterranean Basin, Africa (Mauritius) and Japan.

In the genus *Closterovirus*, the grape-infecting GLRaV-2 showed high genetic variation (Bertazzon et al., 2010b; Jarugula et al., 2010) like the grape-infecting ampeloviruses, with a maximum nucleotide distance between isolates of 0.375 and a nucleotide diversity of 0.128 (**Table 1**). GLRaV-2 sequences were classified into six genetic groups including isolates from: (I) China, USA, Brazil, Italy, Poland, and South Africa, (II) Italy and South Africa, (III) USA, Italy, and Brazil, (IV) Italy and France, (V) USA, and (VI) Italy (**Figure 1C**). Finally, the citrus-infecting CTV, the best studied virus in the family, with CP sequences of almost 600 worldwide isolates (Albiach-Martí et al., 2000; Rubio et al., 2001b; Alavi et al., 2005; Roy et al., 2005; Sorrentino et al., 2005; Papayiannis et al., 2007; Iglesias et al., 2008; Jiang et al., 2008; Černi et al., 2009; Herrera-Isidrón et al., 2009; Oliveros-Garay et al., 2009; Fisher et al., 2010; Harper et al., 2010; Matos et al., 2013), showed a moderate genetic variability. All isolates fell into a single genetic group, albeit this could be divided into five to seven subgroups (**Figure 1C**), some of them very homogeneous (isolates with almost identical sequences). There was no association between the subgroups and the geographic origin of CTV isolates. In our analysis, all biologically characterized isolates in the subgroup 1a were severe and induced stem pitting in sweet orange and/or grapefruit, whereas those characterized in the subgroup two were mild, inducing only weak symptoms in Mexican lime. Other subgroups included mild and severe isolates with no association between symptoms and genetic distance between isolates being observed. For example, about 43, 70, 40, 25, and 19% of the biologically characterized isolates in subgroups 1b, 3b, 3a, 4, and 5a, respectively, were mild, and 57, 30, 60, 75, and 81%, respectively, were severe and incited stem pitting in grapefruit or sweet orange (Alavi et al., 2005; Papayiannis et al., 2007; Černi et al., 2008; Harper et al., 2009; Nolasco et al., 2009; Biswas et al., 2012; Hančević et al., 2013).

RECOMBINATION

Most of the analyzed viruses showed no recombination in the CP (**Table 2**). GLRaV-3 had 14 isolates out of 191 with a recombinant

Table 2 | Recombination events detected in the coat protein genes of viruses of the family *Closteroviridae*.

Genus	Virus	Genome[a]	NI[b]	NR[c]	RE[d]	Sites[e]
Ampelovirus	GLRaV-1	CP	7	0	0	–
	GLRaV-3	CP	191	14	3	X-500, 450-X, 300-X
	GLRaV-4	CP	5	1	1	500-X
	GLRaV-5	CP	79	0	0	–
	GLRaV-11	CP	15	4	1	400–500
	PMWaV-1	CP	6	0		–
Crinivirus	CYSDV	CP	41	0	0	–
	PYVV	CP	9	0	0	–
	SPCSV	CP	39	0	0	–
	ToCV	CP	23	0	0	–
	TICV	CPm	7	0	0	–
	BYVaV	CP	34	0	0	–
Closterovirus	GLRaV-2	CP	55	0	0	–
	CTV	CP	577	63	2	200-X, 400-X

[a] *Genomic region analyzed: CP, coat protein; CPm, minor coat protein.*

[b] *NI, number of isolates analyzed for each virus.*

[c] *NR, number of recombinant isolates.*

[d] *Number of different recombination events.*

[e] *Approximate recombination sites. X means an unknown recombination site outside of the coat protein gene.*

CP which resulted from three different recombination events involving different parental sequences and recombination sites (approximate nucleotide positions 300, 450, and 500 of the CP gene). All recombinations involved isolates from China, except one that had a parental sequence from Chile. GLRaV-11 had four isolates out 15 with the same recombination at nucleotide position 500 involving the same parental sequences. CTV comprised 63 out of 577 isolates with a recombinant CP resulting from two recombination events with respect to the parental sequences and recombination sites (**Table 2**). CTV isolates from South America, North America, Africa, and Asia showed the same recombination indicating that this corresponds to an ancient event that occurred before the recombinants spread worldwide.

NATURAL SELECTION

The ratio of non-synonymous to synonymous substitutions (N/S values in **Table 1**), were low indicating functional or structural constraints for amino acid changes. The selection pressure was particularly strong for CYSDV, with N/S = 0.008 and composed of two genetic groups separated by a genetic distance of about 0.1. Indeed only 0.2% of the nucleotide changes between the two groups produced amino acid changes. In some cases the number of synonymous substitutions was very low (not shown) suggesting also constraints for nucleotide changes that could affect thermodynamic stability of RNA, codon usage bias for translation efficiency, secondary structure, activation of gene silencing, or RNA-RNA or RNA-protein interactions (Cuevas et al., 2012).

MIGRATION (GENE FLOW)

The presence in one geographic region of a diverse virus population with some isolates similar to those of other regions is usually indicative of a possible dispersion center for that virus (Bateson et al., 2002). Our analyses showed that GLRaV-3 had isolates from South Africa in the three genetic groups making plausible to consider South Africa or nearby areas as a dispersion center of this virus. However, this could be a secondary dispersion center since South Africa is far from the host (genus *Vitis*) origin region and likely initial dispersion area. GLRaV-11 had four genetic groups with isolates from Argentina and one group with one isolate from Greece, suggesting that Argentina could be a dispersion center for this virus. Finally, GLRaV-2 had six genetic groups with isolates from Italy distributed in five groups and isolates from USA in three groups which pointed at both countries as possible dispersion zones. Another explanation is that the locations with divergent isolates received them via importation of infected grapevine material from different areas. In most cases, the phylogenetic relationships among viral isolates were not geographically structured and often isolates from distant regions were genetically very close (**Figure 1**), indicating long distance movement probably by international traffic of virus-infected plant material (Rubio et al., 2001b; Angelini et al., 2004; Alabi et al., 2011).

To estimate the degree of virus migration or gene flow, the statistic F_{st} was calculated (see above). The global gene flow was high for GLRaV-3, moderate for GRLaV-5 and GRLaV-2, and low for criniviruses and CTV (**Table 1**). A more detailed analysis was performed by comparing virus subpopulations of different areas two by two (not shown). GRLaV-1 subpopulations from California, Washington, and New York (Alabi et al., 2011) had an infrequent genetic exchange. CYSDV has a null gene flow between the Middle East and the rest of the world, but the gene flow was very high between Spain, Near East, and North America ($F_{st} = 0.000$), albeit this could be due to a unique migration event given the genetic stability of this virus. SPCSV showed also a high geographical structure between the two genetic groups, but availability of only one isolate in one group precluded gene flow analysis between both groups. In group I, the comparison of three neighboring countries of East Africa showed a high gene flow between Uganda and Kenya ($F_{st} = 0.027$) but very low between them and Tanzania ($F_{st} \approx 0.450$). In spite of its low genetic diversity, ToCV showed a low gene flow between Europe and North America ($F_{st} = 0.560$). GRLaV-2 with a moderate global flow showed a puzzling migration pattern which did not correspond to geographic proximity. Thus, extensive gene flow occurred between Poland and China and between Italy, Brazil and USA but this was very low between Italy and Poland. Finally, CTV, the best documented virus with isolates from 47 countries showed different degrees of gene flow which were not correlated with geographic distance. Some subpopulations had a high gene flow ($F_{st} < 0.100$), e.g., California, Mexico, Spain, Italy, and Portugal, or Brazil, Angola, China, and Portugal, whereas other subpopulations were almost isolated ($F_{st} > 0.300$) such as Cuba (although with a moderate gene flow, $F_{st} \approx 0.150$, with Mexico and Guatemala), Trinidad and Tobago, and Argentina. Several reports showed that genetically and biologically divergent isolates of CTV have been introduced one or several times in Iran, Sicily (Italy), Cyprus, and Dominican Republic (Alavi et al., 2005; Davino et al., 2005; Papayiannis et al., 2007; Matos et al., 2013).

DISCUSSION

The genetic variability for each virus analyzed was different, although it must be taken into account that in some cases the number of sequences available was low and/or these were from a few geographical locations. The most variable viruses were the grapevine-infecting ampeloviruses GRLaV-1, 4, and 11 and the closterovirus GLRLaV-2, whereas criniviruses showed very low genetic variation. In general, the viruses of the family *Closteroviridae* showed a low genetic diversity or were comprised of genetic groups with very low within-group nucleotide diversity, as found in many other plant viruses (García-Arenal et al., 2001), due to strong negative selection. The existence of a few genetically homogeneous genetic groups suggests that the sequence space of these viruses may be restricted to a few narrow peaks in the adaptive or fitness landscape (Wright, 1932). A high level of covariation at molecular level (the coordinated change of certain nucleotides in response to the change of other nucleotides to maintain biologically relevant structures and functions) could explain this discontinuous adaptive landscape (Gultyaev et al., 2000). Reduced fitness of chimeras between CTV strains from different genetic subgroups occupying distinct adaptive peaks (Satyanarayana et al., 1999) support this notion. The temporal genetic stability reported for CTV (Albiach-Martí et al., 2000; Rubio et al., 2001b; Silva et al., 2012) and CYSDV (Marco and Aranda, 2005; Rubio et al., 2001a) supports the existence of a strong negative selection. There have been many studies trying to associate genetic relationships and the pathogenicity characteristics of CTV variants (Sambade et al., 2003; Hilf et al., 2005; Nolasco et al., 2009). Although sequence of the CP gene does not appear associated with pathogenicity characteristics in many subgroups, separation between mild and severe stem pitting isolates found in other genomic regions and analysis of phylogenetic networks suggest that pathogenicity has been an important evolutionary force in CTV populations (Martin et al., 2009).

Viruses of the family of *Closteroviridae* are transmitted by insect vectors which favor mixed infections between different viruses or strains of the same virus. Mixed infections may have important evolutionary implications since they can affect the within-isolate population of virus variants (quasispecies) and allow interaction and/or recombination between different virus entities which can affect to pathogenicity and adaptability.

Recombination has played an important role in the evolutionary history of the family *Closteroviridae*. It has been postulated that the ancestor of this family was a smaller filamentous virus composed of three genes encoding replication-associated proteins, the p6-like movement protein and a single coat protein (Dolja et al., 2006). During evolution new genes were incorporated to the genome by two means: (i) recombination with cellular mRNAs, e.g., HSP70h, or with RNAs of other viruses, e.g., the leader proteinase (L-Pro) from potyviruses, and (ii) unequal recombination between two genomic RNA copies (or involving subgenomic RNAs) of the same virus which produced gene duplication, e.g., the genes encoding the ∼60 kD and CPm proteins evolved after being generated as duplicates of the CP gene. Recent cases of recombination-mediated gene gain have occurred in the criniviruses SPCSV and *Beet pseudoyellows virus* (BPYV), both with isolates differing in the number of genes (Tzanetakis and Martin, 2004; Cuellar et al., 2008). Unequal recombination also generated the multipartite genome of criniviruses.

Our analyses indicated homologous recombination in the coat protein gene between divergent genetic variants of GLRaV-3, GLRVaV-11, and CTV. It is clear that more recombination events would be found if additional genomic regions were analyzed. Homologous recombination between divergent isolates of the same virus has been reported for CTV (Rubio et al., 2001b; Sambade et al., 2003; Hilf, 2005; Roy et al., 2005; Vives et al., 2005; Weng et al., 2007; Gomes et al., 2008; Martin et al., 2009; Roy and Brlansky, 2009, 2010; Harper et al., 2010; Melzer et al., 2010; Scott et al., 2013), GLRaV-2 (Alabi et al., 2011) and *Raspberry leaf mottle virus* (McGavin and MacFarlane, 2010); the crinivirus BYVaV (Poudel et al., 2012) and the ampeloviruses GLRaV-3 (Turturo et al., 2005), GLRaV-4 (Thompson et al., 2012) and GLRaV-5 (Turturo et al., 2005; Farooq et al., 2012). Moreover, phylogenetic network analysis showed that homologous recombination must be an important evolutionary force for CTV (Martin et al., 2009). Population analyses showed CTV isolates containing a heterogeneous population of diverged virus strains and recombinants at low frequency (Vives et al., 2005; Weng et al., 2007; Scott et al., 2013), suggesting that these recombinants did not have a selective advantage (more fitness) with respect to the parental sequences. In some cases artificial chimeras of two genetically and biologically divergent CTV isolates failed to infect citrus (Satyanarayana et al., 1999) suggesting that only some recombinants are viable. Also, homologous recombination seems to have occurred between CTV and another unknown closterovirus given the unusual disparity in the divergence of CTV isolates between the two halves of CTV genome: ∼0.1 for the 3′-moiety and ∼0.3 for the 5′-moiety (Mawassi et al., 1996; Vives et al., 1999).

Unequal recombination seems to occur frequently during replication as evidenced by the large number of defective RNAs (D RNAs) detected in the closterovirus CTV (Mawassi et al., 1995; Ayllón et al., 1999a; Mawassi et al., 2000; Che et al., 2002, 2003), the criniviruses *Lettuce infectious yellows virus* (LIYV) (Rubio et al., 2000b, 2002), PYVV (Livieratos et al., 2004; Eliasco et al., 2006) and SPCSV (Kreuze et al., 2002; Cuellar et al., 2008), and the ampelovirus GLRaV-3 (Ling et al., 1998). D RNAs are deletion forms of virus genomic RNAs that retain the replication signals but require the parental virus for replication. The existence of direct repeats, secondary structure or AT-rich regions at the junction site of some D RNAs suggested template-switching as a plausible mechanism for recombination (Ayllón et al., 1999a; Rubio et al., 2000b). In this model the D RNAs are generated by a translocation event in which the polymerase, together with the nascent RNA strand, falls off the template strand probably at regions of secondary structure and RNA synthesis reinitiates at a different site with identical or similar nucleotide sequence to the jumping site. Also a weak base pairing in A/U-rich regions can facilitate the release and/or the re-annealing of the incomplete nascent RNA by formation of a temporary bubble (Nagy and Bujarski, 1997). The junction site of some CTV D RNAs coincided with the transcription start site of subgenomic RNAs (Yang et al., 1997) suggesting their involvement in recombination and

in the genome modular evolution of the family *Closteroviridae* (Bar-Joseph et al., 1997). The stochastic nature of D RNA generation as replication errors was evidenced by the great variety of D RNAs generated after protoplast inoculation with RNA transcripts of the two LIYV genomic segments (Rubio et al., 2002).

The genetic variation and structure of viruses within an infected plant (considered as a virus isolate) also provides important information to understand viral evolution. This has been studied for GRLaV-1 (Alabi et al., 2011), GRLaV-3 (Turturo et al., 2005; Esteves et al., 2012), CYSDV (Rubio et al., 1999, 2001a), ToCV (Lozano et al., 2009), GRLaV-2 (Bertazzon et al., 2010b; Jarugula et al., 2010), and CTV (Ayllón et al., 1999b, 2006; d'Urso et al., 2000; Kong et al., 2000; Rubio et al., 2001b; Davino et al., 2005; Hilf et al., 2005; Melzer et al., 2005; Silva et al., 2007; Gomes et al., 2008; Iglesias et al., 2008; Černi et al., 2009; Oliveros-Garay et al., 2009; Matos et al., 2013; Wu et al., 2013). Analysis of nucleotide sequences or molecular markers such as single-strand conformation polymorphism (Rubio et al., 1996) of a certain number of clones obtained from RT-PCR products showed that most viral isolates had populations composed of a predominant sequence variant and different one- or two-nucleotide mutants in a very low frequency. These mutant clouds are predicted by the quasispecies model as a consequence of the high error frequency in RNA replication and have been described for some animal and plant viruses (Domingo and Holland, 1997). However, some viral isolates had two or more divergent variants, some of which were genetically similar to variants predominant in other viral isolates, suggesting mixed infection by two different strains. These strain mixes that have been found in the closteroviruses CTV (Kong et al., 2000; Rubio et al., 2001b; Sambade et al., 2002; Ayllón et al., 2006; Iglesias et al., 2008) and GLRaV-2 (Bertazzon et al., 2010a), and in the ampeloviruses GLRaV-3 (Farooq et al., 2012) and GLRaV-5 (Esteves et al., 2012), must be common in vector-transmitted viruses infecting long-lived woody hosts. Infection of the same cell with diverged virus strains is a requisite for detectable recombination events to occur.

Co-infection of two viruses or virus strains in mixed infections may have additional evolutionary consequences resulting from their interactions. Sometimes interaction is synergistic, inciting more severe symptoms and increased fitness (virus accumulation) of one or both variants in comparison with single infections. This effect seems to be due to the supression of a host defense mechanism, e.g., gene silencing, by one of the viruses that inhibits accumulation of the other virus in single infections (Palukaitis, 2011). Several cases of synergism have been described between criniviruses and other plant viruses. BYVaV increases concentration of Blackberry virus Y (BVY, genus *Brambyvirus*, family *Potyviridae*) in mixed infections (Susaimuthu et al., 2008). CYSDV enhanced multiplication of *Cucumber vein yellowing virus* (CVYV, genus *Ipomovirus*, family *Potyviridae*) and increased symptom severity in mixed infections (Gil-Salas et al., 2012). In sweet potato, SPCSV increased multiplication of several viruses of the genera *Potyvirus* (*Sweet potato feathery mottle virus*, *Sweet potato latent virus* and *Sweet potato mild speckling virus*), *Ipomovirus* (*Sweet potato mild mottle virus*), *Cucumovirus* (*Cucumber mosaic virus*), and putative members of the genus *Carlavirus* (*Sweet potato chlorotic fleck virus* and C-6 virus) in double and triple infections, which was associated to an increase in the severity of symptoms. In some cases SPCSV titer was reduced indicating an antagonistic interaction (Karyeija et al., 2000; Mukasa et al., 2006; Untiveros et al., 2007). Co-infection of ToCV and *Tomato spotted wilt virus* (TSWV, genus *Tospovirus)* in tomato plants susceptible to both viruses resulted in rapid death of the plants, with a pronounced enhancement of ToCV accumulation, whereas TSWV accumulation was not altered. However, in tomato cultivars carrying the *Sw-5* gene that confers resistance to TSWV, preinfection with ToCV resulted in TSWV resistance breakage, a phenomenon not observed when plants were simultaneously co-inoculated with both viruses. This suggested that a threshold level or a time lapse is needed for ToCV to interfere or downregulate the defense response in the TSWV-resistant plants (García-Cano et al., 2006). Finally, co-infection of the two tomato-infecting criniviruses TICV and ToCV altered accumulation of each virus in a host-specific manner. While in *Nicotiana benthamiana* the TICV titer increased and the ToCV titer decreased, in *Physalis wrightii* the titers of both TICV and ToCV decreased in comparison with the corresponding single infections (Wintermantel et al., 2008). In summary, sinergistic co-infections lead to higher accumulation of at least one of the viruses and may acelerate its adaptation to an initially difficult host.

Other times antagonist interactions may produce a fitness decrease of one or the two viruses. In some cases, previous infection by one viral isolate prevents or hamper subsequent infection by other viral isolate (superinfection exclusion). This phenomenon has been exploited as a disease control strategy named cross protection consisting of preinoculation of the plant with a mild isolate to protect it against damage caused by infection with a virulent isolate. This interaction is most common between genetically related viruses and it has been hypothesized that it might be caused by competition for host resources or because previous infection would trigger the gene silencing antiviral defence of the plant that would impair infection by the second virus (Palukaitis, 2011). CTV cross protection has been efficient for disease control only in some areas (e.g., South Africa and South America) and with some citrus varieties, whereas it has shown limited success in other areas or with other varieties, indicating that (i) cross protection probably depends on the varieties, CTV strains and environmental conditions prevalent in each region (Moreno et al., 2008), and (ii) it is unlikely that CTV cross protection is ruled only by the gene silencing reaction triggered by the pre-inoculated isolate. Indeed preinoculation of citrus plants with artificial hybrid CTV virions containing some genomic segments of the challenging isolate failed to exclude superinfection by this isolate, with only isolates of the same strain being excluded (Folimonova et al., 2010). Demostration that the CTV p33 protein is necessary for superinfection exclusion (Folimonova, 2012) further supports the hypothesis that antiviral silencing reaction triggered by mild isolate pre-inoculation may not be the main mechanism for CTV cross protection. Finally, co-inoculation with a mild and a severe isolate genetically divergent usually resulted in severe symptom expression and predominance of the severe isolate, indicating cross protection failure

between divergent isolates and higher fitness of the severe isolate (Roistacher and Dodds, 1993; Sambade et al., 2007; Velazquez-Monreal et al., 2009). Therefore, whatever the cross protection mechanism will be, massive use of cross protecting CTV isolates in some citrus areas is doubtless an important determinant of the viral population structure and an evolutionary factor.

Interactions with the host are one of the most important factors in virus evolution. The tandem of leader proteases of GLRaV-2 seems to have evolved to facilitate infection of this virus in grapevine, a woody perennial host (Liu et al., 2009). Another host effect is the CTV codon usage adaptation to citrus that has also been found in other closteroviruses infecting woody plants but not in those infecting herbaceous hosts (Cheng et al., 2012). Biological and genetic variations of some CTV isolates have been also observed after host change (Ayllón et al., 2006; Scott et al., 2013). Thus, graft-transmission of the mild isolate T317 from citron to sweet orange originated the virulent isolate T318. When T318 was transmitted back to citron, it remained virulent and had the same population structure as it had in sweet orange (Rubio et al., 2000a). This suggests that a minor severe variant contained in the mild isolate T317 was established in sweet orange and became predominant in the T318 population by genetic drift during a transmission bottleneck. This severe variant was as fit in citron as the mild variant (well-adapted to citron and sweet orange). However, when 3 years latter the mild isolate T317 was transmitted again to sweet orange, the new isolate T317D was also mild, albeit the population structure had changed as detected by SSCP analysis. When T317D was transmitted back to citron, its population structure changed back to become indistinguishable from that of the original isolate T317 (Rubio et al., 2000a). This suggests that the severe variant was not sorted in this occasion but some of the mild variants were positively selected as an adaptation to sweet orange. Predominance of these mutants was reverted after back transmission to citron probably as a result of fitness trade-off by host specialization (Woolhouse et al., 2001).

Finally, interactions with the vector can also have an important effect in viral evolution. The association between phylogenetic relationship among members of the three genera of the *Closteroviridae* family and their type of insect vector (mealybugs for the genus *Ampelovirus*, whiteflies for *Crinivirus* and aphids for *Closterovirus*) likely reflects vector adaptation as a driving force in the diversification in this family (Karasev, 2000). In criniviruses, an emergent group of viruses whose expansion has been linked to the rapid spread of their whitefly vectors (Wisler et al., 1998), the specificity of the association between virus species and whitefly species is a main factor determining the geographical distribution of the different criniviruses. Thus, the displacement of the crinivirus BPYV by CYSDV in Spain has been associated with the increasing populations of *Bemisia tabaci* in comparison with *Trialeurodes vaporariorum* (Berdiales et al., 1999). Also disappearance of LIYV in Southern California has been associated with the displacement of the biotype A of *B. tabaci* by the biotype B, a very poor vector of LIYV (Cohen et al., 1992; McLain et al., 1998; Wisler et al., 1998). Geographical distribution of TICV and ToCV also seems to depend on distribution of the whitefly vectors (Wintermantel, 2008). Interestingly, mixed infections of ToCV and TICV allowed transmission of TICV by the non-vector *T. abutilonea* (Wintermantel, 2008), indicating an interaction of these viruses during transmission. In ampeloviruses, there is no evidence of vector–virus specificity in the mealybug transmission of different grapevine viruses (Tsai et al., 2010). In closteroviruses, changes in aphid transmissibility of the local CTV isolates have been observed along the years. In the early 1950s, the transmission rate of CTV by *Aphis gossypii* Glover was very low but this increased in 1960–1970 in Israel and California. (Bar-Joseph and Loebenstein, 1973; Roistacher et al., 1980), suggesting an adaptation and coevolution of CTV to this vector. Brazil and Dominican Republic severe CTV variants are preferentially transmitted by *Toxoptera citricida* in comparison with the mild components (Brlansky et al., 2011). The absence of this vector in some geographical areas could explain in part why severe CTV isolates are less common in these areas, a situation that may change after introduction of the brown citrus aphid as observed in several countries (Garnsey et al., 2000; Powell et al., 2003). Differences in transmissibility of CTV isolates by different aphid species (Raccah et al., 1976, 1980; Hermoso de Mendoza et al., 1988; Broadbent et al., 1996) indicate the vector exerts a selective pressure and it is an important factor shaping CTV populations. There are evidences that aphid transmission can induce changes in the population structure and/or biological characteristics of individual CTV isolates (Kano and Koizumi, 1991; Ayllón et al., 1999b, 2006; d'Urso et al., 2000; Van Vuuren et al., 2000; Sentandreu et al., 2006; Sambade et al., 2007; Roy and Brlansky, 2009; Velazquez-Monreal et al., 2009; Ananthakrishnan et al., 2010). Comparison of genetic diversity in plants and in aphids showed the occurrence of an important bottleneck for the CTV population during aphid transmission (Nolasco et al., 2008). All these observations indicate that vectors have an important effect in the evolution of closteroviruses by the interplay of natural selection imposed by vector-virus interactions and genetic drift by population bottlenecks during transmission between plants. Also, the movility and dispersibility of vectors determine the level of gene flow between close geographical areas. Thus, the rapid spread of some white-flies which could account in part for the low genetic variation and wide distribution areas of the criniviruses transmitted by them.

CONCLUSIONS

The increased number of nucleotide sequences and the availability of more sophisticated analytical tools allows a better understanding of the evolution, population genetics and epidemiology of the viruses in the family *Closteroviridae*. Analysis of the genetic variability and population structure shows a limited genetic variation as in other plant viruses. This seems to occur mainly by a strong negative selection, indicated by the low number of non-synonymous substutions with respect to the synonymous substitutions in the three genera of this family. Some viruses, had isolate groups with a great genetic divergence between groups but each group being very homogeneous. This suggests that their sequence space is restricted to a few sharp adaptive peaks and that covariation between different nucleotide positions occurs, as suggested by decreased fitness of chimeras of different CTV strains. Long distance movement or gene flow may have contributed in some cases to this low genetic variation.

Although most viral isolates were composed of one major variant and a population of genetically related variants forming a quasispecies structure, some isolates had divergent variants originated from mixed infections of different strains which can affect symptom expression. Interaction between different viruses or variants from the same virus has been found in some cases. The antagonism between close variants of the same virus is the base for cross protection to control damage from severe CTV strains (Moreno et al., 2008). Another element in the virus evolution is the interaction between different virus species, as the synergistic or antagonist interactions observed between the two tomato infecting criniviruses (ToCV and TICV) in different hosts (Wintermantel et al., 2008). Also, viruses of the genus *Crinivirus* produce increased symptom expression and accumulation of viruses from other families in mixed infections (Karyeija et al., 2000; Mukasa et al., 2006; Untiveros et al., 2007).

Changes in the population structure caused by vector transmission or host change, either by selection of viruses or genetic variants of the same virus or by genetic drift, could explain the appearance of virulent CTV isolates, or the emergence of some viruses in new areas, e.g., criniviruses, associated to their whitefly vector spread.

Recombination between divergent genotypes have been described in the genera *Closterovirus* and *Ampelovirus*, which have played an important role in their evolution by increasing genetic diversity and adaptability.

ACKNOWLEDGMENTS

This work was supported by grants AGL2009-08226 and AGL2012-32429, co-financed by FEDER (European Fund for Regional Development) and by the Ministerio de Economia y Competitividad (MIEC), respectively.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 February 2013; accepted: 29 May 2013; published online: 26 June 2013.

*Citation: Rubio L, Guerri J and Moreno P (2013) Genetic variability and evolutionary dynamics of viruses of the family Closteroviridae. Front. Microbiol. **4**:151. doi: 10.3389/fmicb.2013.00151*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 23 May 2013
doi: 10.3389/fmicb.2013.00132

The defective RNAs of Closteroviridae

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The family Closteroviridae consists of two genera, *Closterovirus* and *Ampelovirus* with monopartite genomes transmitted respectively by aphids and mealybugs and the Crinivirus with bipartite genomes transmitted by whiteflies. The Closteroviridae consists of more than 30 virus species, which differ considerably in their phytopathological significance. Some, like *beet yellows virus* and *citrus tristeza virus* (CTV) were associated for many decades with their respective hosts, sugar beets and citrus. Others, like the grapevine leafroll-associated ampeloviruses 1, and 3 were also associated with their grapevine hosts for long periods; however, difficulties in virus isolation hampered their molecular characterization. The majority of the recently identified Closteroviridae were probably associated with their vegetative propagated host plants for long periods and only detected through the considerable advances in dsRNA isolation and sequencing of PCR amplified replicons. Molecular characterization of CTV and several other Closteroviridae revealed that, in addition to genomic and subgenomic RNAs, infected plants contain several different subviral defective RNAs (dRNAs). The roles and biological functions of dRNAs associated with Closteroviridae remain terra incognita.

Keywords: citrus viruses, RNA viruses, RNA recombination, viral replicase, template-switching, non-replicative RNAs, virus replication, defective RNA

LARGE RNA GENOMES AMONG THE CLOSTEROVIRIDAE: MEETING THE CHALLENGE OF SURVIVAL

Viruses are among the smallest biological entities and, because of the small size of their genomes, their survival depends on the use of a variety of molecular strategies that allow them to stretch their genome-coding capacities to the limit. For example, the single-stranded DNA Geminiviridae that are ∼3 kb in length, a length that would generally allow transcription and translation of a less than 100-kDa polypeptide, employ a sophisticated multiframe reading of their plus-genome and negative-genome strands that amplifies their coding capacity several-folds, considerably increasing the number and size of the translation products that can be obtained (Hull, 2002). Similarly, the coding capacities of many single-stranded RNA viruses are enhanced by the use of read-through products. An even more common strategy is for a few viral expression products to play multiple roles. For example, the p25 gene of the *citrus tristeza closterovirus*, family Closteroviridae, (i) encodes the coat protein that encapsidates most (∼97%) of the viral particles (Febres et al., 1996), (ii) acts as a suppressor of RNA silencing (RSS; Lu et al., 2004), and (iii) is most probably involved in vector adaptation for the natural transmission of this virus from infected to uninfected hosts. Similarly, the p23 protein of CTV serves as (i) an RSS (Lu et al., 2004), (ii) a regulator of RNA strand synthesis (Satyanarayana et al., 2002), and (iii) the inducer of CTV symptoms in certain CTV-sensitive host plants (Flores et al., 2013).

Viral genomes are equipped with a complex and versatile toolbox that allows them to survive and spread, despite a number of serious limitations, such as (i) dependence on just a few insect species for natural transmission, (ii) dependence on a sometimes restricted range of host plants or on only certain types of tissues, and (iii) the challenge of a potent generic host defense mechanism, the RNA-silencing system. Despite the sophisticated replication machinery that viruses have developed for their survival, it is difficult to understand how some large, single-stranded, single-component RNA genomes, such as the ∼30-kb genome of animal-infecting coronaviruses and most of the 15- to 20-kb genomes of Closteroviridae members, manage to survive in the face of incidental degradation and targeted dicing by the active host defense silencing machinery within their host cells and/or as they are carried by phloem-feeding aphid vectors. The biological cost of such a situation, in which every single fracture at each of the c. 20,000 possible fragile targets could lead to RNA disruption and to total genetic and energetic loss, could have been detrimental to the continuous survival of such big genomes. In addition, CTV must also contend with the grave consequences of high error rates of viral RNA replication (Lauring and Andino, 2010), which for the large CTV genome is expected to result in average of at least two nucleotide changes per each genome/generation. For CTV tolerant *Citrus* sp. trees, which often survive tens and even up to 100 years, such a mutation rate could had been expected to result in a considerable genetic diversity. Surprisingly, however, analyses of CTV strains from spatially and temporally separated citrus trees revealed a highly conserved genome (Albiach-Martí et al., 2000). Further evidence for the remarkable genetic stability of closterovirus genomes (Dolja and Koonin, 2013), especially of the CTV, is the CTV-based vectors infecting citrus plants. Some of the CTV-based vectors infected citrus trees continued to express the inserted green fluorescent protein (GFP) gene for up to 7 years (Dawson, 2011). The genetic stability of CTV holds apparently only when plants are infected by a monotypic closterovirus isolate (Weng et al., 2007). Genetic analysis of a Floridian CTV

isolate from citrus tree infected by three major CTV genotypes revealed numerous variants generated by promiscuous recombination between the major genotypes and additional divergence further increased genotypic complexity of the initial recombinants (Weng et al., 2007; Zhongguo Xiong, personal communication). These results raise the possibility of an unknown mechanism to limit accumulation of point mutation CTV mutants while preserving those generated through recombination. The presence of multiple defective dRNAs in trees infected with many CTV isolates further supports this possibility (Batuman et al., 2010).

DEFECTIVE AND OTHER SUBVIRAL RNAs

In addition to genomic and subgenomic RNAs, virus-infected hosts often contain two different types of subviral RNAs: (i) satellite RNAs, with sequences that are mostly or completely unrelated to their "helper" viruses and (ii) dRNAs that do not interfere with their helpers, or defective interfering RNAs (DI-RNAs) whose presence results in less virus accumulation and often in milder symptoms. Interestingly, however, the DI-RNA of *turnip crinkle virus* (TCV) that reduces virus accumulation causes increased rather than decreased severity of disease symptoms (Li et al., 1989; White and Morris, 1995). These different types of virus-associated molecules are distinguished from viral genomic RNA by the fact that they are not required for normal virus propagation.

Only the replication of satellite RNAs and some dRNAs and DI-RNAs is dependent upon enzymes encoded by their helper viruses. However, a few other dRNAs are able to replicate autonomously in inoculated cells, although their ability to spread and move is restricted in intact plants. DI-RNAs and dRNAs have been reported for many animal and plant viruses and the characterization of their sequences has revealed a mosaic of truncated forms, suggesting a variety of situations that could have lead to their emergence. All dRNAs possess some of the *cis*-acting elements necessary for replication of the parent virus and all are missing some of the genetic elements necessary for some essential virus functions, such as replication, encapsidation, or the ability to spread within a host.

A SHORT INTRODUCTION TO CTV AND THE CLOSTEROVIRIDAE

The Closteroviridae family includes viruses that have been known for at least seven to eight decades, such as *beet yellows virus* (BYV; Duffus, 1973) and CTV (Bar-Joseph et al., 1989). Other viruses of this family, particularly among those belonging to the *Crinivirus* genus, appear to have emerged more recently. Low virus titer and association with deciduous fruit tree and grapevine (*Vitis vinifera*) woody hosts that are polyphenol rich handicapped purification of many of these viruses and delayed their characterization until finding that Closteroviridae infections are associated with large amounts of dsRNA (Dodds and Bar-Joseph, 1983). The considerable progress achieved toward elucidation of the genomic functions of Closteroviridae has been widely reviewed in different journals and book chapters

Members of the genus *Closterovirus* are mainly transmitted by aphids; whereas members of the genus *Ampelovirus*, which also have monopartite genomes, are mainly transmitted by mealybugs (Martelli et al., 2012). The *Crinivirus*, and a recently proposed new genus with monopartite particles named *Velarivirus* (Al Rwahnih et al., 2012) are mostly transmitted by whiteflies. Most of the members of the genus *Crinivirus* (transmitted by whiteflies) have bipartite genomes, although a virus with a tripartite genome, *potato yellow vein virus* (PYVV), was assigned to this genus (Livieratos et al., 2004). Interestingly, the virus–vector relationships of members of the three Closteroviridae genera appear to have had far stronger effects on the diversification of a range of genomic properties than on the adaptation of these viruses to different hosts (Karasev, 2000).

A schematic presentation of the CTV genomic RNA is shown in **Figure 1**. Among the interesting features of this virus are the arrangement of the genome and the high level of similarity among the sizes of the full-length genomes of the CTV strains that have been characterized to date. The sequences of the 3′-halves of most strains of CTV are very similar. However, the 5′ end of the T36 strain differs from that of the VT and most of the other strains to the extent that, at a certain stage, virologists considered referring to these strains as two different species. In addition to the genomic RNA, which is also found in isolated virus particles, CTV-infected cells contain RF (replicative-form) RNA molecules that consist of full-length plus and minus strands and a large number (up to 30 or more) of 5′- and 3′-sgRNAs. Interestingly, while the 5′-sgRNAs are all single-stranded and positive, the 3′-sgRNAs include both positive- and minus-strand molecules. Readers are referred to several other chapters in this series, which provide considerably more detailed discussions of the CTV genome and the replication strategies of this virus.

CLASSES OF CTV-dRNAs

One of the most prominent features of CTV, which was revealed soon after its genome was first characterized (Karasev et al., 1995), is that dRNA molecules are present in most isolates of this virus (Mawassi et al., 1995a,b; and for a recent review, see Batuman et al., 2010). Interestingly, T36, the first CTV strain to have its genome characterized, differs in this aspect. Unlike VT, in which almost every subtype contains major dRNAs, dRNAs appear to be less common in the T36 strain. This difference was probably responsible for one of the main bottlenecks in the early attempts to obtain a full-length genomic sequence of CTV-VT, despite partial cloning and efficient utilization of cDNA clones to distinguish between CTV strains (Rosner and Bar-Joseph, 1984).

Many CTV isolates contain one or more dRNAs of various sizes. Most of these dRNA molecules consist of two genomic termini, with extensive internal deletions (Mawassi et al., 1995a,b; Yang et al., 1997; Ayllon et al., 1999). It is interesting to note that only certain dRNAs are regularly transferred by mechanical transmission (Che et al., 2003) and most are not passed along during aphid transmission (P. Moreno, personal communication).

For convenience, CTV-defective RNAs (dRNAs) can be grouped into different classes. These classes are described below (see **Figure 1**).

CLASS 1: CTV-dRNAs

Class 1 CTV-dRNAs possess different-sized 5′ and 3′ sequences that are not significantly homologous with one another (Mawassi et al., 1995a,b) and are thought to be the result of erroneous

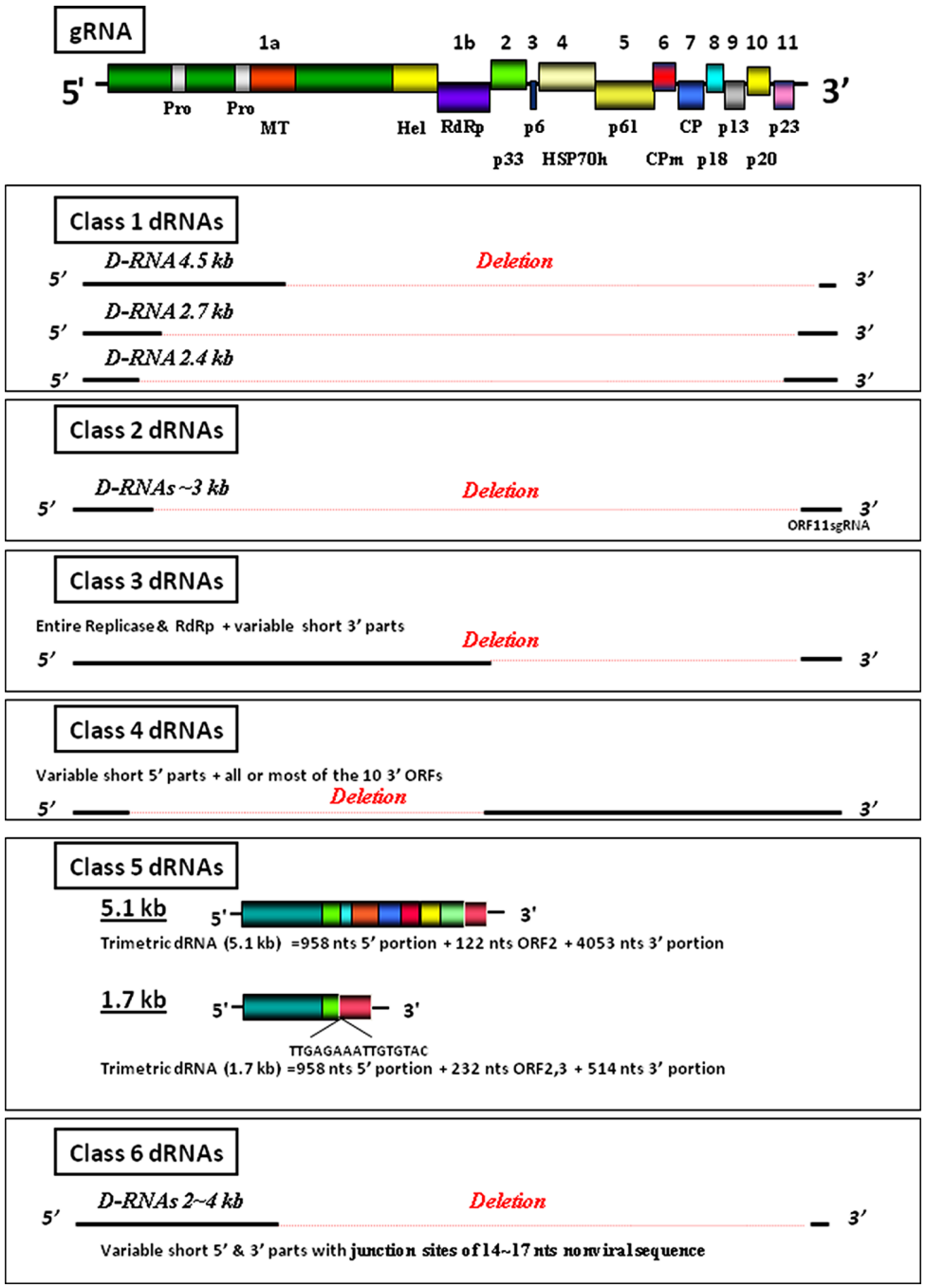

FIGURE 1 | A diagram of the *Citrus tristeza virus* (CTV) genomic RNA (gRNA). The 5′ and 3′-termini of the genome, the ORFs with respective numbers and encoded proteins and protein domains are indicated. Pro, papain-like protease; MT, methyltransferase; Hel, RNA helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, a heat shock protein 70 homolog; CP, major coat protein; CPm, minor coat protein. Proteins are named according to their relative molecular masses. The figure shows also diagrams of Classes 1–6 defective (D) RNAs associated with CTV gRNA.

replication involving non-homologous recombination. The junction sites of the 2.3- and 4.5-kb dRNAs from CTV-VT (Mawassi et al., 1995b) are not flanked by direct repeats in the genomic RNA. Other dRNAs (Ayllon et al., 1999) contain direct repeats of 4–5 nt near their junction sites, supporting the possibility that they were generated via a replicase-driven template-switching mechanism.

CLASS 2: CTV-dRNAs

Class 2 CTV-dRNAs possess 3′ moieties that are similar in size and structure to the full-length sgRNA of ORF11 (Yang et al., 1997). The extra cytosine at the junction sites of several of the dRNAs of this class corresponds to the extra guanine reported on the 3′ ends of minus strands of sgRNA and RF molecules (Karasev et al.,

1997). These characteristics indicate that: (i) CTV-dRNA synthesis may have taken place through a process of template-switching of 5′ plus-strand molecules toward distal positions after the completion of minus-strand ORF11 sgRNA transcription and (ii) the 5′ of the ORF11 sgRNA might serve as a highly specific hotspot for RNA recombination (Bar-Joseph et al., 1997; Yang et al., 1997).

CLASS 3: CTV-dRNAs

Class 3 CTV-dRNAs possess large (∼12 kb) encapsidated dRNA molecules that are infectious when mechanically transmitted to citrus plants and *Nicotiana benthamiana* protoplasts. Their 5′ termini are identical to or slightly larger than the 5′ large, single-stranded sgRNA of ORF1a + 1b (LaMT) that have been reported in CTV-infected plants (Gowda et al., 2001; Che et al., 2002). The 3′ moiety of these large dRNAs varies and some have a truncated ORF11 (Che et al., 2002). Two of these junction sites start at the first nucleotide of the ORF10–11 intergenic region and one other coincides with the full-length sgRNA that codes for ORF10.

Artificially constructed large dRNAs that have intact ORF1a + b reading frames, but lack the translation products of all of the 3′ ORFs were found to self-replicate in protoplasts (Satyanarayana et al., 1999). Although these artificially constructed, defective molecules have not been found *in planta* and there is no evidence for their ability to spread systemically within inoculated plants, they have become useful genetic platforms for the study of sgRNA transcription (Gowda et al., 2001). The presence of insertions, deletions and inversions of 3′ sequences, including ORFs and their intergenic regions, in this self-replicating construct, allowed Gowda et al. (2001) to demonstrate that the production of a 5′-terminal positive strand and 3′-terminal positive- and negative-stranded sgRNA is permitted by each of the 3′ CTV controller elements.

CLASS 4: CTV-dRNAs

Class 4: CTV-dRNAs are large, dRNAs, which retain all or most of the ten 3′ ORFs and appear to be analogous to the genomic RNA 2 of criniviruses. These large, dRNA molecules (LD-RNA2) can be transmitted to citrus plants by mechanical inoculation. However, the transmission of LD-RNA2 to protoplasts has been shown to be limited and cannot be detected by RT-PCR until 4 days after inoculation (Che et al., 2002, 2003).

CLASS 5: CTV-dRNAs

Class 5: CTV-dRNAs vary in size (1.7–5.1 kb) and contain sequences that point to double-recombination events (DR). These sequences are comprised of two termini and a non-contiguous internal sequence from ORF2. Interestingly, LD-RNA2 and DR-dRNAs from three different CTV isolates all contain an identical 948-nt 5′ region.

CLASS 6: CTV-dRNAs

Class 6: CTV-dRNAs have variable regions between the 5′ and 3′ termini and inserts of short (14–17 nt) sequences that have no homology with the CTV genome (Mawassi et al., 1995a,b; Yang et al., 1997; Che et al., 2003). The question of why these heterologous double-recombinants are so small has been raised. CTV-dRNA homologous-sequence double-recombinants (Class 5 dRNAs) are more than 100 nucleotides in length; whereas the heterologous inserts of Class 6 dRNAs are at least 10-fold shorter. One possible explanation for this could be that a naturally occurring selection process eliminates any CTV-dRNAs with inserts of host genes of 21–25 nt, which might silence the normal expression of the respective host genes. Such a mechanism could also explain the limited amount of non-self recombinants among RNA viruses, in general, and suggest a new function for RNA silencing of RNA viruses, namely reducing the possibility that virus genomes might amplify RNA segments derived from the mRNA of their hosts.

DEFECTIVE RNAs ASSOCIATED WITH OTHER CLOSTEROVIRIDAE

Compared with CTV, the presence of dRNA in other viruses in this family has received far less attention. The few reports that have been published in this area include occasional observations of dRNA associated with the criniviruses *lettuce infectious yellows virus* (LIYV; Rubio et al., 2000), *lettuce chlorosis virus* (LCV; Mongkolsiriwattana et al., 2011), and PYVV (Eliasco et al., 2006) and the closterovirus *carrot yellow leaf virus* (CYLV; Menzel et al., 2009; W. Menzel, personal communication). In addition, a rather large dRNA (∼6.0 kb) was reported in pineapple plants infected with the ampelovirus *pineapple mealybug wilt-associated virus-1* (PMWaV-1; Melzer et al., 2008). Thus, the presence of dRNA is a common feature of all three genera of the Closteroviridae family.

DISCUSSION: STILL MORE QUESTIONS THAN ANSWERS

RNA recombination is the key process in the formation of the dRNA molecules associated with most animal and many plant viruses (White and Morris, 1995; Simon, 1999). Three different events have been suggested to lead to the recombination of viral RNA: breakage and ligation of incomplete RNA molecules, replicase-driven template-switching and breakage-induced template-switching (Nagy and Simon, 1997). Information gathered from Class 2 and 3 CTV-dRNAs led researchers to suggest a fourth mechanism involving the recombination of the 3′ termini of sgRNAs with different-sized pieces from the 5′ end of the CTV genome (Yang et al., 1997; Che et al., 2003). More recent studies have reported recombination involving the 5′ termini of sgRNAs and different-sized molecules from the 5′ part of the virus (see **Figure 1**, Classes 2 and 4). Components of the replication-associated machinery were found to be involved in the evolution of other viruses and intergenic regions are known to be the preferred crossover sites for brome mosaic virus (BMV) recombinants (Nagy and Bujarski, 1996).

The dRNAs of CTV provide us with some tentative answers to questions about virus evolution. First, the finding that populations of naturally self-replicating dRNAs (Class 3) and Class 4 dRNAs harboring the entire battery of ORFs analogous to the RNA 2 of criniviruses suggests that similar forms of dRNA may have led to the evolution of the bipartite criniviruses from a monopartite velarivirus parent. In addition, dsRNA molecules corresponding to one or more major CTV-dRNAs have been found to account for substantial amounts of the total dsRNA found in CTV-infected plants. Since dsRNA molecules can be considered the dead ends

of the viral replication process, their abundance in Closteroviridae (Dodds and Bar-Joseph, 1983) naturally raises evolutionary questions. One possibility that we would like to suggest is that, in addition to the three suppressor genes, CTV and other Closteroviridae with large, very fragile genomes use the abundant dsRNA in their genomic, subgenomic, and dRNA as a buffering system, to protect their large RNA genomes against the risk of being targeted by the active host defense RNA silencing.

In our early work, we could not associate any specific biological phenomena with the 2.4-kb dRNA or some of the other dRNA molecules. After two decades of continuous transmission of the VT isolate in Mexican lime and Alemow seedlings, the symptoms observed on the Alemow plants remained unchanged. However, among the SO (sour orange) plants that used to test for SY (seedling yellows), we noticed that some failed to exhibit the typical SY symptoms. The examination of the dRNAs of Alemow plants infected with CTV-VT subisolates that exhibited SY and non-SY reactions revealed the presence of one major dRNA population. The main difference between both subisolate groups was the association of 4.5- and 2.4-kb dRNAs with non-SY- and SY-reacting VT isolates, respectively. Furthermore, VT isolate #12, which contains a large dRNA with a complete 5′ moiety of the gRNA, did not induce the stem-pitting symptoms typically observed in infected Alemow plants. However, the possibility of differences between SY and non-SY, or stem pitting(SP) and non-SP, isolates in other genomic regions was not ruled out and the considerable progress that has been made recently in the field of sequencing techniques is expected to clarify this issue. In conclusion, the roles and biological functions of the numerous dRNAs associated with Closteroviridae remain *terra incognita*.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 February 2013; accepted: 06 May 2013; published online: 23 May 2013.

Citation: Bar-Joseph M and Mawassi M (2013) The defective RNAs of Closteroviridae. Front. Microbiol. ***4****:132. doi: 10.3389/fmicb.2013.00132*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 06 March 2013
doi: 10.3389/fmicb.2013.00038

Beet yellows virus replicase and replicative compartments: parallels with other RNA viruses

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In eukaryotic virus systems, infection leads to induction of membranous compartments in which replication occurs. Virus-encoded subunits of the replication complex mediate its interaction with membranes. As replication platforms, RNA viruses use the cytoplasmic surfaces of different membrane compartments, e.g., endoplasmic reticulum (ER), Golgi, endo/lysosomes, mitochondria, chloroplasts, and peroxisomes. *Closterovirus* infections are accompanied by formation of multivesicular complexes from cell membranes of ER or mitochondrial origin. So far the mechanisms for vesicles formation have been obscure. In the replication-associated 1a polyprotein of *Beet yellows virus* (BYV) and other closteroviruses, the region between the methyltransferase and helicase domains (1a central region (CR), 1a CR) is marginally conserved. Computer-assisted analysis predicts several putative membrane-binding domains in the BYV 1a CR. Transient expression of a hydrophobic segment (referred to here as CR-2) of the BYV 1a in *Nicotiana benthamiana* led to reorganization of the ER and formation of ~1-μm mobile globules. We propose that the CR-2 may be involved in the formation of multivesicular complexes in BYV-infected cells. This provides analogy with membrane-associated proteins mediating the build-up of "virus factories" in cells infected with diverse positive-strand RNA viruses (alpha-like viruses, picorna-like viruses, flaviviruses, and nidoviruses) and negative-strand RNA viruses (bunyaviruses).

Keywords: RNA virus replication, membrane vesicles, virus replication factory, endoplasmic reticulum modification, intracellular traffic

Eukaryotic viruses from disparate groups, both DNA and RNA containing ones, induce in cells drastic rearrangement of the membranes leading to formation of "virus organelles" or "virus factories". It is suggested that these compartments protect virus nucleic acids from nucleases and specific cell defense mechanisms, along with creating sufficiently high concentration of interacting templates, replication proteins, and substrates. Recent excellent reviews cover the topic in full (den Boon and Ahlquist, 2010; Netherton and Wileman, 2011; Verchot, 2011). In this work, we attempted to reconcile the ultrastructural data available for several RNA virus groups with our findings of the membrane-modifying activity of a hydrophobic segment of the 1a polyprotein of beet yellows closterovirus (BYV).

OPEN ULTRASTRUCTURES: BUNYAVIRUSES

Bunyamwera virus (BunV) is an enveloped virus with a negative-sense RNA genome (~12 kb) divided among three segments. In infected mammalian cells, BunV infection leads to formation of tubular structures (up to 50 per cell) encompassing the Golgi membranes, actin, myosin I, and viral non-structural protein NSm (Fontana et al., 2008). The tubes are in close contact with mitochondria and rough endoplasmic reticulum (ER), possibly serving as sources of host factors (e.g., translation elongation factor eEF-2 and ribosomal proteins) aiding the virus replication. Transcription and replication of BunV occur inside the "globular domain," a U-like structure at one end of the tubes. The replicative complexes consisting of BunV nucleoproteins and RNA replicase, concentrate on the inner surface of the globular domain. BunV transcription yields mRNAs that are transferred to rough ER for translation, and replication produces the progeny nucleoproteins transported to the Golgi stacks modified by inserted BunV surface glycoproteins, for particle maturation (Fontana et al., 2008).

The model by Fontana et al. (2008) implies dynamic changes of, and communication between, the cell membranous compartments induced by bunyavirus infection, driven mainly by actin filaments and that the viral NSm. Apparently, the primary transcription of the gene encoding NSm must occur prior to changes in Golgi. The BunV replication-associated globular domains are open structures, unlike the vesicles and spherules induced by positive-sense RNA viruses (see below). This might reflect a nuclease-protected state of the BunV genomic and antigenomic RNA templates, the absence of dsRNA (which might trigger RNA interference in cells) in negative-sense RNA viruses replication, and employment of strategies against host defense mechanisms (Léonard et al., 2006; Habjan et al., 2008).

"CLOSED" ULTRASTRUCTURES: NIDOVIRUSES

Nidoviruses are enveloped viruses with positive-sense RNA genomes of 13–16 kb (arteriviruses) and ~30 kb (coronaviruses). The replication-associated proteins are encoded in overlapping

5′-open reading frames (ORFs) 1a and 1b, and translation of the genomic RNA yields polyproteins 1a and 1ab autocatalytically processed into non-structural proteins forming the replication complex (reviewed in Gorbalenya, 2008). Using ER membranes as the main source, nidoviruses induce in cells double-membrane vesicles (DMVs, 150–300 nm in diameter), convoluted membranes (CMs), and vesicle packets (VPs) of merged DMVs. These structures accumulate dsRNA and replication-associated proteins. The coronavirus nsp3, nsp4, and nsp6 encompass transmembrane domains and are plausibly the key factors for membrane remodeling. Recent EM tomography analysis of the severe acute respiratory syndrome (SARS) virus-infected cells allowed refinement of the topology of SARS ultrastructures (Knoops et al., 2008). DMVs and VPs apparently form a network with connections to each other and to the ER; however, no openings to the cytosol were detected (Knoops et al., 2008). The apparently "closed" state of the DMV network poses a yet unresolved question as to how the coronavirus factory exchanges ribonucleotide triphosphates (rNTP) substrates and newly synthesized RNA with the cytosol (Knoops et al., 2008).

Picornaviruses, small non-enveloped viruses with (+)RNA genome of ∼8 kb, induce heterogeneous (50–500 nm) DMVs of the ER, Golgi and lysosomal origin (Bienz et al., 1990; Schlegel et al., 1996). Some commonality of the picornavirus and coronavirus ultrastructures (particularly, the absence of apparent bridges to cytosol) has been noted (den Boon and Ahlquist, 2010; Netherton and Wileman, 2011). However, the question of whether picornaviruses indeed produce a "closed" network of DMVs awaits further study.

ULTRASTRUCTURES WITH NECKS: ALPHA-LIKE VIRUSES, NODAVIRUSES, FLAVIVIRUSES

The alpha-like superfamily unites positive-sense RNA viruses of animals (alphaviruses, rubiviruses, hepeviruses), and plants (e.g., bromoviruses, tobamoviruses, tymoviruses), whose genomes encode the conserved domains of methyltransferase (MTR), NTPase/helicase (HEL), and RNA polymerase (POL; Goldbach, 1987). The replication system of Brome mosaic virus (BMV) has been studied in considerable detail. BMV has a tripartite genome (∼8.2 kb), with RNA-1 and RNA-2 coding, respectively, for proteins 1a (MTR–HEL) and 2a (POL). Early in infection, 1a binds to perinuclear ER membranes via an amphipathic helix located in non-conserved region between the MTR and HEL (Liu et al., 2009). It should be noted parenthetically that in the capping enzyme of Semliki Forest alphavirus, the equivalent membrane-binding function is governed by an unrelated amphipathic helix within the MTR (Ahola et al., 1999). The BMV 1a protein causes membrane invaginations and engages $2a^{Pol}$ and viral RNA templates (rendering them non-sensitive to nucleases) to the membrane (den Boon and Ahlquist, 2010). Each mature vesicle retains a thin neck (∼8 nm) to cytosol. The vesicle encompasses hundreds of 1a molecules forming inner layer, 10–20 $2a^{Pol}$ molecules, and a few molecules of genomic and antigenomic RNAs (Schwartz et al., 2002). Other alpha-like viruses (with the exception of closteroviruses, see Section 5 of this paper) apparently induce morphologically similar ultrastructures, the line-up of 50–100 nm single-membrane vesicles, often with detectable necks to cytosol, originating from endosomes and lysosomes (alphaviruses), ER (tobamoviruses), tonoplasts (alfamoviruses), and chloroplasts (tymoviruses; reviewed in Netherton and Wileman, 2011; Verchot, 2011).

Flock house nodavirus (FHV) has compact bipartite (+)RNA genome (∼4.5 kb). RNA-1 encodes protein A, a multifunctional RNA replicase (Venter and Schneemann, 2008). The replicase molecules, via the N-terminal mitochondrial targeting signal and transmembrane domain, attach to the outer mitochondrial membrane and cause its invaginations, thus producing numerous 50-nm vesicles (spherules) with 10-nm necks into cytosol (Kopek et al., 2007). The interior of the vesicles is lined by ∼100 copies of replicase (Kopek et al., 2007). Hence, FHV and BMV, albeit distantly related evolutionarily, employ similar mechanisms of membranes modification and replication factory build-up.

Dengue flavivirus (DenV) is an enveloped virus with a monopartite (+)RNA genome (∼11 kb) encoding a single polypeptide precursor (Bartenschlager and Miller, 2008). Non-structural proteins NS2A, NS4A, and NS4B bear transmembrane domains and are responsible for transformation of ER membranes into a network of interconnected VPs (∼90-nm single-membrane vesicles surrounded by common membrane), CVs, and virion budding sites (Welsch et al., 2009). The VPs retain dsRNA and viral replication proteins. Noteworthy, the DenV-induced network has ∼8-nm neck-like openings to the cytosol (Welsch et al., 2009). Hence, the flavivirus factory combines features of the coronavirus network and the *bromovirus* and nodavirus necked ultrastructures.

INTRACELLULAR TRANSPORT OF REPLICATION COMPLEXES

After entry of one or a few virus particles or viral nucleic acid molecules into the cell, these must move to the compartments where genome expression and replication proceed. The intracellular transport of viral particles and replication complexes is rather an active process than mere diffusion, as cytosol is a highly viscous matter where translocation of molecules or complexes exceeding a ∼500-kDa limit is impeded (Luby-Phelps, 2000; Greber and Way, 2006). Microinjection of fluorescently labeled tobacco mosaic virus (TMV) RNA into tobacco trichome cells rapidly leads to formation of granules associated with the ER, that are translocated along the actin network (Christensen et al., 2009). Using TMV particles where RNA and coat protein were labeled with different fluorescent dyes, it was found that that both signals initially co-localized on the same granules, indicating that the virus may become attached to the ER/actin prior to uncoating (Christensen et al., 2009).

There is emerging evidence that the replication complexes and/or the associated membranous ultrastructures of (+)RNA viruses are transported along the cytoskeleton. Thus, the replication factories of turnip mosaic potyvirus (TuMV) are represented by heterogeneous vesicles of 0.6 to 4.3 μm in diameter accumulating in the perinuclear zone. Interestingly, some vesicles are highly motile with an average velocity of 0.45 μm/s. Their movement is unidirectional and occurs in "stop and go" mode (Cotton et al., 2009; Grangeon et al., 2010, 2012). Likewise, the distribution of *tobamovirus* replication-associated complexes in

cells is dynamic and cytoskeleton-dependent (Más and Beachy, 1999; Szecsi et al., 1999). The *tobamovirus* 126-kDa (MTR–HEL) protein and the 126-kDa-induced vesicles bind to and traffic along the actin microfilaments (Liu et al., 2005). In the hepatitis C flavivirus system, interaction of two replication proteins, NS3 (RNA HEL – serine proteinase) and NS5A (phosphoprotein), provides for binding and movement of the replication complex along microtubules and actin filaments (Lai et al., 2008). Mouse *norovirus* appears to utilize microtubules during the early stages of replication to establish localization of the replicative complexes proximal to the microtubule organizing center (Hyde et al., 2012). There is a significant overlap in the function and regulation of microtubule and actin networks in animal and plant systems (Goode et al., 2000; Barton and Overall, 2010; Sampathkumar et al., 2011). Many proteins, including molecular motors, have been demonstrated to associate with both networks to coordinate intracellular trafficking and movement of organelles (Petrásek and Schwarzerová, 2009; Viklund et al., 2009; Mucha et al., 2011; Meiri et al., 2012). A number of disparate viruses, including Semliki forest virus, vaccinia virus, and respiratory syncytial virus, have been shown to utilize, in a coordinated manner, both the microtubule and actin networks to facilitate replication (Newsome et al., 2004; Kallewaard et al., 2005; Spuul et al., 2011).

Plant viruses often utilize cytoskeleton for the cell-to-cell movement (Harries et al., 2009, 2010). The movement proteins interact with replication complexes as well as with actin microfilaments and microtubules (Grangeon et al., 2012; Solovyev et al., 2012; Tilsner et al., 2012). Both cytoskeletal systems may act as conduits for individual viral RNAs, transported ribonucleoproteins, as well as large replication complexes to reach plasmodesmata and thus to assist intercellular trafficking (Bamunusinghe et al., 2009; Harries et al., 2010; Schoelz et al., 2011; Grangeon et al., 2012; Pena and Heinlein, 2012; Solovyev et al., 2012; Tilsner and Oparka, 2012; Tilsner et al., 2012). These data indicate that diverse (+)RNA viruses of plants may use cytoskeleton for intracellular trafficking of replication complexes or the components thereof, to plasmodesmata.

MULTIVESICULAR COMPLEXES OF CLOSTEROVIRUSES

Members of the *Closteroviridae* family are related to alpha-like viruses with respect to conservation of key replication-associated protein domains (MTR–HEL–POL), but strikingly resemble nidoviruses in the genome size, layout, and expression pattern (Agranovsky, 1996; Karasev, 2000; Dolja et al., 2006). The beet yellows closterovirus (BYV) 15.5-kb genome encodes the replication-associated proteins in 5′-proximal ORFs 1a and 1b (**Figure 1A**). Translation of these ORFs is expected to yield NH_2-coterminal 1a and 1ab polyproteins encompassing, respectively, the arrays of papain-like cysteine proteinase (PCP)–MTR–central region(CR)–HEL and PCP–MTR–CR–HEL–POL (L-PCP, leader PCP domain; CR, non-conserved CR; **Figure 1A**; Agranovsky et al., 1994). The autocatalytic cleavage of BYV polyproteins by the PCP at Gly^{588}/Gly^{589} releases the 66-kDa leader protein (Zinovkin et al., 2003) which activates amplification of the BYV RNA (Peremyslov et al., 1998; Peng and Dolja, 2000). The 1a and 1ab polyproteins are further processed by a yet unknown proteolytic activity(-ies) into at least three fragments, of which the 63-kDa MTR-containing and 100-kDa HEL-containing proteins were identified in infected plants (Erokhina et al., 2000). The ~70-kDa protein(s) corresponding to the 1a CR (**Figure 1A**) has not been yet detected.

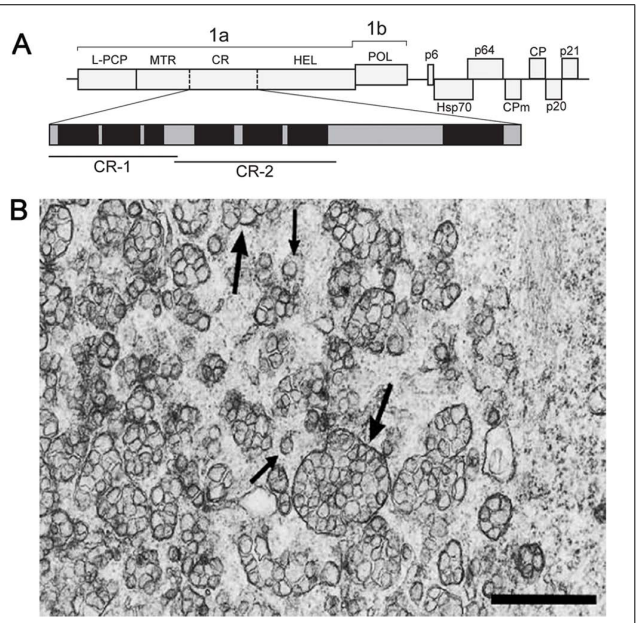

FIGURE 1 | (A) Schematic representation of the BYV genome and the encoded proteins. 1a polyprotein encompasses the leader papain-like cysteine proteinase (L-PCP), methyltransferase (MTR), unique central region (CR), and NTPase/helicase (HEL). Vertical solid bar, established cleavage site of the L-PCP; broken bars, arbitrary cleavage sites within the 1a polyprotein calculated from the apparent molecular masses of the MTR- and HEL-containing proteins. Enlarged map of hydrophobic domains (black) and hydrophilic hinges (gray) in the 1a CR is shown below. The portions used for cloning and expression as GFP C-terminal fusions (CR-1 and CR-2) are indicated by bars. **(B)** Electron microscopy of the BYV-induced ultrastructures in leaf parenchyma cells of *Tetragonia expansa* (21 days p.i.). Double-membrane vesiles (DMVs, small arrows) and vesicle packets (VPs, large arrows) on a tissue section embedded in Epon after fixation with glutaraldehyde and OsO_4. Scale bar, 1 μm.

In plant cells, closteroviruses induce the formation of ~100-nm DMVs and multivesicular complexes (single-membrane vesicles surrounded by a common membrane; **Figure 1B**; Cronshaw et al., 1966; Esau et al., 1967; Esau and Hoefert, 1971; Lesemann, 1988). The multivesicular complexes often neighbor with stacks of aligned filamentous BYV particles (Cronshaw et al., 1966; Esau et al., 1967). These ultrastructures broadly resemble the DMVs and VPs produced by nidoviruses and flaviviruses (see Sections 2 and 3 in this paper), and are referred to here as DMVs and VPs for simplicity. The BYV replication-associated proteins (L-PCP, MTR, and HEL) co-localize with the DMV and VP membranes, supporting the role of these ultrastructures as replication platforms (Erokhina et al., 2001; Zinovkin et al., 2003). The membranes in *closterovirus* DMVs and VPs are likely to be derived from ER (*Crinivirus*; Wang et al., 2010) or mitochondria (*Ampelovirus*; Kim et al., 1989; Faoro et al., 1992; Faoro and Carzaniga, 1995). Whether these ultrastructures have "closed" or "necked" state, remains unknown.

Inspection of the BYV 1a CR sequence (approximately aa 1100 to 1800; **Figure 1A**) using hydropathicity plot drawing software (protScale; Kyte and Doolittle, 1982) revealed several hydrophobic stretches longer than 20 aa forming putative alpha helixes, which resembled membrane-binding domains. Two segments of the 1a CR predicted to form separate hydrophobic domains, CR-1 (aa 1114–1301), and CR-2 (aa 1301–1498; **Figure 1A**), were cloned as green fluorescent protein (GFP) fusions in a binary vector. Upon transient expression in *Nicotiana benthamiana* leaves the fusions showed distinct distribution of the fluorescence. The GFP:CR-1 produced aggregates of heterogeneous shape and size (0.2–1 μm, average 0.5 μm) accumulated at the cell periphery (**Figure 2A**), whereas the GFP:CR-2-induced uniform globules ∼1 μm in diameter mostly concentrated around the nucleus (**Figures 2B,C**). Some CR-2-induced globules were apparently motile (**Figure 2B**). Further, we found that the CR-2 globules co-localized with actin filaments (**Figure 2D**), suggesting that the globules might be translocated along the actin network. In cells expressing the GFP:CR-2, the ER network transformed into diffuse membrane reservoirs partially co-localized with the perinuclear groups of GFP:CR-2 globules (cf. **Figures 2E,F–H**). These data corroborate the recent findings by Bryce Falk and colleagues for lettuce infectious yellows virus (genus *Crinivirus* of the *Closteroviridae*), i.e., the rearrangement of perinuclear ER in *N. tabacum* protoplasts inoculated with LIYV RNA1 transcripts, specifically the R1-322 transcript encoding only the 1a and 1ab replicative proteins (Wang et al., 2010).

With due caution in interpreting the results presented in **Figure 2**, it is tempting to speculate that the phenotypes induced by the BYV CR-2 segment might reflect the formation of BYV replication-associated ultrastructures. It is possible that the build-up of *closterovirus* replication platforms depends on the ER membranes and is accompanied by essential changes in perinuclear ER, and that the BYV 1a protein contains a membrane anchor (CR-2) in the region between MTR and HEL, as is the case with BMV 1a protein (Liu et al., 2009). Further study is necessary to elucidate the fine structure of the BYV CR-2-induced globules

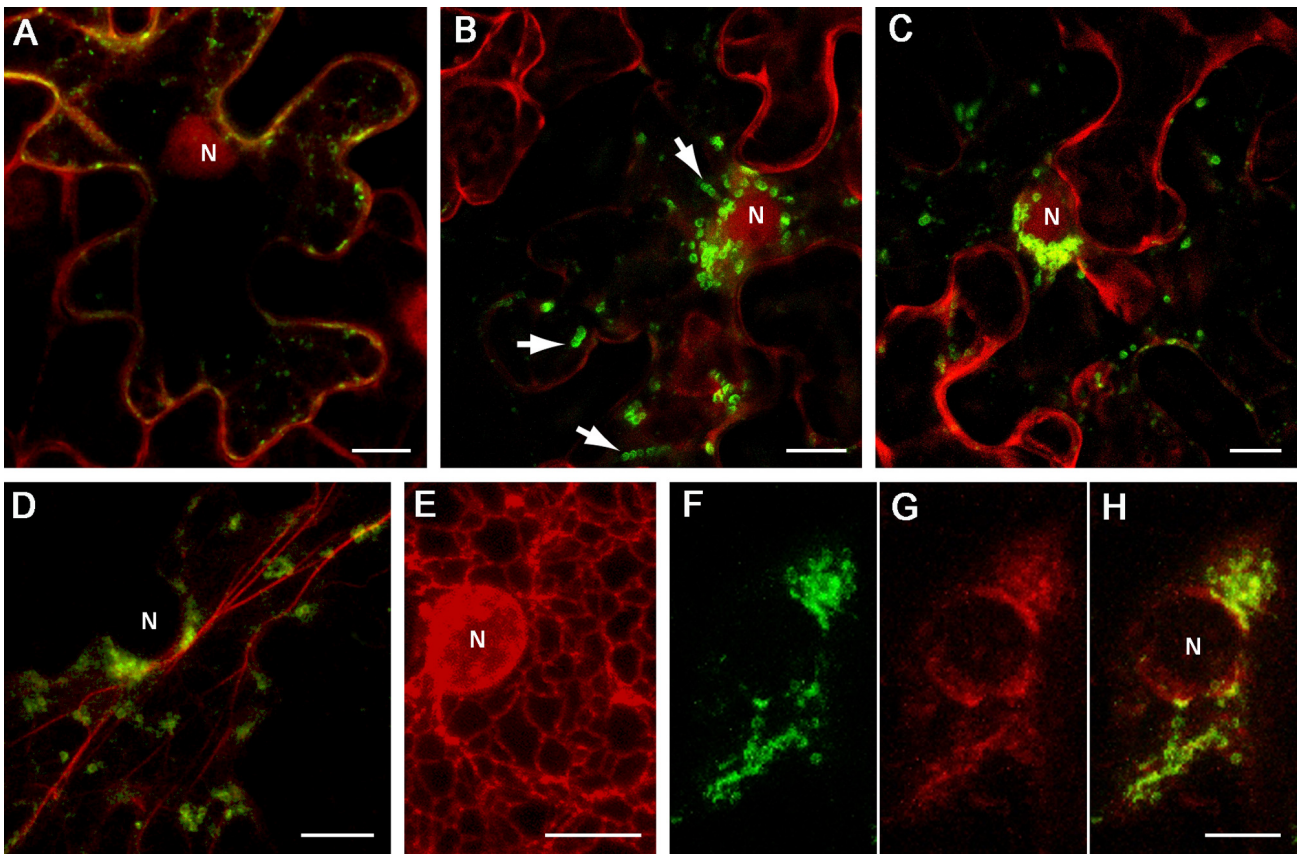

FIGURE 2 | Localization of GFP-fused CR-1 and CR-2 in epidermal cells of *N. benthamiana* leaves. Proteins were expressed by agroinfiltration and visualized at 48 h post infiltration by confocal laser scanning microscopy. **(A)** Co-expression of GFP:CR-1 with the red fluorescent marker protein mCherry, which localizes to the cytoplasm and the nucleoplasm in plant cells (Lee et al., 2008). **(B)** and **(C)** Co-expression of GFP:CR-2 with mCherry in two individual cells. Arrows indicate the motile CR-2 globules revealed in frame captures. **(D)** Co-expression of GFP-CR-2 with YFP-Tal (red channel), a fluorescent marker for actin cytoskeleton (Shemyakina et al., 2011). **(E)** Expression of ER-mRFP, the protein targeted to the ER lumen by N-terminal signal peptide and C-terminal ER-retention signal (Haseloff et al., 1997), in the perinuclear region of a plant cell. **(F–H)** Co-expression of GFP:CR-2 with ER-mRFP. **(F)** Perinuclear groups of GFP:CR-2-containing globules. **(G)** Modified perinuclear ER representing diffuse membrane reservoirs. **(H)** Overlap of images **(F)** and **(G)**. All images represent the superpositions of series of confocal optical sections. N, nucleus. Scale bar, 10 μm.

and their relationship to DMVs and VPs produced in naturally infected cells, as well as to verify the significance of the actin network in transport of the *closterovirus* factory components within the cell.

ACKNOWLEDGMENTS

This study has been supported by grant 10-04-00805 from the Russian Foundation for Basic Research. The authors are thankful to Joseph Atabekov for the critical reading of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 December 2012; paper pending published: 28 January 2013; accepted: 14 February 2013; published online: 06 March 2013.

Citation: Gushchin VA, Solovyev AG, Erokhina TN, Morozov SY and Agranovsky AA (2013) Beet yellows virus replicase and replicative compartments: parallels with other RNA viruses. Front. Microbiol. ***4****:38. doi: 10.3389/fmicb. 2013.00038*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 05 March 2013
doi: 10.3389/fmicb.2013.00039

Molecular characterization of closteroviruses infecting *Cordyline fruticosa* L. in Hawaii

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GenBank Accessions: JQ599282, JQ599283, JQ599284

In Hawaii, common green ti plants (*Cordyline fruticosa* L.) have been shown to harbor Cordyline virus 1 (CoV-1) which, along with Little cherry virus 1 (LChV-1), and Grapevine leafroll-associated virus 7 (GLRaV-7), form a distinct clade within the family *Closteroviridae*. Preliminary work has indicated that, aside from CoV-1, three additional closteroviruses may infect common green ti plants in Hawaii. In this study, pyrosequencing was used to characterize the genomes of closteroviruses infecting a single common green ti plant. The sequence data confirmed the presence of CoV-1 as well as three additional closteroviruses. Although all four viruses had the same general genome organization, the sequence divergence between the RNA-dependent RNA polymerase, heat shock protein 70 homolog, and coat protein ranged from 22 to 61%, indicating these represent four distinct closterovirus species. The names CoV-2, CoV-3, and CoV-4 are proposed for the three new viruses. Phylogenetic analyses placed CoV-2, CoV-3, and CoV-4 in the same clade as CoV-1, LChV-1, and GLRaV-7.

Keywords: pyrosequencing, *Closteroviridae*, *Velarivirus*, *Cordyline*, ti ringspot

INTRODUCTION

The family *Closteroviridae* represents a related group of mono- and multipartite, single-stranded, positive-sense RNA plant viruses with long, flexuous virions (Dolja et al., 1994). There are currently three genera in the family which are segregated largely based on vector species. Members of the genera *Closterovirus*, *Crinivirus*, and *Ampelovirus* are, in general, transmitted by aphids, whiteflies, and mealybugs, respectively (Karasev, 2000). Members of the genera *Closterovirus* and *Ampelovirus* have monopartite genomes, while members of the genus *Crinivirus* have multipartite genomes. Little cherry virus 1 (LChV-1) and Grapevine leafroll-associated virus 7 (GLRaV-7) are two members of the family that have not been assigned to a genus (Martelli et al., 2002). Molecular phylogenies and sequence similarity values associate them most closely to members of the genus *Crinivirus*, however, their monopartite genomes and their lack of a known insect vector have precluded their inclusion into this genus.

The ti plant, *Cordyline fruticosa* (L.), belongs to the plant family Agavaceae and has considerable cultural and economic importance in Hawaii and throughout most of Polynesia. In Hawaii, the common green variety was introduced by early Polynesian settlers and is a popular ornamental in residential settings that has also become naturalized in Hawaii's forests. Vegetatively propagated due to sterility (Hinkle, 2007), it is also the most prominent variety grown commercially. In 2009, ti farmers on the island of Oahu reported ringspot symptoms on their common green ti plants. These ringspot symptoms were subsequently observed on commercial and residential ti plants on the islands of Maui and Hawaii. In a search for a causal agent of the ringspot symptoms, it was recently discovered that Hawaiian ti plants harbored multiple putative members of the plant virus family *Closteroviridae* (Melzer et al., 2011). The complete nucleotide sequence for one of these viruses, Cordyline virus 1 (CoV-1), was determined. Its 16.9 kb genome was organized similar to LChV-1 and GLRaV-7 (Melzer et al., 2011). Together, LChV-1, GLRaV-7, and CoV-1 form a monophyletic clade distinct from the other three genera in the family *Closteroviridae*. This has led to proposals for the creation of a fourth genus, "*Velarivirus*," to represent these viruses (Al Rwahnih et al., 2012; Martelli et al., 2012).

A reverse-transcription polymerase chain reaction (PCR) assay revealed that CoV-1 is widespread in Hawaii and is present in ti plants with and without ringspot symptoms, making it unlikely to be involved in the etiology of the disease (Melzer et al., 2011). Based on partial sequence data, three additional closteroviruses were identified in ti plants. The objectives of this study are to further characterize these additional closteroviruses in common green ti plants and determine whether they represent distinct species (or strains of CoV-1) as well as their placement within the family *Closteroviridae*.

MATERIALS AND METHODS

LIBRARY SEQUENCING AND ASSEMBLY

A previously described randomly primed complementary DNA (cDNA) library generated from double-stranded (ds) RNA isolated from a common green ti plant was used as the input material for multiplex pyrosequencing (Melzer et al., 2011). The most common cDNA length was estimated to be approximated 550 bp based on agarose gel electrophoresis. MID7 (5′-ACGTACACACT-3′) was ligated to the cDNAs which then underwent pyrosequencing using a 454 GS FLX Titanium platform (Roche, Branford, CT, USA) at the University of Hawaii's Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) laboratory. Following

pyrosequencing, the MID7 and random primer sequences as well as low quality basecalls at the end of reads were trimmed. Short length (<65 nt) and low quality reads as well as those that mapped to the CoV-1 genome were purged from the dataset. The remaining reads underwent *de novo* assembly using Geneious® Pro 5.6.5 (Biomatters Ltd., Auckland, New Zealand). To validate low coverage regions or where unexpected stop codons or frameshifts occurred, and to bridge sequence gaps between contiguous sequences (contigs), primers were designed flanking the region in question and PCR was performed using the cDNA library as template. PCR products were either directly sequenced following treatment with ExoSAP-IT® (USB/Affymetrix, Santa Clara, CA, USA) or ligated into pGEM®-T Easy (Promega, Madison, WI, USA) followed by Sanger-based sequencing at the ASGPB.

The 3′-terminal sequences were obtained by polyadenylating heat-denatured dsRNA using yeast poly(A) polymerase (USB/Affymetrix) following the manufacturer's instructions. An oligo d(T) primer [5′-CACTCCCTATTATCCAGG$(T)_{16}$-3′] was used to initiate cDNA synthesis and also used in the subsequent PCR reaction along with a virus-specific primer designed to anneal near the 3′-end of the available virus sequence. Amplification products were cloned using pGEM®-T Easy and at least five clones underwent Sanger-based sequencing at the ASGPB.

PHYLOGENETIC ANALYSES

The combined helicase domain (HEL), RNA-dependent RNA polymerase (RdRp), heat shock protein 70 homolog (HSP70h), p61 (PF03225), and coat protein (CP) amino acid sequences of members and tentative members of the family *Closteroviridae* were aligned using ClustalX 2.0.12 (Larkin et al., 2007). With this alignment, the phylogenetic relationships of the sequences were inferred using neighbor-joining (NJ) and maximum likelihood (ML) algorithms. The NJ algorithm was performed using ClustalX 2.0.12 and bootstrapped with 1000 replications. The ML algorithm was performed with PhyML 3.0 (Guindon et al., 2010) using the WAG model and bootstrapped with 1000 replications.

RESULTS

LIBRARY SEQUENCING AND ASSEMBLY

A total of 107,655 high quality reads >64 nt were generated from the cDNA library, with maximum, minimum, and mean lengths of 772, 65, and 392.1 nt, respectively. Of these reads, 4,424 mapped to the CoV-1 genome. The majority of the remaining reads assembled into three contigs, each in excess of 10 kb in length (**Table 1**). Based on comparisons with accessions in GenBank, all three contigs represented closterovirus-based genomes. The first contig was found to be essentially identical to the previously identified Contig5 (Melzer et al., 2011) where the two sequences overlapped, and was thus designated Contig5. Similarly, the second and third contigs were found to be essentially identical in overlapping regions with Contig8 and CloneH11, respectively, and were designated as such. The 15,031 nt Contig5 was extended to 15,107 nt following the addition of the 3′-terminal sequence. The 14,941 nt Contig8 was extended to 16,274 nt following the addition of the 3′-terminal sequence and a contig in the 5′-region of the genome. The 10,684 nt CloneH11 was extended to 14,620 nt with the addition of the 3′-terminal sequence and a contig in the 5′-region of the genome. The 5′-terminal sequences were not obtained for any of the contigs.

Table 1 | Summary of pyrosequencing results and assembly of reads into contiguous sequences (contigs).

	# of reads (% of total)	Length of contig[1]	Mean coverage
CoV-1	4,424 (4.1)	n/a	n/a
CoV-2 (Contig5)	11,395 (10.6)	15,031	342.8
CoV-3 (Contig8)	79,593 (73.9)	14,941	2410.8
CoV-4 (CloneH11)	3,537 (3.3)	10,684	168.7
Unincorporated	8,728 (8.1)	n/a	n/a

[1] *Post-editing.*

GENOME ORGANIZATION

The overall genome organization of Contig5, Contig8, and CloneH11 were similar to that of CoV-1 (**Figure 1**), GLRaV-7, and LChV-1 (Jelkmann et al., 1997, 2012; Melzer et al., 2011). Although incomplete at their 5′-terminal regions, open reading frame (ORF)1a of Contig5, Contig8, and CloneH11 encoded a polyprotein containing protease (PRO; PF05533), methyltransferase (MTR; PF01160), and HEL (PF01443) domains. ORF1a of Contig5 and Contig8 terminated with the sequence 5′-UUUGA-3′ with the stop codon underlined. This is also the terminal sequence of CoV-1 and GLRaV-7 (Melzer et al., 2011; Jelkmann et al., 2012) and may initiate a +1 ribosomal frameshift allowing expression of ORF1b. ORF1a of CloneH11 terminated with the sequence 5′-UUUAA-3′ that may also allow expression of ORF1b via the same frameshift mechanism. For all contigs, however, a start codon was present near the ORF1a termination sequence in the same reading frame of ORF1b that may also allow its expression. This was also observed for CoV-1 and LChV-1 (Jelkmann et al., 1997; Melzer et al., 2011). ORF1b of all three contigs encoded all the typical motifs of an RdRp (Koonin, 1991). Small transmembrane proteins 4 and 7 kDa in weight were present between ORF1b and the HSP70h ORF for Contig5 and Contig8, respectively. No such protein was present at this location in CloneH11, however, a 4 kDa protein with transmembrane properties was present in the +1 reading frame within C-terminal region of the HSP70h ORF (which is in the +3 reading frame). For all contigs, downstream of the HSP70h ORF was a 9–10 kDa ORF that is also present in CoV-1 and GLRaV-7, followed by ORFs encoding a 60–61 kDa protein common to all closteroviruses (PF03225), the major CP (PF01785), and then the minor CP (CPm). An ORF encoding a 25–26 kDa homolog of CoV-1 p26 was present in Contig5, Contig8, and CloneH11. The final ORFs encoded by Contig5, Contig8, and CloneH11 were 28–29 kDa proteins. While p29 of Contig5 and p28 of Contig8 shared sequence homology with p29 of CoV-1, p29 of CloneH11 did not appear to be a homolog of these putative proteins, and did not have significant sequence similarity with any viral sequences currently in GenBank. The 3′-untranslated regions (UTRs) of Contig5, Contig8, and CloneH11 were 259, 154, and 186 nt, respectively. The exact 3′-termini of Contig5 and

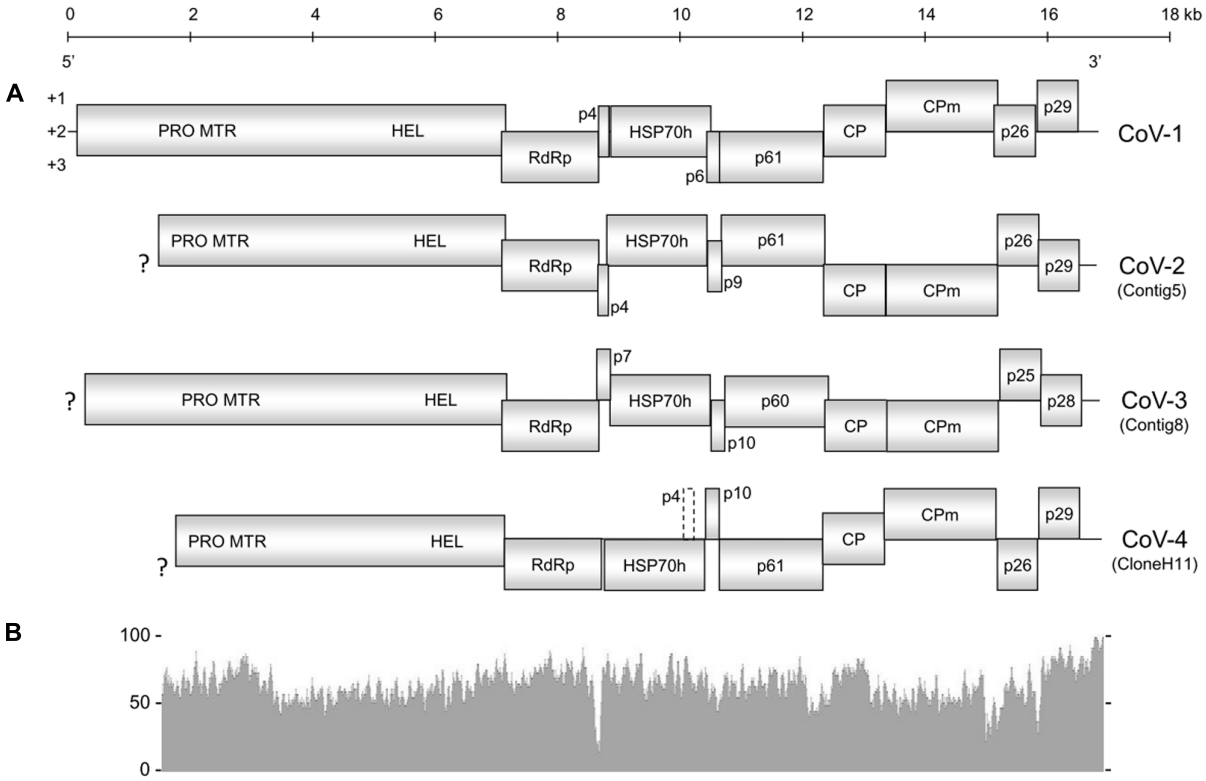

FIGURE 1 | (A) Genome organization of Cordyline virus 1 (CoV-1), CoV-2, CoV-3, and CoV-4. Solid boxes represent putative open reading frames (ORFs) with their vertical position indicating reading frame. The dashed box in CoV-4 represents a possible ORF that encodes a small hydrophobic protein within the HSP70h ORF. PRO, protease domain; MTR, methyltransferase domain; HEL, helicase domain; RdRp, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homolog; CP, coat protein; CPm, minor coat protein; ?, incomplete terminus. **(B)** Graphical comparison of the percent nucleotide identity between CoV-1 and CoV-2 where the two genomes overlap. The above scale gives approximate size in kilobases for both **(A)** and **(B)**.

CloneH11 were identical to that of CoV-1, having the sequence 5′. . .AAAGGUGCG-3′. Contig8 also ended with this sequence, but appeared to lack the terminal guanine residue.

DIVERSITY AND PHYLOGENETIC PLACEMENT

The amino acid identity between Contig5, Contig8, CloneH11, and CoV-1 for their respective RdRp, HSP70h, and CP sequences was under 70% in all cases except for the RdRp sequences of Contig5 and CoV-1 which were 78% identical (**Table 2**). Using the current criteria for closterovirus species demarcation recently revised by the International Committee on Taxonomy of Viruses (Martelli et al., 2011), these contigs would represent distinct closterovirus species. As such, Contig5, Contig8, and CloneH11 were tentatively designated CoV-2, CoV-3, and CoV-4, respectively.

Cordyline virus 1 and CoV-2 appear to be the most closely related of the CoVs characterized in this study with an overall nucleotide identity of 63.7%. Toward the 3′-termini of their genomes, however, the similarity gradually increased. This similarity peaked in the 3′UTR of CoV-1 and CoV-2 which shared a 90.8% nucleotide identity.

Over 8% of the total sequence reads did not map to the genomes of CoV-1, CoV-2, CoV-3, or CoV-4 (**Table 1**). The majority of these appeared to be either of plant or prokaryotic origin, or did not share significant similarity to any of the sequence accessions in the current databases. Approximately 31% of these reads, however, represented closterovirus sequences. One thousand and ninety-five of these reads were selected for further investigation. One hundred and seventy-four of these reads, when translated to amino acid sequences, were similar to the N-terminal region of a closterovirus ORF1a. Reverse-transcription PCR revealed this region was part of CoV-4. The remaining reads, when translated to amino acid sequence, had high similarity (between 80 and 92% identity) to proteins encoded by CoV-1.

Phylogenetic analyses of the combined HEL domain, RdRp, HSP70h, p60/61, and CP amino acid sequences using distance-based (NJ) and character-based (ML) algorithms inferred almost identical relationships between CoV-2, CoV-3, and CoV-4

Table 2 | RNA-dependent RNA polymerase/heat shock protein 70 homolog/coat protein amino acid percent identities between the CoVs infecting common green ti plants.

	CoV-1	CoV-2 (Contig5)	CoV-3 (Contig8)
CoV-2 (Contig5)	78/69/67		
CoV-3 (Contig8)	62/50/44	62/51/41	
CoV-4 (CloneH11)	59/53/39	57/51/40	55/55/39

and members of the family *Closteroviridae*. Both analyses clearly placed these four viruses along with CoV-1, GLRaV-7, and LChV-1 in a distinct clade within the family (**Figure 2**; data not shown).

DISCUSSION

We have previously reported the presence of one closterovirus, CoV-1, infecting common green ti plants in Hawaii, and provided preliminary evidence for the presence of additional closterovirus species using a Sanger-based sequencing approach (Melzer et al., 2011). In this study we used a massively parallel sequencing approach to identify, in addition to CoV-1, three new closterovirus species which we have tentatively designated CoV-2, CoV-3, and CoV-4.

Cordyline virus 2 and CoV-3 share the same general genome organization as CoV-1, and differ from each other only in the molecular weight of their small hydrophobic proteins and the small protein encoded by the ORF located between their HSP70h and p61 ORFs. CoV-4, however, is unusual by lacking an ORF encoding a small transmembrane protein between the ORFs of the RdRp and HSP70h. The small transmembrane protein of *Beet yellows virus* (BYV) associates with the host endoplasmic reticulum and is involved in the cell-to-cell movement of BYV and presumably other closteroviruses as well (Peremyslov et al., 2004). An ORF which could encode a small protein possessing a transmembrane domain does exist in CoV-4, although it is located within the HSP70h ORF in a +1 reading frame relative to the HSP70h ORF. If this ORF is not expressed, it is possible that CoV-4 requires co-infection with another closterovirus for cell-to-cell movement. Since all four CoVs were present in a single host plant, multiple infections in a single host plant may not be uncommon.

Overall, the genomes of CoV-1 and CoV-2 had a moderate sequence similarity. Near the 3′-terminus, however, this similarity gradually increased to the point where the 3′UTRs of these viruses were nearly identical, indicative of a potential recombination

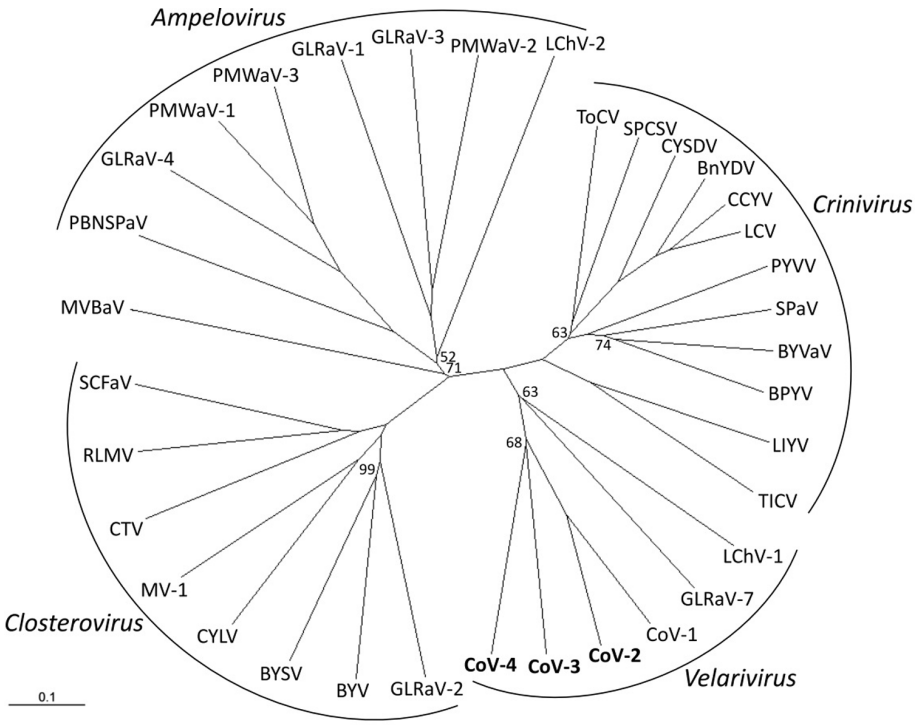

FIGURE 2 | Phylogenetic placement of Cordyline virus 2 (CoV-2), CoV-3, and CoV-4 within the family *Closteroviridae* using the combined RNA-dependent RNA polymerase, heat shock protein 70 homolog, p60, and coat protein amino acid sequences with a neighbor-joining algorithm and 1000 bootstrap replications. Branch support is indicated in percentage support, and is 100% unless indicated, and the scale provides branch distance for the given number of substitutions. Virus abbreviations (GenBank accession numbers) are: Genus *Ampelovirus*: GLRaV-1, *Grapevine leafroll associated virus 1* (AF195822); GLRaV-3 (NC_004667); GLRaV-4 (NC_016416); LChV-2 (AF416335); PBNSPaV, *Plum bark necrosis stem pitting associated virus* (YP_001552326); PMWaV-1, *Pineapple mealybug wilt associated virus 1* (AF414119); PMWaV-2 (AF283103); PMWaV-3 (DQ399259); Genus *Closterovirus*: BYSV, *Beet yellow stunt virus* (U51931); BYV, *Beet yellows virus* (AF190581); CTV, *Citrus tristeza virus* (NC_001661); CYLV, *Carrot yellow leaf virus* (NC_013007); GLRaV-2 (AF039204); MV-1, *Mint virus-1* (NC_006944); RLMV, Raspberry leaf mottle virus (NC_008585); SCFaV, Strawberry chlorotic fleck-associated virus (DQ860839); Genus *Crinivirus*: BnYDV, *Bean yellow disorder virus* (NC_010560/NC_010561); BPYV, *Beet pseudoyellows virus* (NC_005209/NC_005210); BYVaV, *Blackberry yellow vein-associated virus* (NC_006962/NC_006963); CCYV, Cucurbit chlorotic yellows virus (AB523788/AB523789); CYSDV, *Cucurbit yellow stunting disorder virus* (NC_004809/NC_004810); LCV, *Lettuce chlorosis virus* (NC_012909/NC_012910); LIYV, *Lettuce infectious yellows virus* (NC_003617/NC_003618); PYVV, *Potato yellow vein virus* (NC_006062/NC_006063), SPaV, *Strawberry pallidosis-associated virus* (NC_005895/NC_005896); SPCSV, *Sweet potato chlorotic stunt virus* (NC_004123/NC_004124); TICV, *Tomato infectious chlorosis* virus (NC_013258/NC_013259); ToCV, *Tomato chlorosis virus* (NC_007340/NC_007341); Genus "*Velarivirus*" (proposed): CoV-1 (HM588723); CoV-2 (JQ599282); CoV-3 (JQ59983); CoV-4 (JQ599284); LChV-1, Little cherry virus 1 (NC_001836); GLRaV-7 (NC_016436); unassigned: MVBaV, Mint vein banding associated virus (AY548173).

event. Putative examples of closterovirus recombination are abundant (Cuellar et al., 2008; Melzer et al., 2010; Farooq et al., 2013). The gradual increase in sequence similarity is comparable to that proposed for Citrus tristeza virus (CTV) strain T36, and suggests the potential recombination event was not recent (Mawassi et al., 1996). The presence of multiple CoVs in a single host plant would provide an environment conducive for such recombination events.

The family *Closteroviridae* Subcommittee to the International Committee on Taxonomy of Viruses (ICTV) has recently amended a commonly used criterion for species demarcation of closteroviruses. To be considered a distinct species, the level of sequence divergence in a phylogenetically informative protein (RdRp, HSP70h, or CP) was raised from 10 to 25% (Martelli et al., 2011). This increase in stringency was undertaken to address the proliferation of closteroviruses that had a similar genome organization, host range, and biological properties but, in some cases, only marginally exceeded the previous 10% sequence divergence criterion, thereby elevating them to species status (Martelli et al., 2012). Following the implementation of this more stringent criterion, a group of seven GLRaVs species and their "variants" (GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-6 DE, GLRaV-9, GLRaV-Car, and GLRaV-Pr) were condensed into a single species, GLRaV-4 (Martelli et al., 2012). Based on the amino acid identities of the RdRp, HSP70h, and CP sequences it is clear that CoV-1, CoV-3, and CoV-4 are distinct species under the new criterion. The two most closely related CoVs, CoV-1 and CoV-2, have amino acid divergence values for the RdRp, HSP70h, and CP of 22, 31, and 33%, respectively. Although the sequence divergence between the CoV-1 and CoV-2 RdRp does not exceed the 25% threshold, the average sequence divergence for these three phylogenetically informative proteins is 29%, and we therefore contend that CoV-1 and CoV-2 should represent two distinct species. Additional closterovirus-like sequences were also identified in the library. The majority of these, when translated into amino acid sequences, were only 10–20% divergent from CoV-1 and are likely to have come from a second strain of CoV-1 that also infects common green ti.

The discovery of four related closterovirus species co-infecting the same host which share a similar genome organization and perhaps similar biological properties presents a situation reminiscent to the GLRaV-4 group. Since these four CoVs were discovered through the intense study of a single ti plant, it is also plausible that additional CoV species exist. Some members of the GLRaV-4 group, however, were found to be serologically related (Ghanem-Sabanadzovic et al., 2012). There are currently no antisera raised against any of the CoVs that would allow experiments to be conducted to determine their serological relationships. Given the amount of sequence divergence between the currently known CoVs, particularly in their respective structural proteins, it seems unlikely that they will be serologically related.

Within the family *Closteroviridae*, LChV-1, GLRaV-7, and CoV-1 form a distinct monophyletic clade for which the genus "*Velarivirus*" has been proposed (Al Rwahnih et al., 2012; Martelli et al., 2012). Phylogenetic analyses placed CoV-2, CoV-3, and CoV-4 within this clade. We therefore propose that CoV-2, CoV-3, and CoV-4 also be members of the proposed genus "*Velarivirus*," should it be ratified by the ICTV.

ACKNOWLEGMENTS

The authors would like to thank Julie Coughlin, Dr. Shaobin Hou, Kenneth Hu, Chris Kadooka, James Kam, and Christina Liu for their assistance with this project. This project was supported, in part, by Award #59955 from the Hawaii Department of Agriculture.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 December 2012; paper pending published: 25 January 2013; accepted: 15 February 2013; published online: 05 March 2013.

Citation: Melzer MJ, Sugano JS, Uchida JY, Borth WB, Kawate MK and Hu JS (2013) Molecular characterization of closteroviruses infecting Cordyline fruticosa L. in Hawaii. Front. Microbiol. ***4****:39. doi: 10.3389/fmicb.2013.00039*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 11 April 2013
doi: 10.3389/fmicb.2013.00083

The closterovirus-derived gene expression and RNA interference vectors as tools for research and plant biotechnology

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Edited by:
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Reviewed by:
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Important progress in understanding replication, interactions with host plants, and evolution of closteroviruses enabled engineering of several vectors for gene expression and virus-induced gene silencing. Due to the broad host range of closteroviruses, these vectors expanded vector applicability to include important woody plants such as citrus and grapevine. Furthermore, large closterovirus genomes offer genetic capacity and stability unrivaled by other plant viral vectors. These features provided immense opportunities for using closterovirus vectors for the functional genomics studies and pathogen control in economically valuable crops. This review briefly summarizes advances in closterovirus research during the last decade, explores the relationships between virus biology and vector design, and outlines the most promising directions for future application of closterovirus vectors.

Keywords: viral vector, closteroviruses, RNAi, *Beet yellows virus*, *Citrus tristeza virus*, *Grapevine leafroll-associated virus-2*

INTRODUCTION

The family *Closteroviridae* has a special place in molecular and evolutionary virology. Together with animal coronaviruses, closteroviruses explore the upper size limit for the RNA-based genomes (Dolja et al., 2006; Gorbalenya et al., 2006). The relatively large genetic capacity of these viruses likely requires higher fidelity of RNA replication than is typical for RNA viruses (Denison et al., 2011), but also allows them to acquire new beneficial genes. On a more practical side, genetic plasticity of closteroviruses makes them attractive vehicles for the delivery and expression of recombinant genes engineered into viral genomes. While generation of coronavirus-based expression vectors seems to be in its infancy, several well-developed closteroviral vectors are available and show strong potential for application in functional genomics and pathogen control (Prokhnevsky et al., 2002; Folimonov et al., 2007; Kurth et al., 2012). Because RNA viruses do not normally integrate their genomes into host chromosomes, utilization of RNA virus vectors provides a useful alternative to transgenic technology helping to bridge the divide between a science-based perspective and the more emotionally charged public perception of genetic engineering and biotechnology.

As is the case for any positive-strand RNA virus, engineering a closterovirus into a vector requires generation of a biologically active cDNA clone. Such full-length genomic clones so far have been reported for *Lettuce infectious yellows virus* (LIYV; Klaassen et al., 1996), *Beet yellows virus* (BYV; Peremyslov et al., 1998), *Citrus tristeza virus* (CTV; Satyanarayana et al., 1999), *Grapevine leafroll-associated virus-2* (GLRaV-2; Liu et al., 2009), and *Lettuce chlorosis virus* (Mongkolsiriwattana et al., 2011). Although the ability of LIYV to express recombinant proteins has been confirmed (Wang et al., 2009), only BYV, CTV, and GLRaV-2 were developed into gene expression vectors capable of full-fledged systemic infection of the host plants. Furthermore, it was recently shown that the GLRaV-2-derived vector has a capacity to trigger RNA interference (RNAi) that targets host endogenous genes (Kurth et al., 2012), a capacity traditionally called virus-induced gene silencing (VIGS; Baulcombe, 1999).

Admittedly, unlike the *Tobacco mosaic virus* (TMV)-based vectors (Pogue et al., 2002; Gleba et al., 2007), closterovirus vectors are not well suited for rapid mass production of the recombinant proteins. This is the case because of the slower infection cycle and tissue-specific tropism of most closteroviruses whose replication is limited to the phloem (Bar-Joseph et al., 1979). However, closterovirus vectors fill very important niches that are inaccessible to most other plant virus vectors. These niches include fruit-producing specialty crops such as citrus and grapevine, genetic capacity and stability that allow long-term expression of the large recombinant genes, and ability to combine protein expression and VIGS in the same vector. It seems that the scientific base for closterovirus vector application in research and biotechnology is mature. Thus, realization of a strong commercial potential of these vectors depends primarily on the availability of the proper investment.

GENOME STRUCTURE, REPLICATION, AND EXPRESSION

Currently, the family *Closteroviridae* includes three approved (*Closterovirus, Crinivirus*, and *Ampelovirus*; Karasev, 2000) and one proposed (*Velarivirus*; Al Rwahnih et al., 2012) genera. All

closteroviruses share two large, conserved gene modules one of which is responsible for genome replication, whereas the other one functions in genome packaging and intercellular transport (Dolja et al., 2006). The composition of the 3′-proximal genome region varies between and often within the genera. Furthermore, crinivirus genomes are split between two RNAs in contrast to a single genomic RNAs in other genera. Despite the large, up to 19.3 kb size of their genomes, closteroviruses are rank-and-file members of the Alphavirus-like superfamily of the positive-strand RNA viruses (Koonin and Dolja, 1993; Dolja and Koonin, 2011) with capped genomic RNAs that are directly translated to produce an RNA replicase (Karasev et al., 1989; Agranovsky et al., 1994b).

Because BYV is the prototype member of the family (Bar-Joseph et al., 1979; Dolja, 2003), this and the following sections of the article are focused on BYV with other viruses being evoked as needed. The ∼15.5 kb BYV genome encompasses nine open reading frames (ORFs; Agranovsky et al., 1991b, 1994b). The conserved replication gene module includes ORFs 1a and 1b that encode a polyprotein containing methyltransferase (MET), superfamily 1 RNA helicase (S1H), and RNA-dependent RNA polymerase (RdRp; expressed from ORF 1b via +1 translational frameshift) domains (**Figure 1A**). A large central portion of this polyprotein is less conserved, but is functionally important because several alanine-scanning mutations introduced into this region decreased or abolished RNA amplification (D. V. Alzhanova and V. V. Dolja, unpublished data). It seems plausible that this region contributes to the relatively high fidelity of RNA replication required for the reproduction of RNA viruses with the largest genomes, as shown to be the case for coronaviruses (Denison et al., 2011). However, extensive database searches failed to identify significant sequence similarity between the central parts of the closterovirus polyproteins and any other proteins. Moreover, examination of the alignment of the sequences of the closterovirus polyproteins between the MET and the S1H domains failed to identify any conserved motifs resembling those in the catalytic sites of any known nucleases, making it unlikely that enzymes functionally analogous to the proofreading nucleases of coronaviruses lurk in the uncharacterized parts of closterovirus polyproteins (E. V. Koonin, unpublished observations).

The 5′-terminal region of ORF 1a encodes a papain-like leader protease (L-Pro) that is autocatalytically released from the polyprotein; optimal RNA amplification requires functionally intact L-Pro (Agranovsky et al., 1994b; Peremyslov et al., 1998). Interestingly, several closteroviruses including CTV and GLRaV-2 encode a tandem of leader proteases that have evolved via gene duplication and functional divergence (Peng et al., 2001). Although the exact composition of the RNA replication complex of BYV is not known, it has been shown that this complex localizes to endomembrane vesicles that contain ORF 1a and 1b products including L-Pro released from polyprotein via auto-processing (Erokhina et al., 2001; Zinovkin et al., 2003). It was also found that formation of the vesicular complexes occurs via recruitment and reorganization of the endoplasmic reticulum (ER) by the ORF 1a product (e.g., **Figure 1E**), similar to many other positive-strand RNA viruses (den Boon and Ahlquist, 2010).

In addition to the 5′-proximal replication gene module, efficient amplification of BYV requires p21, a 21-kDa protein encoded by the 3′-proximal ORF 8 (Peremyslov et al., 1998). It has been shown that p21 is a strong suppressor of RNAi that non-specifically binds and sequesters double-stranded form of the small interfering RNAs (siRNAs), and micro RNAs (miRNAs; Reed et al., 2003; Chapman et al., 2004). The homologs of p21 are conserved throughout the genus *Closterovirus* (Chiba et al., 2006), but not in more distantly related viruses; however, RNAi suppressors unrelated to p21 were identified throughout the family (Lu et al., 2004; Kreuze et al., 2005).

Typical of the Alphavirus-like superfamily, expression of the ORFs downstream of the replication gene module occurs via generation of the positive-strand subgenomic RNAs (sgRNAs). These sgRNAs are collinear and 3′-coterminal with the genome, and are functionally monocistronic, expressing only the 5′-terminal ORF. The BYV genome transcription produces seven sgRNAs that have minus-strand counterparts (Dolja et al., 1990). The proteins encoded by the ORFs 2–7 are involved in virion assembly and virus transport within plants (see below).

The transcription start site mapping for the five BYV sgRNAs revealed a somewhat lax sequence conservation pattern in the upstream regions presumed to form sgRNA promoters (Agranovsky et al., 1994a; Peremyslov and Dolja, 2002; Vitushkina et al., 2007). It was also shown that the sgRNA synthesis in BYV is regulated both quantitatively and temporally by several early and late promoters (Hagiwara et al., 1999). The promoter controlling production of the major capsid protein (CP) directs gene expression early in the replication cycle and to the highest level.

More extensive analysis of the genome transcription in CTV produced a complex picture suggesting that each sgRNA promoter can also act as a terminator. As a result, each "normal" positive-strand sgRNA has not only a minus-strand, but also a plus-strand counterpart that expands to the 5′-terminus of genome (Gowda et al., 2001). Furthermore, additional, ∼800 nts-long, plus-strand, 5′-coterminal sgRNAs were also described (Che et al., 2001). The exact mechanisms whereby such a complex population of sgRNAs is produced are difficult to interpret in functional terms or to fit into any of the three major expression strategies employed by other positive-strand RNA viruses, namely: (i) internal initiation on a minus-strand; (ii) premature termination of the minus-strand synthesis followed by use of this strand to produce plus-strands; (iii) common leader-initiated, discontinuous synthesis of minus-strand templates for plus-strand sgRNAs typical of coronaviruses (Miller and Koev, 2000; Pasternak et al., 2006; Sztuba-Solińska et al., 2011). The unusually complex pattern of expression in CTV is exacerbated by the promiscuous initiation of the 3′- and 5′-coterminal sgRNAs that appear to use distinct controlling elements within the same promoter region (Ayllón et al., 2003, 2004).

VIRION MORPHOLOGY, VIRUS TRANSPORT AND TRANSMISSION

The flexuous filamentous virions of closteroviruses are the longest currently known, reaching the length of ∼2,000 nm; these virions are built of CPs that are helically arranged around genomic RNA. The overall morphology of the closterovirus capsids is

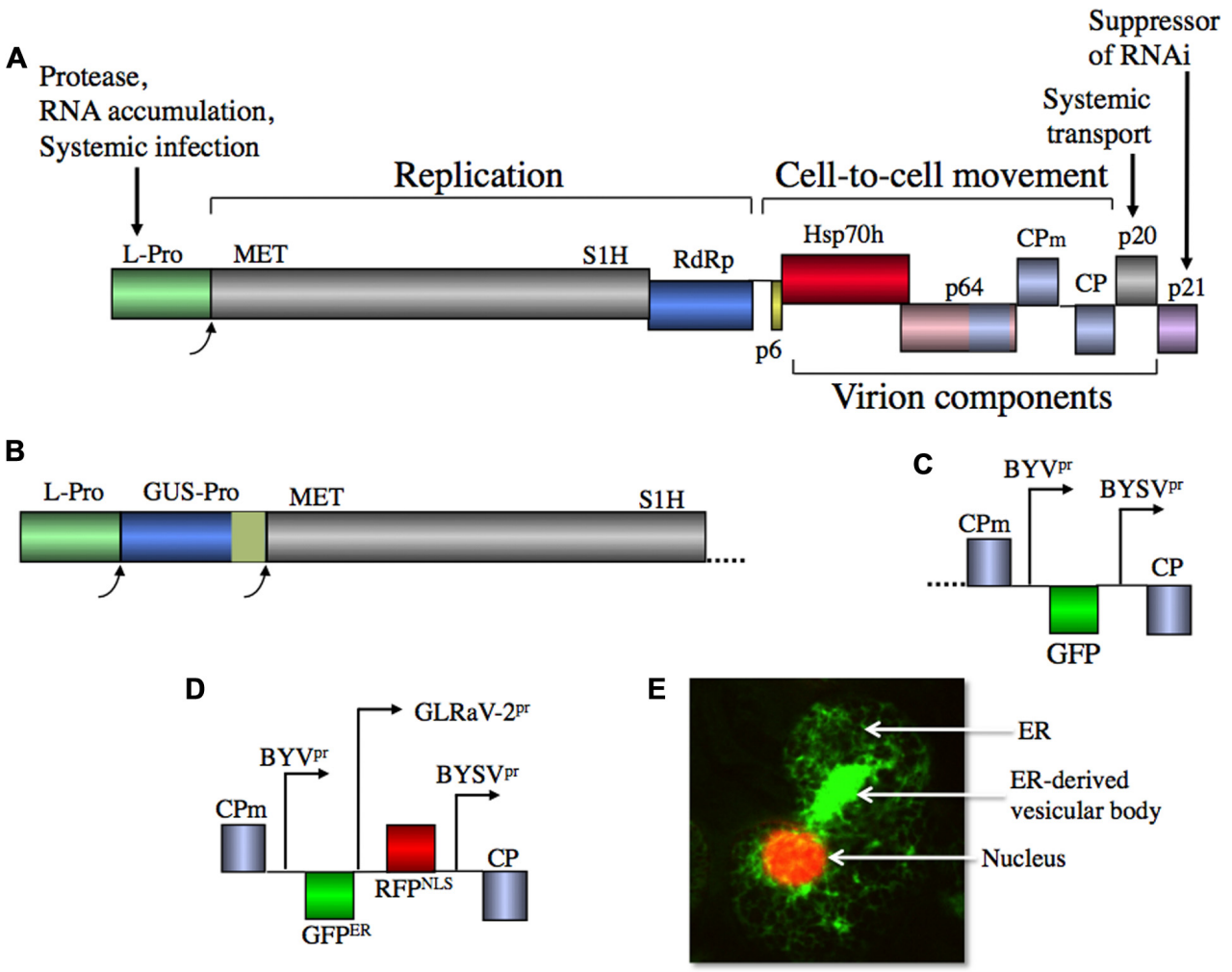

FIGURE 1 | (A) Diagram of BYV genome with gene functions shown. L-Pro, papain-like leader protease; MET, methyltransferase (capping enzyme); S1H, superfamily I helicase; RdRp, RNA-dependent RNA polymerase; p6, 6-kDa protein; Hsp70h, Hsp70 homolog; p64, 64-kDa protein; CPm, minor capsid protein; CP, capsid protein; p20, 20-kDa protein; p21, 21-kDa protein. Homologous CP, CPm, and the C-terminal domain of p64 are shown in the same color. **(B)** Proteolytic gene expression cassette including β-glucuronidase ORF (GUS) fused in frame to papain-like, self-cleaving domain of potivirus HC-Pro. **(C)** Recombinant gene expression cassette including native BYV promoter, green fluorescent protein ORF (GFP) and heterologous BYSV promoter. **(D)** Dual expression cassette including heterologous BYSV and GLRaV-2 promoters, ER-targeted GFP and red fluorescent protein containing nuclear localization signal. **(E)** Confocal laser scanning microscopic image of the *N. benthamiana* leaf cell infected with BYV vector that expresses GFP^{ER} and RFP^{NLS}. Note the virus-induced, ER-derived, multivesicular body likely containing viral RNA replication complexes.

similar to that of the other filamentous viruses in the families α-, β-, and γ-*Flexiviridae* (Martelli et al., 2007), and *Potyviridae* (Adams et al., 2012), all of which encode homologous CPs (Dolja et al., 1991). However, closteroviruses are distinguished by a remarkable structure that caps one end of the virion and was unwittingly called the "tail" by its discoverers (Agranovsky et al., 1995). Subsequently, it was shown that this ~100 nm-long structure encapsidates the 5′-terminal ~650 nts (4%) of the genome and accordingly rather represents a "snout" that measures ~8 nm in diameter compared to the 12 nm virion "body" (Peremyslov et al., 2004). Nevertheless, to avoid confusion, we will continue to use the term "tail" throughout the article. The main building block of the virion tail is the minor CP (CPm; Agranovsky et al., 1995; Satyanarayana et al., 2004) that is a divergent duplicate of the major CP which forms the long virion body (Boyko et al., 1992).

In addition to CP and CPm, the virions of closteroviruses contain at least two more structural proteins. The third one is a ~60-kDa protein (p64 in BYV) whose C-terminal domain is yet another divergent duplicate of the CP (**Figure 1A**; Tian et al., 1999; Satyanarayana et al., 2000; Napuli et al., 2003). The fourth and most unusual virion protein is a homolog of cellular molecular chaperones of the heat shock protein 70 (Hsp70) family, Hsp70 homolog (Hsp70h; Agranovsky et al., 1991a). The viral Hsp70h, however, is a "misbehaving chaperone" that does not leave the scene following successful matchmaking. It was shown that Hsp70h is an integral virion component (Tian et al., 1999; Napuli et al., 2000) that is required for proper virion tail

assembly (Satyanarayana et al., 2000; Alzhanova et al., 2001, 2007). Although CPm alone can initiate virion assembly, coordinated incorporation of CPm, the ~60-kDa protein and Hsp70h is required for efficient assembly of the tails of the correct length (Satyanarayana et al., 2000, 2004; Alzhanova et al., 2001, 2007; Napuli et al., 2003).

As was shown for BYV, the complexity of the closterovirus particles does not stop at four structural proteins and includes a fifth, ~20-kDa protein (p20) that incorporates into virions via interaction with Hsp70h (Prokhnevsky et al., 2002). Moreover, analysis of the BYV tail morphology and composition indicated that p20 most likely forms the pointed tip segment of the three-segment tail, with two other segments assembled of CPm, the ~60-kDa protein and Hsp70h (Peremyslov et al., 2004).

The cell-to-cell movement of closteroviruses turned out to be a no less engaging story than that of virion assembly. The closteroviruses possess a conserved dedicated movement protein of ~6-kDa (p6 in BYV) that is targeted to ER via its N-terminal transmembrane domain (Alzhanova et al., 2000; Peremyslov et al., 2004). However, each of the CP, CPm, Hsp70h, and p64 is also indispensable for the cell-to-cell movement of BYV (Peremyslov et al., 1999; Alzhanova et al., 2000; Napuli et al., 2003). Taken together, tight functional coupling of the virion assembly and cell-to-cell movement (Alzhanova et al., 2001) and an ability of Hsp70h to target plasmodesmata in association with microfilaments and class VIII myosin motors (Medina et al., 1999; Prokhnevsky et al., 2005; Avisar et al., 2008) prompted a hypothesis that the virion tail is a movement device (Dolja, 2003; Dolja et al., 2006). The encapsidation of 5′-terminal region of viral genome by the tail is compatible with this hypothesis implying that the Hsp70h-containing tails guide viral genomes to and through plasmodesmata to allow directional transport and translation of viral genomes entering the neighboring cell.

Another twist of the "tail as a transport device" concept was the finding that the virion tip component p20 is required for the long-distance transport of BYV through the phloem (Prokhnevsky et al., 2002). Because BYV p20 shows little if any sequence similarity to proteins in other closteroviruses, it is not clear if these viruses also encode the analogous long-distance transport factors. In contrast, the leader proteases that also are implicated in the long-distance transport of BYV and GLRaV-2 (Peng et al., 2002, 2003; Liu et al., 2009), are conserved throughout the family (Peng et al., 2001).

The semi-persistent vector transmission of closteroviruses relies on three distinct taxa of insects, aphids (*Closterovirus*), mealybugs (*Ampelovirus*), and whiteflies (*Crinivirus*; Karasev, 2000; Ng and Falk, 2006). It is not known which viral proteins mediate aphid- or mealybug-dependent transmission of the viruses in two former genera. For criniviruses, there is strong experimental support for the critical role of CPm in transmission (Tian et al., 1999; Stewart et al., 2010; Chen et al., 2011) suggesting that CPm and/or other tail components are the transmission determinants in other closteroviruses as well.

CLOSTEROVIRUS BIOLOGY AND VECTOR DESIGN

It should be emphasized that the infection cycle of closteroviruses is relatively slow, with BYV moving from cell-to-cell at a rate of ~1 cell per day (Peremyslov et al., 1999), not every 2–4 h as is the case for TMV (Kawakami et al., 2004). Similarly, the onset of BYV systemic infection occurs at 2–3 weeks post inoculation (Prokhnevsky et al., 2002) compared, for instance, to 3 days for *Tobacco etch potyvirus* (Dolja et al., 1992). The pace of systemic infection for closteroviruses that infect woody plants, such as CTV or GLRaV-2, is even slower, reaching 1 month or longer (Folimonov et al., 2007; Kurth et al., 2012). Furthermore, whereas BYV is able to infect leaf mesophyll and epidermal cells, most of the other closteroviruses are strictly limited to phloem where they are acquired by vectoring insects and deposited by viruliferous insects at the beginning of each infection cycle (Ng and Falk, 2006). These aspects of the virus biology have major impact on development of the proper inoculation techniques for closterovirus-derived gene vectors. Similarly, virus biology has to be taken into account when the utility of the viral vectors or safety measures preventing vector escape during propagation are considered.

The genome organization and molecular biology of closteroviruses are also of paramount importance for the vector design. The recombinant sequence could be either spliced into the virus vector genome, or used to replace part of it. Due to the "wall-to-wall" organization of viral genomes, the replacement strategy almost inevitably results in a loss-of-function phenotype. For instance, there are no non-essential genes in BYV (Dolja, 2003); replacement of any gene will result in a loss of replication or systemic infectivity. In contrast, the larger CTV genome contains genes that are required for infection of some citrus species but not others (Tatineni et al., 2011). These genes, albeit expressed to low levels, are potential replacement targets for vector design.

The mechanisms whereby Alphavirus-like viruses including closteroviruses express their proteins involve polyprotein processing by proteases and expression of sgRNAs (Dolja and Carrington, 1992; Miller and Koev, 2000). Because the closterovirus leader proteases appear to cleave only *in cis*, the proteolytic expression of recombinant protein can be ensured either by fusing the protein to L-Pro, or by inserting a new proteolytic cassette, similar to original designs of the potyviral vectors (**Figure 1B**; Dolja et al., 1992; Carrington et al., 1993).

Engineering of an autonomous expression cassette controlled by an additional sgRNA promoter is the preferable approach to closterovirus vector design. This approach allows one to choose sgRNA promoter of desired strength and to place the cassette into an optimal genomic location. The source of the additional promoter, however, is of paramount importance. If the homologous promoter is duplicated, an added expression cassette can be readily eliminated via homologous recombination. An elegant solution to this problem, utilization of a heterologous sgRNA promoter from a related virus, was advanced originally for a TMV vector (Donson et al., 1991; Dawson, 2011). Because the heterologous promoter has a distinct nucleotide sequence, homologous recombination is effectively eliminated and vector stability increases. Obviously, the activity of the heterologous promoter could be lower than it is in the natural background, so picking the right promoter is a matter of trial and error.

Two other aspects of closterovirus biology important for the vector design are the virion morphology and the inherent large size of the genomes. Unlike icosahedral virions with their limited genome packaging capacity, elongated virions do not set an

upper limit to the size of the expression cassette. It also stands to reason that due to their large genomes, closterovirus vectors are better suited for accommodating recombinant expression cassettes than more rigidly organized genomes of smaller RNA viruses such as TMV.

The utility of closteroviruses as VIGS vectors seemed uncertain from general considerations. Unlike well established *Potato virus X* (PVX)- or *Tobacco rattle virus* (TRV)-based VIGS vectors (Baulcombe, 1999; Bachan and Dinesh-Kumar, 2012), closteroviruses encode RNAi suppressors that are among the strongest characterized so far (Reed et al., 2003; Lu et al., 2004; Chiba et al., 2006). However, this did not preclude development of the powerful VIGS vector from at least one closterovirus (see the GLRaV-2 section below).

BYV-DERIVED VECTORS

Development of the first BYV vectors rapidly followed the generation of the biologically active cDNA clone (Peremyslov et al., 1998; Hagiwara et al., 1999). In these vectors, the β-glucuronidase (GUS) reporter was fused to three different BYV proteins. In addition, a minireplicon that produced only the replication-associated proteins and a free GUS reporter controlled by the CP sgRNA promoter was engineered. Interestingly, accumulation of GUS sgRNA expressed by this minireplicon was ∼3.5-fold higher than that of CP sgRNA in the wild type genome background (Hagiwara et al., 1999). In general, relocation of the sgRNA promoter closer to the 3′-end of the closterovirus genome increases its expression levels, an important consideration for optimal vector design.

A more advanced BYV vector capable of expressing recombinant protein from an autonomous cassette has become a prototype for the subsequent designs of vectors based on other closteroviruses (Peremyslov et al., 1999). In this vector, the recombinant ORF encoding green fluorescent protein (GFP) was inserted downstream from the native CP sgRNA promoter, whereas a heterologous CP promoter derived from a closely related *Beet yellow stunt virus* (BYSV; Karasev et al., 1996) was used to express the BYV CP (**Figure 1C**). The infectious RNA transcripts for plant inoculation were obtained *in vitro* using bacteriophage SP6 RNA polymerase and plasmid linearized near the 3′-end of the viral cDNA (Peremyslov and Dolja, 2007). This vector was useful for mechanical inoculation of a highly susceptible BYV local lesion host *Claytonia perfoliata*, whereas systemic infection of a convenient systemic host, *Nicotiana benthamiana*, using RNA transcripts was inefficient.

The next generation of BYV vectors suited for systemic infection of a host plant involved replacement of the SP6 promoter with the 35S *Cauliflower mosaic virus* promoter active in plants, and insertion of a ribozyme downstream from the viral cDNA to ensure proper processing of the resulting viral transcript (Prokhnevsky et al., 2002). This design, originally proposed by Leiser et al. (1992) allowed the use of *Agrobacterium* for efficient delivery of viral cDNA to plant cells mediated by T-DNA-containing binary vectors. The resulting agroinoculation procedure, further improved by vacuum infiltration of the bacterial suspension (Marillonnet et al., 2005), remains the method of choice for introducing RNA viral vectors back to plants.

In general, transient expression of recombinant genes via *Agrobacterium*-mediated transformation is highly efficient because the leaf infiltration procedure delivers large numbers of gene transfer-competent bacteria per each plant cell. In *N. benthamiana*, this technique results in high-level production of a recombinant protein in virtually all exposed cells. Surprisingly, when agroinfiltration is used to deliver the viral vector, only very few cells become infected (Marillonnet et al., 2005; Chiba et al., 2006). Two strategies were proposed to improve the cell infection rate following agroinoculation: (i) labor-extensive vector modifications aimed at suppression of the accidental splicing of the viral transcripts in the transfected plant nuclei (Marillonnet et al., 2005) and (ii) co-expression of the strong RNAi suppressors during agroinoculation (Chiba et al., 2006). Each strategy resulted in a drastic, three-orders of magnitude increase of the infection rate. Interestingly, when a combination of both strategies was attempted for GLRaV-2-derived vectors, it was found that RNAi suppression overrides the need for splicing modification. Thus, the simple use of an RNAi suppressor to supplement agroinoculation appears to be the method of choice for improving vector infectivity.

Further elaboration of the BYV vectors involved engineering of tandem expression cassettes. In a dual expression vector, the GLRaV-2-derived CP sgRNA promoter was inserted upstream from BYSV promoter to allow simultaneous production of the monomeric red fluorescent protein (mRFP) targeted to nucleus and the ER-targeted GFP (**Figures 1D,E**). An alternative vector design included a proteolytic expression cassette introduced downstream from L-Pro; this cassette encompassed a fusion of GUS to the proteolytic domain of the potyvirus helper component-protease (**Figure 1B**). Thus, BYV was proven to provide a facile platform for various vector designs showing genetic plasticity so far unmatched by other plant virus-derived vectors.

At the time of their generation, the genetic stability of the BYV vectors was greater than that of vectors based on other plant viruses. The potyvirus-based vectors could maintain reporter expression for up to 1 month when propagated in the same plant (Dolja et al., 1993), whereas BYV vectors were at least twice as stable (V. V. Peremyslov and V. V. Dolja, unpublished data). For comparison, the PVX-based vectors did not maintain reporter expression even within one cycle of systemic infection lasting around 2 weeks (Chapman et al., 1992). Thus, reporter-expressing BYV vectors provided a facile experimental model for the identification of the genes involved in virus replication, assembly, cell-to-cell movement and systemic transport (Dolja et al., 2006). Regrettably, the utility of these vectors for gene expression or VIGS in the economically relevant BYV hosts such as sugar beet or spinach has not been so far assessed.

CTV-DERIVED VECTORS

The generation of the full-length, biologically active cDNA clone of CTV was more challenging than it was for BYV. This was mainly because CTV genome is larger than the BYV genome and because unlike BYV, CTV does not normally infect herbaceous plant species. Due to the low infectivity of full-length transcripts of the CTV cDNA in protoplasts, most of the initial experimentation was performed with minireplicons (Satyanarayana et al., 1999). To overcome this limitation, a laborious procedure of cyclic

virion transfer in protoplasts initially transfected with RNA transcripts was developed (Satyanarayana et al., 2000). This procedure was also adapted for slash-inoculation of citrus trees with virions propagated in protoplasts (Satyanarayana et al., 2001).

The later development of CTV-based vectors expressing the GFP reporter produced the best results with the design mirroring that of the BYV vector; a short variant of the BYV CP sgRNA promoter was used to drive GFP expression (Peremyslov et al., 1999; Folimonov et al., 2007). Most remarkably, the genetic stability of CTV vector in citrus proved to be much higher than the stability of the BYV vector in *N. benthamiana*. Although gradual loss of the expression cassette occurred in some of the vector-infected trees, many trees maintained GFP expression for over 2 years (Folimonov et al., 2007) and some even longer, up to 7 years (Dawson, 2011).

Recently, an agroinoculation procedure to introduce CTV to *N. benthamiana* has been developed (Ambrós et al., 2011). Surprisingly, a CTV vector launched by an *Agrobacterium* was not only systemically infectious in this presumed non-host plant, but was able to exit the phloem to which it is strictly limited in the citrus hosts. Similar to BYV, the efficiency of agroinoculation was increased by addition of RNAi suppressors (Chiba et al., 2006; Ambrós et al., 2011). Although an agroinoculation technique to infect citrus is not yet available, the ability to propagate CTV-derived gene expression vectors in *N. benthamiana* rather than in protoplasts will facilitate investigation of CTV gene functions. It will be interesting to determine if genetic stability of the CTV vectors in a herbaceous host matches that in citrus.

The CTV vector has a significant potential not only for the research on the gene functions or virus population dynamics in the infected citrus (Folimonova et al., 2010; Tatineni et al., 2011), but also in the development of pathogen-resistant citrus trees (Dawson, 2011). However, this potential might be jeopardized by the concerns due to the CTV transmission by its natural insect vectors, aphids. Even if the CTV transmission factors are identified and disabled without affecting vector infectivity, transmissibility of such disarmed vectors could be restored via recombination with the wild CTV isolates that are ubiquitous in the agricultural settings.

GLRaV-2-DERIVED VECTORS

The latest addition to the assortment of closterovirus gene vectors were the vectors based on the GLRaV-2 (Liu et al., 2009; Kurth et al., 2012). The first generation of these vectors was used to dissect functions of the two leader proteinases (L1 and L2) in the experimental host *N. benthamiana* and to determine that both of them provided varying contributions to the establishment and systemic spread of virus infection (Liu et al., 2009). Unexpectedly, the significance of these proteases was much greater for the infection of grapevine leaf cells compared to that in *N. benthamiana*, attesting to the host-specific roles of L1 and L2 in virus infection. These vectors, however, failed to systemically infect grapevine; it took us several years of sustained effort to identify the culprit and to find a solution of this problem.

There turned out to be two major impediments to the development of a virus vector for grapevine. Unlike CTV whose ability to infect citrus trees upon slash-inoculation was established long ago (Garnsey et al., 1977), to the best of our knowledge, there have been no reports of successful mechanical inoculation of grapevine with any virus. Thus, we had to rely on agroinoculation without knowing if this technique was suitable for virus launching to phloem tissue where it naturally reproduces. Paradoxically, GLRaV-2 can be mechanically transmitted to *N. benthamiana* (Goszczynski et al., 1996), seemingly a blessing that turned to be a curse.

The initial full-length cDNA clone was obtained using *N. benthamiana*-propagated GLRaV-2; this clone was readily launched to this host plant via agroinoculation, and exhibited primarily phloem-limited systemic distribution as one would expect (Liu et al., 2009). To overcome the lack of systemic infectivity in grapevine, we tested a number of potential solutions: (i) addition of homologous and heterologous RNAi suppressors (Chiba et al., 2006); (ii) improving vector infectivity via eliminating potential sites of aberrant splicing and adding plant-specific introns (Marillonnet et al., 2005); (iii) a combination of (i) and (ii); (iv) all possible means of mechanical inoculation from rubbing to pinching to slashing to bombarding with microparticles to vacuum infiltrating; (v) testing agroinoculation of grapevine roots (Muruganantham et al., 2009), young seedlings, or micropropagated plantlets; and, (vi) screening for the optimal *A. tumefaciens* and *A. vitis* strains.

When all these possibilities were exhausted, we reasoned that there must have been a problem with the cDNA clone itself. Because the viral RNA genomes are prone to rapid mutation accumulation and thus rapidly evolve to adapt to a new host (Roossinck and Schneider, 2006), propagation of GLRaV-2 in *N. benthamiana* could result in the loss of infectivity in grapevine. Accordingly, we embarked on a wholesale reassembly of our vector with cDNA fragments derived from GLRaV-2-infected grapevine. It is important to emphasize that only cDNAs corresponding to a consensus sequence of an isolate that were likely to represent the dominant infectious variant were used. Compared to the complete consensus sequence of the grapevine isolate, the original vector had 75 point mutations some or all of which could contribute to the loss of vector infectivity in grapevine.

Using the optimized procedure of vacuum agroinfiltration of the whole, micropropagated plantlets, we obtained consistent systemic infection of several grapevine varieties with this second generation, "grapevinized," GFP-expressing, GLRaV-2 vector dubbed vLR2-GFP (**Figure 2A**; Kurth et al., 2012). It was found that the vector-infected cells appeared first in the stem bark phloem and then colonize leaf petioles, midrib, and smaller veins between 3 and 6 weeks post inoculation. After several months of propagation, vLR2-GFP started to accumulate in the root phloem. Conspicuously, when the berries were formed, infection invaded some of them spreading initially through the phloem bundles and then exiting into mesocarp cells (**Figure 2B**; Kurth et al., 2012). Once established, the vector infection can be readily transmitted by grafting to apparently any variety of table or wine grapes. Similar to CTV vectors, vLR2-GFP is genetically highly stable: only some of the infected plants exhibited deterioration of the insert after 1 year-long propagation in grapevine (Kurth et al., 2012).

The most unexpected and important ability of vLR2 was to elicit powerful systemic VIGS despite the fact that GLRaV-2

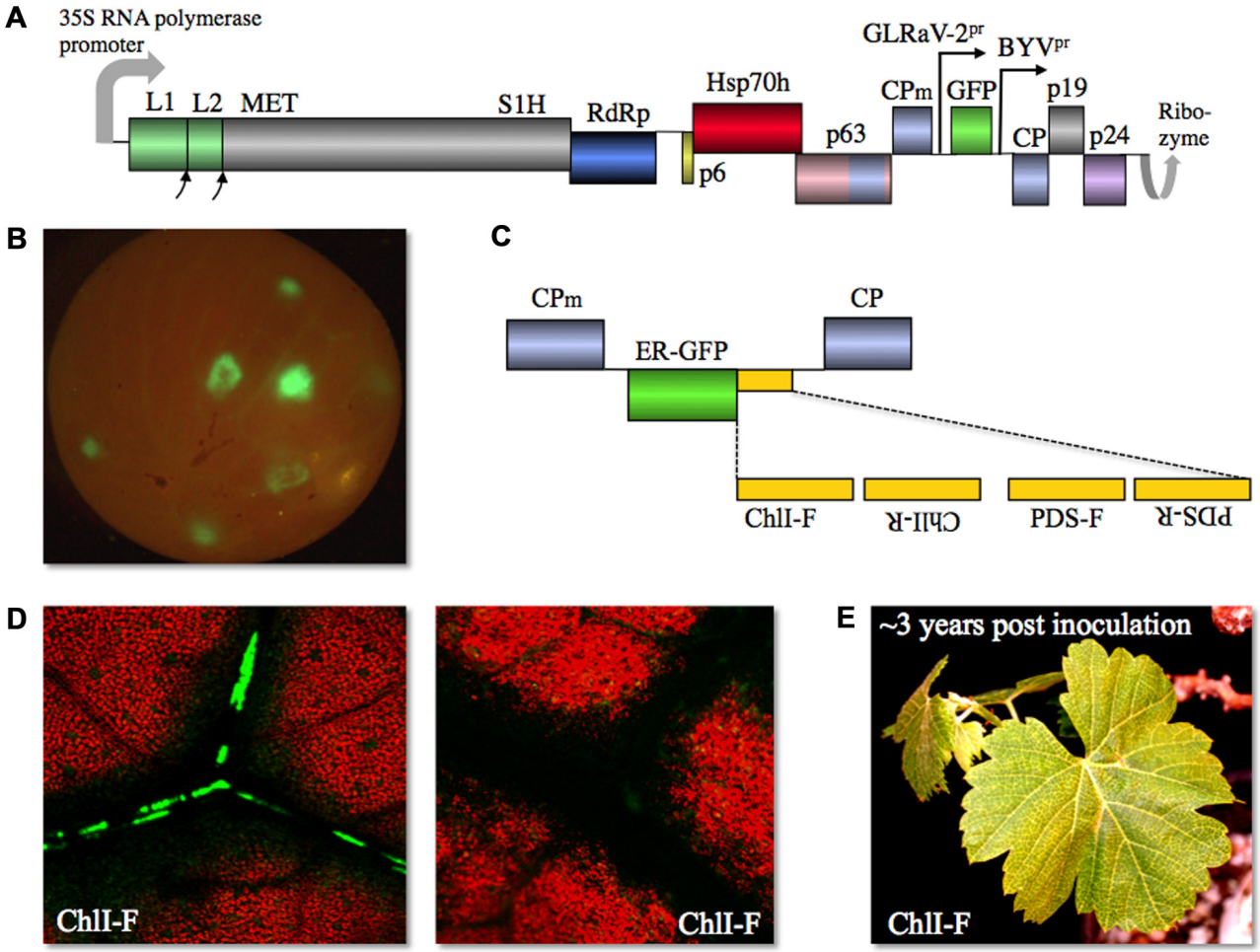

FIGURE 2 | (A) Diagram of GLRaV-2-based gene expression vector vLR2-GFP with gene functions shown. L1 and L2, papain-like leader proteases 1 and 2; p19, 19-kDa protein; p24, 24-kDa protein; other designations as in **Figure 1A**. **(B)** vLR2-GFP-mediated GFP expression in grapevine (*Vitis vinifera*) berry. **(C)** Diagram of vLR2-based dual vector in which four variants of ChlI- or PDS-derived sequences in forward (F) or reverse (R) orientation were inserted downstream of ER-GFP ORF. **(D)** Spread of the dark, chlorophyll-less areas starts around the *V. vinifera* leaf cells infected with vLR2-ER-GFP-ChlI-F (green) as shown in the left panel, and later expands along the veins into the areas that contain no virus-infected cells (right panel). **(E)** Grapevine leaves showing chlorotic symptoms due to the RNAi targeting ChlI ~3 years post inoculation with vLR2-ER-GFP-ChlI-F.

encodes a strong RNAi suppressor p24 (Chiba et al., 2006). This ability was validated using two endogenous grapevine genes involved in chlorophyll metabolism as VIGS targets. These genes were phytoene desaturase (PDS) and subunit I of magnesium-protoporphyrin IX chelatase (ChlI); nucleotide sequences derived from each of the corresponding ORFs were inserted into vLR2-GFP in the positive or negative orientations either downstream of the GFP ORF or as replacement of the GFP ORF (**Figure 2C**). Each of these vector variants was inoculated to grapevine and each induced a strong VIGS response manifested as leaf discoloration due to chlorophyll loss (Kurth et al., 2012). The chlorophyll-less cells appeared first nearby the virus-infected cells and then VIGS spread along the veins systemically and into leaf mesophyll and epidermis (**Figure 2D**) as is typical for VIGS elicited by other vectors (Baulcombe, 1999). The PDS and ChlI VIGS phenotypes proved to be long-lasting; they were maintained in most of the infected plants for over 1.5 years (Kurth et al., 2012). Furthermore, some of the plants exhibited the VIGS phenotype after nearly 3 years of propagation (**Figure 2E**).

Thus, the vLR2 vector has a dual capacity for recombinant gene expression in the phloem and systemic VIGS targeting endogenous host genes or, potentially, genes of pathogens or pests that parasitize the grapevine. Thus, this vector provides powerful tools for functional genomics and pathogen control in grapevine. Because GLRaV-2 is known to be transmitted only by grafting, potential genetically modified organism (GMO) safety concerns are greatly reduced promoting commercial application of this vector.

CURRENT AND FUTURE CHALLENGES

Over a decade of research into generation of the closterovirus-derived gene vectors taught us several valuable lessons. One of these is the paramount significance of the meticulous reconstruction of the viral cDNA representing the genome variant that is the

most fit within the virus population in a systemically infected natural host plant. Another lesson is the importance of development of the optimal plant inoculation technique. Although agroinfiltration remains by far the most efficient and broadly applicable among these techniques, it needs to be tailored for each virus–host combination, particularly for the woody hosts.

It is unlikely that closterovirus vectors will ever over-compete TMV or TRV for the tasks of facile protein production or VIGS in common herbaceous plants such as tobacco. However, neither TMV nor TRV are capable of infecting citrus or grapevine, or maintaining the recombinant gene expression cassette for years. Therefore, closterovirus vectors provide unique and extremely valuable tools for citrus and grapevine biotechnology. The VIGS capability of vLR2 is an excellent example of the power of closterovirus vectors. This vector is immediately applicable to the functional genomics of grapevine whose complete genome has been sequenced (Jallion et al., 2007; Velasco et al., 2007). Compared to other tools of functional genomics such as plant transformation, VIGS is much less time- and labor-consuming and thus is the method of choice for mapping the grapevine genes that control pathogen resistance, berry physiology, or nutrient content. It seems all but certain that the use of vLR2 will greatly facilitate the quest for more environmentally friendly and sustainable viticulture, as well as for the grapes that are more nutritious, beneficial for health, or make for better wines.

Another potential application of closterovirus vectors is development of RNAi-mediated resistance to the RNAi-susceptible pathogens such as viruses and fungi, or pests, such as insects or nematodes. For vLR2-based VIGS vectors, the obvious targets are mildew-causing fungi, phylloxera, mealybugs, and glassy-winged sharpshooters. It should be emphasized that mealybugs and sharpshooters are not only pests, but also vectors that transmit viruses causing leafroll disease and bacterium *Xylella fastidiosa* causing Pierce's disease, respectively. Obviously, to be useful for disease protection, viral vectors themselves need to exhibit as low pathogenicity as possible. The GLRaV-2 infection causes relatively mild disease facilitating the use of vLR2 vectors as a "lesser evil" to fight devastating diseases, e.g., GLRaV-3 infection or Pierce's disease. Perhaps, an even better virus vector for grapevine could be generated using GLRaV-7 that causes symptomless infections in many grape varieties (Al Rwahnih et al., 2012).

A major strength of closterovirus vectors is their exceptional genomic stability unmatched by other plant virus vectors. The causes of this stability, however, remain enigmatic. One possible explanation is the viral population dynamics related to strict tissue tropism of most closteroviruses including GLRaV-2 and CTV. An initial phase of infection by a phloem-limited virus could involve massive loading to sieve elements from one or a few initially inoculated companion of phloem parenchyma cells. Such loading would avoid multiple genetic bottlenecks associated with the cell-to-cell movement of other viruses that traverse many epidermal and mesophyll cells before reaching the phloem. Accordingly, the recombinant cassette-possessing vector that initiates the infection faces less competition from the more fit deletion variants that lose the cassette. Another explanation is higher RNA replication fidelity provided by the unusually large closterovirus replication polyprotein; elucidation of the molecular mechanisms behind this high replication fidelity remains an interesting challenge for further work on closteroviruses. Virus population dynamics and replication fidelity could act in synergy resulting in the sustained years-long expression of the recombinant proteins or RNAi triggers.

We would like to conclude this brief overview on a somewhat personal note. Since we have started to investigate closteroviruses over two decades ago, we continuously enjoyed finding many surprising features that distinguish these viruses from their smaller and less sophisticated kin. These features included the first virus-encoded molecular chaperone that turned to be a dispatcher of virion assembly and virus transport, triplication of the CP gene that provided building blocks for the formation of unusual polar virions, discovery of several diverse RNAi suppressors, extreme versatility and stability of closteroviral gene vectors and more. However, several important problems including the exact function of the unique domains in the RNA replicase, mechanisms of insect transmission, cooperation between Hsp70h, myosins, plasmodesmata, and ER-targeted movement protein that empowers cell-to-cell movement, molecular functions of the leader proteases or AlkB domain present in some closteroviruses, remain unsolved. It is our hope that the available advanced models including BYV, CTV, GLRaV-2, and LIYV will be used to address these and other outstanding problems of molecular plant virology.

ACKNOWLEDGMENTS

The authors are grateful to Ellie Kurth and Valera Peremyslov for the images. The GLRaV-2 vector work in Valerian V. Dolja lab has been supported by contract 2010-744 with Vinoculate, Inc., Soledad, CA, subcontract with USDA-NIFA (award No. 2009-04401), and BARD award No. IS-4314-10C.

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Conflict of Interest Statement: The Research in Valerian V. Dolja lab is supported in part by Vinoculate, Inc. (Soledad, CA).

Received: 03 February 2013; paper pending published: 22 February 2013; accepted: 22 March 2013; published online: 11 April 2013.

*Citation: Dolja VV and Koonin EV (2013) The closterovirus-derived gene expression and RNA interference vectors as tools for research and plant biotechnology. Front. Microbiol. **4**:83. doi: 10.3389/fmicb.2013.00083*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 16 April 2013
doi: 10.3389/fmicb.2013.00082

Grapevine leafroll-associated virus 3

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Grapevine leafroll disease (GLD) is one of the most important grapevine viral diseases affecting grapevines worldwide. The impact on vine health, crop yield, and quality is difficult to assess due to a high number of variables, but significant economic losses are consistently reported over the lifespan of a vineyard if intervention strategies are not implemented. Several viruses from the family *Closteroviridae* are associated with GLD. However, *Grapevine leafroll-associated virus 3* (GLRaV-3), the type species for the genus *Ampelovirus*, is regarded as the most important causative agent. Here we provide a general overview on various aspects of GLRaV-3, with an emphasis on the latest advances in the characterization of the genome. The full genome of several isolates have recently been sequenced and annotated, revealing the existence of several genetic variants. The classification of these variants, based on their genome sequence, will be discussed and a guideline is presented to facilitate future comparative studies. The characterization of sgRNAs produced during the infection cycle of GLRaV-3 has given some insight into the replication strategy and the putative functionality of the ORFs. The latest nucleotide sequence based molecular diagnostic techniques were shown to be more sensitive than conventional serological assays and although ELISA is not as sensitive it remains valuable for high-throughput screening and complementary to molecular diagnostics. The application of next-generation sequencing is proving to be a valuable tool to study the complexity of viral infection as well as plant pathogen interaction. Next-generation sequencing data can provide information regarding disease complexes, variants of viral species, and abundance of particular viruses. This information can be used to develop more accurate diagnostic assays. Reliable virus screening in support of robust grapevine certification programs remains the cornerstone of GLD management.

Keywords: grapevine leafroll disease, GLRaV-3, ampelovirus, *Closteroviridae*, genetic variants

INTRODUCTION

Grapevine leafroll disease (GLD) is one of the most important viral diseases affecting wine, juice, and table grape cultivars, as well as rootstocks. It currently ranks as one of the most important diseases affecting wine grape cultivars, comparable with several fungal diseases (Naidu et al., 2008). While it is generally accepted that the etiology of GLD is complex, with a number of closteroviruses associated with the disease, it is *Grapevine leafroll-associated virus 3* (GLRaV-3) in the genus *Ampelovirus* that carries the mantle as the "main etiological agent" contributing to the disease.

Here, we review the current state of knowledge on this ubiquitous pathogen. The review is comprehensive in covering most aspects of GLRaV-3 research, but pays special attention to the more recent molecular characterization. The virus genome organization and gene functions of the 13 ORFs (12 ORFs in the case of group VI variants), which are based on the comparative sequence analysis, the expression of the encoded proteins and the replication of the genome are discussed. The genetic variability between GLRaV-3 isolates is addressed in detail, and a proposal is made to standardize the naming of the genetic variant groups identified to date. A summary of diagnostic assays employed to detect the virus is also presented, with a special emphasis on the application of next-generation sequencing technologies and its potential to revolutionize our understanding of the metagenomic nature of virus infections in grapevine varieties. The review is concluded with a discussion of the various levels of host-pathogen

interactions, highlighting a very intriguing potential role of small RNAs (sRNAs) in this complex plant virus interplay.

A HISTORICAL OUTLINE OF GRAPEVINE LEAFROLL DISEASE

Grapevine leafroll disease is one of the major virus diseases of grapevine (*Vitis vinifera* L.) that may have originated in the "Old World," from where it spread, primarily through commercial trading of propagation material, to attain its current worldwide geographical distribution. Evidence that leafroll occurred in Europe and in other regions of the Mediterranean basin and Near East before the introduction of phylloxera (*Dactulosphaira vitifoliae*) from the eastern United States in the mid nineteenth century (Gale, 2002), rests on a number of observations: (i) Description in the old European literature of "reddening," an abnormal condition of red-berried grapevine cultivars consisting of early discoloration of the leaves, which accumulated carbohydrates, and showed downward rolling of the laminae. This condition was frequently attributed to physiological disorders and referred to as "rougeau" in France (Ravaz and Roos, 1905; Pacottet, 1906) and "rossore" in Italy (Arcangeli, 1907). (ii) Presence of dried grapevine shoots in a herbarium, collected in north-eastern Sicily between 1880 and 1888, that display various degrees of leaf discoloration (Martelli and Piro, 1975). The leaves of one specimen, in particular, were black, thicker and heavier than normal, and brittle, as indicated by their extensively cracked surface. This and other specimens were labeled "*Malattie della vite. Rossore. Foglie quasi nere o rosso-nere. Vitigno nero. Settembre 1885–1886*" (Grapevine diseases. Reddening. Leaves almost black or reddish-black. Red-berried cultivar. September 1885–1886). (iii) Presence of GLD-infected vines in an abandoned vineyard established between 1889 and 1891 by the University of California as a varietal test block in a secluded locality at the foothills of the Sierra Nevada Mountains in Amador County (California), prior to the wide use of rootstocks made necessary by the spread of phylloxera around 1900 (Luhn and Goheen, 1970). (iv) Occurrence of some of the leafroll-associated viruses, especially *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) in own-rooted vines from countries where grapes have been grown for centuries, e.g., Cyprus (Ioannou, 1993), Yemen (Martelli et al., 1994), parts of China (Pio Ribeiro et al., 2004), Armenia, and southern Turkey (P. La Notte and G. P. Martelli, pers. comm.), which are still phylloxera-free.

The etiology of GLD remained undetermined until the successful transmission by grafting from grape to grape provided evidence of its infectious nature (Scheu, 1935). Although it was established that GLD was an infectious disease of possible viral origin, its causal agent was still unknown. The breakthrough came in the late 1970s when Namba et al. (1979) found closterovirus-like particles in thin sections of phloem tissue and in leaf dips from GLD affected vines, suggesting that this type of viruses could be the disease agent. This finding was soon confirmed by ultrastructure studies of leaf tissues of GLD affected vines (Faoro et al., 1981; Castellano et al., 1983).

A second breakthrough came when Gugerli et al. (1984) identified two serologically unrelated closterovirus-like viruses with particle length of 2,200 and 1,800 nm, respectively, in purified preparations from symptomatic grapevine leaves. These two viruses were denoted grapevine leafroll-associated viruses 1 and 2 (GLRaV-1 and GLRaV-2). A third serologically unrelated species, that was speculated (Rosciglione and Gugerli, 1986) and then proven to be transmitted by pseudococcid mealybugs (Rosciglione and Gugerli, 1987), was added when the nomenclature of viruses associated with GLD was revised (Boscia et al., 1995). Subsequent studies in Europe (Gugerli et al., 1984; Rosciglione and Gugerli, 1986; Zimmermann et al., 1990) and the USA (Hu et al., 1990) have identified five serologically unrelated clostero-like viruses associated with the GLD complex. The introduction of molecular technologies, and especially nucleotide sequencing, increased the number of closteroviruses associated with leafroll disease to over10, before a sensible consolidation was proposed, reducing the number to five (Martelli et al., 2012). Nonetheless, GLRaV-3 remains the uncontested primary agent associated with GLD. Milestones in GLRaV-3 research in recent years include the production of antibodies and subsequent development of diagnostic assays; sequencing of the genome; transmission and metagenomics-based epidemiological studies, confirming GLRaV-3 as the major causative agent of GLD. Details of these aspects are discussed below.

GRAPEVINE LEAFROLL DISEASE

SYMPTOMATOLOGY

Symptoms of GLD can vary greatly with the season, grape cultivar, and climatic conditions. Additionally, some varieties can be completely symptomless, like some rootstocks and certain white *V. vinifera* cultivars, which can serve as a reservoir from where GLD can be transmitted to cultivars that would display a range of symptoms. In spring, bud break and shoot development is often delayed in GLD affected vines. This is usually a short-lived phenomenon, lasting for only a few weeks. Leaf symptoms first become apparent in early to mid-summer, often appearing earlier on vines which are water stressed. These symptoms increase in number and severity until late autumn (**Figure 1**). In most red cultivars, GLD causes reddening of the interveinal areas while the primary and secondary veins remain green. Leaves of some red cultivars, particularly those with deeply pigmented fruit, develop uniform red color without green veins. In white cultivars, the interveinal area may become chlorotic. This symptom is often subtle and may not be recognizable. In late autumn the leaf margins roll downward however, the extent of leaf-rolling varies considerably among infected cultivars. White cultivars, like Chardonnay, show pronounced leaf-rolling by harvest time (**Figure 1**), while Thompson Seedless and Sauvignon Blanc, show little or no leaf-rolling at all. In these white cultivars, GLD is nearly impossible to detect visually. As the growing season progresses, more and more leaves display GLD symptoms, progressing from the base of the shoot to the tip. American rootstocks are usually asymptomatic carriers of the associated viruses except for a variable decrease in vigor. Hence, the risk of disseminating the disease is great if untested rootstocks are used for propagation and grafting (Weber et al., 1993; Martelli and Boudon-Padieu, 2006; Martelli et al., 2012).

ASSOCIATED VIRUSES

To date a number of different viruses in the family *Closteroviridae* have been reported to be associated with GLD. These viruses include *Grapevine leafroll-associated viruses* (GLRaV) 1–9 and a

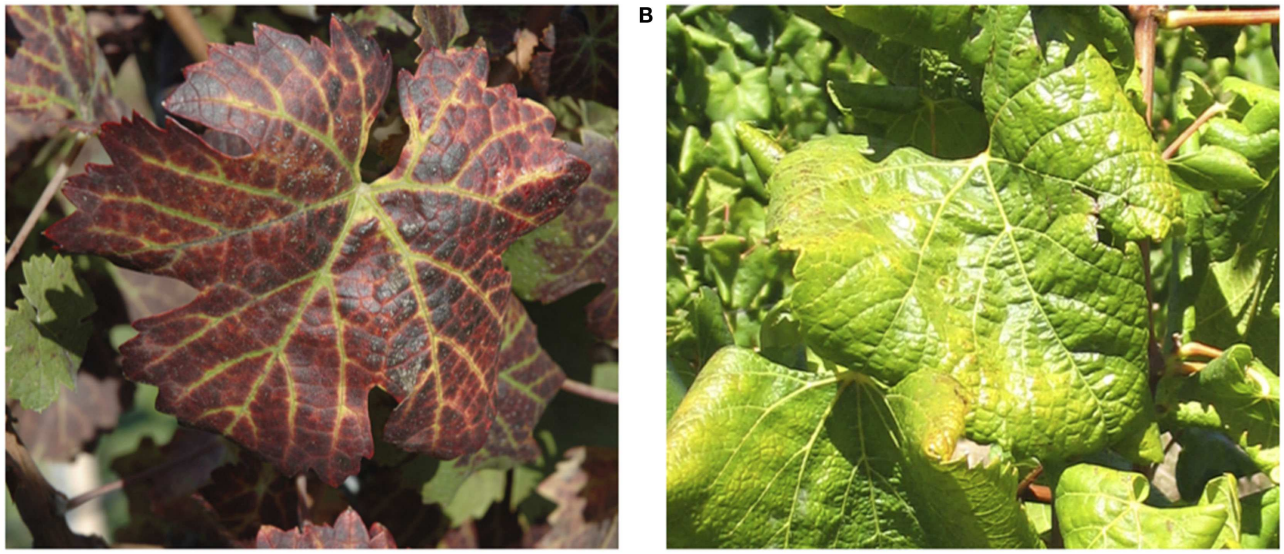

FIGURE 1 | Typical leafroll diseased vines: (A) a red cultivar, *Vitis vinifera* cv Cabernet Franc; (B) a white cultivar, *Vitis vinifera* cv Chardonnay.

group of more recently described viruses (GLRaV-Pr, GLRaV-De, and GLRaV-Car). All these viruses belong to the genus *Ampelovirus* except for GLRaV-2, which is in the genus *Closterovirus*, and GLRaV-7, which is in the tentative genus *Velarivirus* (Al Rwahnih et al., 2011) (**Table 1**). GLRaV-8 sequences (GenBank: AF233936) are not of viral origin, but rather similar to the sequences of the *V. vinifera* host; therefore, GLRaV-8 is no longer recognized as a virus species (Bertsch et al., 2009; Martelli et al., 2012). GLRaV-1, -3, and most strains of -2 usually induce stronger leaf symptoms compared to other leafroll-associated viruses. All known isolates of GLRaV-7 show very mild or uncertain GLD symptoms.

Analysis of the biological and molecular criteria of GLRaVs in the genus *Ampelovirus* suggested that these viruses belong to two different subgroups: subgroup I includes GLRaV-1 and GLRaV-3 along with *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) and *Little cherry virus 2* (LChV-2). Subgroup II includes GLRaV-4 plus PMWaV-1, PMWaV-3, and *Plum bark necrosis stem pitting-associated virus* (PBNSPaV). Further biological, serological and molecular data showed that GLRaV-4, -5, -6, -9, -Pr,-De, and -Car are closely related and all could be considered as different strains of GLRaV-4 (Martelli et al., 2012). The proposed taxonomic modification is in process to be examined by the International Committee on Taxonomy of Viruses (ICTV) (**Figure 2**).

GEOGRAPHICAL DISTRIBUTION

Grapevine leafroll disease has a significant impact on grape-growing regions worldwide, resulting in significant losses. Among all viruses associated with GLD, GLRaV-3 is by far the most noticeable and widely distributed in different regions of the world, including Europe, South and North America, Middle East, Northern and Southern Africa, Asia, and Oceania (Pio Ribeiro et al., 2004; Martin et al., 2005; Cabaleiro and Segura, 2006; Charles et al., 2006a; Pietersen, 2006; Sharma et al., 2011). Evidently, this virus has been introduced to most grape growing regions by exchange and propagation of infected plant material and subsequent local spread by vegetative propagation and insect vectors (Cabaleiro and Segura, 2006; Martelli and Boudon-Padieu, 2006; Sharma et al., 2011; Tsai et al., 2012).

RESISTANT GRAPEVINE VARIETIES

Severity of symptoms and yield losses due to GLD depend on the combination of viruses, cultivars, rootstocks, climate, soil, and viticultural practices. Although some varieties are asymptomatic no sources of GLD resistance have yet been found in *V. vinifera* cultivars and clones (Weber et al., 1993; Martelli, 2000).

Responses to infection by different GLRaVs, or combinations of these, by different grape rootstocks vary significantly. For example, it has been observed that grapevines propagated on the rootstocks Freedom and Harmony were severely affected by these viruses, in contrast to those grafted on AxR, which remained unaffected (Golino et al., 2003). The RG and PN strains of GLRaV-2 have been reported to cause lethal graft incompatibility in certain scion and rootstock combinations. The combination of *V. vinifera* and rootstocks: Couderc 1616, Kober 5BB, Teleki 5C, Couderc 3309, and Paulsen 1103 were shown to be most sensitive (Bertazzon et al., 2010; Alkowni et al., 2011). The cause of this lethal effect remains to be elucidated.

DISEASE MANAGEMENT

Clean stock and certification programs have been established in several countries in order to produce, maintain, and distribute healthy grapevines. These programs test for GLD and other diseases for the maintenance and production of clean stocks. These clean stocks can be generated by virus elimination strategies that include heat therapy, meristem tip culture (Savino et al., 1991), somatic embryogenesis (Gambino et al., 2006), and even chemotherapy of *in vitro*-grown explants (Panattoni et al., 2007).

Table 1 | Current classification and some properties of Grapevine leafroll-associated viruses (GLRaVs).

Virus	Genus	Coat protein (kDa)	Genome size (nt)	GenBank access. No.	ORFs	Vectors	First record *fide* [Boscia et al. (1995), Martelli et al. (2012)]
GLRaV-1	Ampelovirus	34	18659	JQ023131	9	Mealybugs and soft scale insects	Gugerli et al. (1984)
GLRaV-2	Closterovirus	22	16494	AY88162	8	Unknown	Zimmermann et al. (1990)
GLRaV-3	Ampelovirus	35	18498	EU259806	12	Mealybugs, soft scale and scale insects	Zee et al. (1987)
GLRaV-4	Ampelovirus	35	13830	FJ467503	6	Mealybugs	Hu et al. (1990)
GLRaV-5[a]	Ampelovirus	35	13384[b]	FR822696	6	Mealybugs	Walter and Zimmermann (1991), Zimmermann et al. (1990)
GLRaV-6[a]	Ampelovirus	35	13807	FJ467504	6	Mealybugs	Gugerli and Ramel (1993), Gugerli et al. (1997)
GLRaV-7	Velarivirus[c]	37	16496	HE588185	10	Unknown	Choueiri et al. (1996)
GLRaV-9[a]	Ampelovirus	35	12588[b]	AY29781	6	Mealybugs	Alkowni et al. (2004)
GLRaV-Pr[a]	Ampelovirus	30	13696	AM182328	6	Mealybugs	Maliogka et al. (2009)
GLRaV-Car[a]	Ampelovirus	29	13626	FJ907331	6	Unknown	Abou Ghanem-Sabanadzovic et al. (2010)

[a] *Future classification might list these as strains of GLRaV-4.*

[b] *Nearly complete sequence.*

[c] *Tentative classification.*

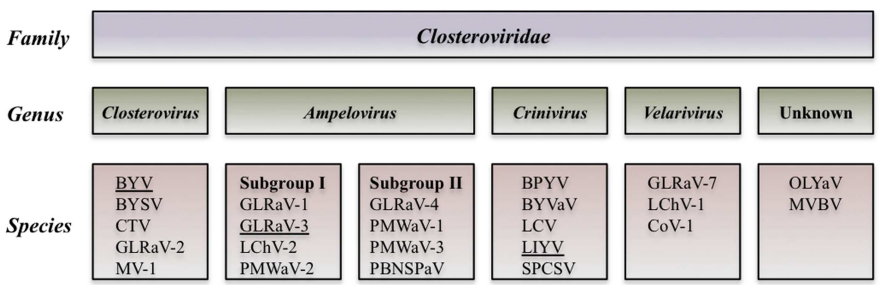

FIGURE 2 | Diagram of the proposed taxonomic modification that is in process to be examined by the International Committee of Taxonomy of Viruses (Martelli et al., 2012).

As a disease management strategy growers are currently advised to plant certified material derived from virus-tested stocks when establishing new vineyards. In areas where this is not possible due to winemaker preferences or other factors, propagating stocks should be carefully screened for viruses using rigorous laboratory tests. Maximizing the distance between new plantings and virus-infected old plantings should reduce the rate of spread. Roguing of infected vines diagnosed with the GLD associated viruses should also reduce spread if done once symptoms are present, especially in new plantings. It may be helpful to minimize the movement of farm equipment between vineyards since this practice may assist mealybug dispersal in vineyards. The use of pesticide sprays to control the mealybug vectors of leafroll may be useful in regional control programs but are not always effective in controlling spread (Golino et al., 2002, 2008; Pietersen et al., 2009). Disease management practices currently used in different world regions are discussed in more detail elsewhere in this research topic (Almeida et al., 2013).

TAXONOMY AND PHYSICAL PROPERTIES OF GLRaV-3

Grapevine leafroll-associated virus 3 is the type species of the genus *Ampelovirus*, family *Closteroviridae*, and a member of the proposed subgroup I of this genus (Martelli et al., 2011, 2012). GLRaV-3 particles are flexuous filaments, 1,800 × 12 nm in size, showing distinct cross banding (**Figure 3**). They are helically constructed and contain approximately 10 protein subunits per turn of the helix, which has a pitch of about 3.5 nm (Martelli et al., 2011). The 34 kDa major coat protein (CP) coats the whole length

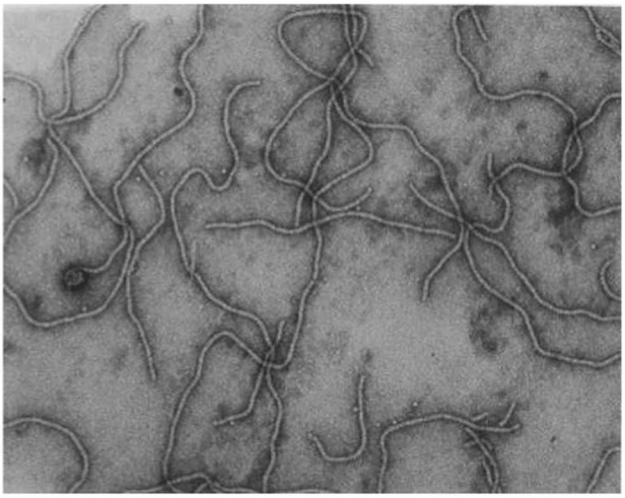

FIGURE 3 | Transmission electron micrograph of negatively stained, purified GLRaV-3 particles, using 1% (w/v) aqueous uranyl acetate staining. Picture taken by G. G. F. Kasdorf.

of the virions, except for 5′ extremity (*ca.* 100 nm). The 5′ end of the viral genome is likely to be encapsidated by the virion tail structure, similar to that of other members of the family *Closteroviridae*, which comprises proteins coded for by ORF4 (HSP70h), ORF5 (p55), and ORF7 (CPm) of the viral genome, and might be instrumental in determining cell-to-cell and systemic transport (Dolja et al., 2006). However, no research has been conducted on the composition of a virion tail/head structure at the 5′ extremity of the GLRaV-3 virion; and the proteins associated with such a structure are inferred from homologous proteins for other viruses (e.g., BYV and CTV). The reference to a virion tail at the 5′ end of the genome is suboptimal and should ideally be referred to as the virion head, as suggested in the ninth report of the International Committee on Virus Taxonomy (ICTV) (2009). To avoid confusion, and to be in line with published data on BYV and CTV, the virion structure at the 5′ extremity will be referred to as the virion tail in this review. The genome is a single-stranded, positive-sense RNA molecule constituting *ca.* 5% of the particle weight. Its 5′ end is likely to be capped and the 3′ end is not polyadenylated.

GLRaV-3 GENOME ORGANIZATION AND FUNCTIONS OF ENCODED PROTEINS

GLRaV-3 has a mono-partite, positive-strand RNA genome of ∼18,500 nucleotides. The first complete, 18,498 nucleotide-long, genome sequence of GLRaV-3 was determined for isolate GP18 from South Africa (Maree et al., 2008). This genome has a 737 nucleotide-long 5′UTR with a very high uracil content (48.5%) (Maree et al., 2008). The large size and U-rich composition of the GLRaV-3 5′UTR are unusual features among members of the family *Closteroviridae* and likely explain technical problems that resulted in the incomplete 5′UTR sequence presented by Ling et al. (2004) and Engel et al. (2008). This issue was unequivocally resolved in the following work by using 5′RACE for the molecular cloning of the 5′-proximal part of the GLRaV-3 genome (Maree et al., 2008; Jarugula et al., 2010; Jooste et al., 2010).

Currently, the complete genomes of 10 distinct GLRaV-3 isolates representing four major groups of genetic variants are available (**Table 2**). All these genomes possess very long 5′UTRs of 510–802 nts and shorter, more conserved 3′UTRs; the features of these UTRs are further discussed in Section "Genetic Variants of GLRaV-3." The consensus genome organization of the GLRaV-3 isolates from groups I-III encompassing 13 open reading frames (ORFs) is shown in **Figure 4**. The ORFs are designated 1a, 1b, and 2–12 according to the convention set out by Agranovsky et al. (1994). There is also a large, GC-rich intergenic region between ORFs 2 and 3 that is atypical of members of the family *Closteroviridae*. The genomes of isolates in variant group VI that have been characterized so far lack ORF2 (Bester et al., 2012a; Seah et al., 2012). Isolates from groups IV and V have yet to be fully sequenced.

The putative functions of the GLRaV-3 proteins encoded by ORFs 3–7 could be inferred by comparison to the homologous ORFs in the genomes of other positive-strand RNA viruses that contain a conserved "core" of replication genes and a "shell" of more variable genes encoding structural and accessory proteins (Dolja and Carrington, 1992). As is typical of the Alphavirus-like superfamily of viruses to which the family *Closteroviridae* belongs, the conserved core includes capping/methyltransferase, superfamily 1 RNA helicase, and RNA dependent RNA polymerase domains (Koonin and Dolja, 1993; Dolja et al., 2006) encoded by GLRaV-3 ORFs 1a and 1b (Ling et al., 2004). Indispensability of these ORFs for RNA replication was demonstrated using reverse genetics for two closteroviruses, LIYV (Klaassen et al., 1996) and BYV (Peremyslov et al., 1998). In addition, ORF1a of GLRaV-3 contains a papain-like leader protease (L-Pro) (Ling et al., 2004) that is implicated in RNA accumulation, virus invasiveness, and systemic spread of BYV (Peng and Dolja, 2000; Peng et al., 2003) and GLRaV-2 (Liu et al., 2009). Remarkably, GLRaV-3 ORF1a also harbors an AlkB domain (Maree et al., 2008) capable of RNA demethylation that is present in many RNA viruses infecting woody plants and proposed to repair viral RNA (Van den Born et al., 2008). Nevertheless, the functional role of different proteins encoded by GLRaV-3 can be studied using a biologically active, full-length cDNA clone that was recently reported (Jarugula et al., 2012).

There are no detectable homologs of the small protein putatively encoded by GLRaV-3 ORF2. The expression of this ORF is uncertain; as mentioned above, it is also missing in GLRaV-3 group VI isolates and seems unlikely to carry an essential function (Bester et al., 2012a; Seah et al., 2012). In contrast, the following five ORFs 3–7 comprise a quintuple gene module that is a conserved hallmark of the family *Closteroviridae* (Dolja et al., 2006). Of these, ORF3 codes for a small transmembrane protein for which the analogous protein of BYV is a cell-to-cell movement protein targeted to the endoplasmic reticulum (Peremyslov et al., 2004a).

As shown for several other closteroviruses, the ORF 4-encoded homolog of cellular HSP70 molecular chaperones (HSP70h) functions in cell-to-cell movement (Peremyslov et al., 1999) and assembly of the short virion tails typical of closteroviruses (Tian et al., 1999; Satyanarayana et al., 2000; Alzhanova et al., 2001; Peremyslov et al., 2004b). This protein is autonomously targeted to

Table 2 | Complete and near complete genomes of GLRaV-3.

Isolate	GenBank accession #	Country	*Vitis vinifera* cultivar	Genome size (nt)	5′UTR	3′UTR	Group	Reference
NY-1	NC_004667	USA	Pinot Noir	17919*	158*	277	I	Ling et al. (2004)
621	GQ352631	South Africa	Cabernet Sauvignon	18498	737	277	I	Jooste et al. (2010)
WA-MR	GU983863	USA	Merlot	18498	737	277	I	Jarugula et al. (2010)
CL-766	EU344893	Chile	Merlot	17919*	158*	277	I	Engel et al. (2008)
GP18	EU259806	South Africa	Cabernet Sauvignon	18498	737	277	II	Maree et al. (2008)
623	GQ352632	South Africa	Ruby Cabernet	18498	737	277	II	Jooste et al. (2010)
PL-20	GQ352633	South Africa	Cabernet Sauvignon	18433	672	277	III	Jooste et al. (2010)
LN	JQ423939	China	Venus Seedless	18563	802	277	III	Fei et al. (2012)
CA7246	JQ796828	USA	Merlot	18552	737	274	VI	Seah et al. (2012)
GH11	JQ655295	South Africa	Cabernet	18671	737	264	VI	Bester et al. (2012a)
GH30	JQ655296	South Africa	Cabernet	18576	642	264	VI	Bester et al. (2012a)
139	JX266782	Australia	Sauvignon Blanc	18475	510	250	ND	Rast et al. (2012)

**Near complete genomes.*

ND, Not determined.

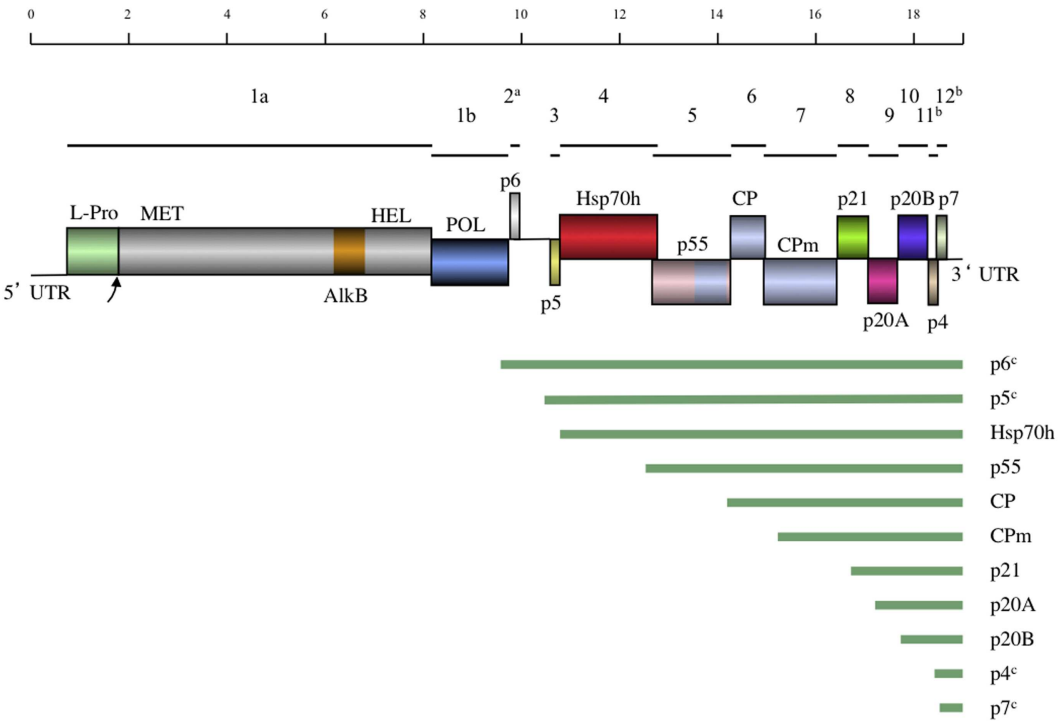

FIGURE 4 | A schematic diagram of the GLRaV-3 genome to scale. Lines above the genome map indicate the positions of the ORFs and their respective corresponding numbers. In the genome map, boxes indicate positions of genes with gene products and domains indicated. Homology between the CP and p55 and CPm is indicated by the same coloring. UTR, Untranslated region; L-Pro, Leader papain-like protease; MET, Methyltransferase; AlkB, AlkB domain; [Fe (II)/2-oxoglutarate-dependent dioxygenase], HEL, Helicase; POL, RNA dependent RNA polymerase; Hsp70h, Heat shock protein 70 homolog; CP, Coat protein; CPm, minor coat protein. Below the genome map the predicted sgRNAs are indicated by lines. [a]Not present in Group VI variants. [b]Highly divergent in Group VI and VI-like variants. [c]Putative sgRNA.

plasmodesmata in a myosin VIII-dependent manner (Avisar et al., 2008). The function of the ∼60 kDa protein encoded by ORF5 is similar to that of HSP70h; these two proteins likely cooperate in virion tail assembly and cell-to-cell movement (Alzhanova et al., 2007). The ORF6 encodes the *bona fide* CP that forms the long virion body, which is also required for cell-to-cell movement (Alzhanova et al., 2000). The last protein of this conserved quintet is a minor capsid protein (CPm) that is actually a main component of the virion tail (Agranovsky et al., 1995; Satyanarayana et al., 2004). Conspicuously, the C-terminal domain of ∼60 kDa

protein, CP, and CPm all belong to a large family of proteins forming filamentous virions of plant viruses (Dolja et al., 1991; Boyko et al., 1992; Napuli et al., 2003). It is interesting to note that the order of the CP- and the CPm-encoding ORFs in GLRaV-3 is the same as in the bi-partite criniviruses (e.g., LIYV) but reversed compared to viruses in the genus *Closterovirus* (e.g., BYV and CTV) (Karasev, 2000). Although these proteins have not been completely characterized for GLRaV-3 it is clear that the functions of the HSP70h, ∼60 kDa protein, and CPm in the virion tail assembly and cell-to-cell movement of closteroviruses are genetically inseparable, and the tail assembly can be conceptualized as a closterovirus-specific movement device (Dolja, 2003; Peremyslov et al., 2004b).

The functions of the remaining ORFs 8–12 could not be inferred by sequence analysis because their products are not conserved outside the genus *Ampelovirus* (Ling et al., 1998). However, by analogy to similarly located ORFs of other members of the family *Closteroviridae*, GLRaV-3 ORF 8, 9, and 10-encoded proteins could be involved in suppression of the host RNA interference defense (Reed et al., 2003; Lu et al., 2004; Chiba et al., 2006) and viral long-distance transport (Prokhnevsky et al., 2002). The recent work by Gouveia et al. (2012) provided experimental support for the suppressor activity of the ORF10 product p19.7 (p20B) in *N. benthamiana*. This protein was also proposed to be a viral pathogenicity determinant (Gouveia and Nolasco, 2012), an activity rather typical of viral suppressors of RNA interference (Voinnet, 2005). The small ORFs 11 and 12 are unique to GLRaV-3 and are not present in other members of the family *Closteroviridae*. Because these ORFs are very diverse among GLRaV-3 variant groups, they are unlikely to specify conserved functions. The functional characterization of ORFs 8–12 and the AlkB domain is a major challenge for future research. The recent development of a biologically active, full-length cDNA clone will aid in determining the functions of these GLRaV-3 proteins (Jarugula et al., 2012).

GLRaV-3 GENOME EXPRESSION AND REPLICATION

The replication-associated proteins of GLRaV-3 encoded by ORFs 1a and 1b are translated directly from the capped genomic RNA, analogously to BYV (Karasev et al., 1989). Translation of the ORF 1b-encoded RdRp likely involves a translational +1 frameshift (Agranovsky et al., 1994; Ling et al., 2004). The resulting products of ORF 1a and ORF 1a + 1b translation are likely processed by a papain-like L-Pro; this processing is critical for the RNA replication in BYV and GLRaV-2 (Peng and Dolja, 2000; Liu et al., 2009). Interestingly, the BYV L-Pro co-localizes with the vesicular network derived from the endoplasmic reticulum (Zinovkin et al., 2003), which, similar to other positive-strand RNA viruses, is recruited by replicase proteins to form viral RNA replication complexes (Den Boon and Ahlquist, 2010). Although not confirmed experimentally, the GLRaV-3 RNA replication likely occurs via recognition of the structural promoter elements formed by the 3′- and 5′UTRs present in the positive and negative strands of the viral RNA as was shown for CTV (Satyanarayana et al., 2002; Gowda et al., 2003).

Similar to other characterized members of the family *Closteroviridae*, the GLRaV-3 ORFs localized downstream of the ORF 1b are expressed via formation of a nested set of sgRNAs that are 3′-colinear with the gRNA (Jarugula et al., 2010; Maree et al., 2010). Each of these sgRNAs serves as a monocistronic messenger for translation of the corresponding 5′-proximal ORF. The sgRNAs are likely transcribed from a genomic RNA by the viral replicase that recognizes internal sgRNA promoters (Miller and Koev, 2000), although the exact mechanism of this process seems to be more complicated in closteroviruses than previously anticipated (Ayllón et al., 2004). Early studies of GLRaV-3 infection suggested the production of multiple sgRNAs (Hu et al., 1990; Rezaian et al., 1991; Saldarelli et al., 1994; Ling et al., 1997), but only recently have these RNAs been characterized in some detail for two different isolates (Jarugula et al., 2010; Maree et al., 2010). In particular, a Northern blot analysis of dsRNA was used to demonstrate the 3′-co-terminal structure of the three sgRNAs corresponding to ORFs 4, 5, and 6 (Maree et al., 2010). The study by Jarugula et al. (2010) showed that sgRNAs expressing ORF6 (CP), ORF8 (p21), ORF9 (p20A), and ORF10 (p20B) are the most abundant viral RNAs present in a GLRaV-3-infected grapevine (*V. vinifera* cv. Merlot). Among these, the sgRNA corresponding to ORF10 (p20B) accumulated to the highest level, followed by sgRNAs encoding products of the ORF8 (p21), ORF9 (p20A), and ORF6 (CP). These results suggest that temporal and quantitative regulation of GLRaV-3 sgRNA transcription occurs during the virus infection cycle, leading to differential expression, and/or accumulation of sgRNAs in a distinct regulation pattern.

The 5′-transcriptional start sites (TSS) for several GLRaV-3 sgRNAs were determined for isolates GP18 and WA-MR that belong to two different genetic variant groups (Jarugula et al., 2010; Maree et al., 2010). Although the techniques used were different between the two studies (RLM-RACE and 5′RACE respectively), identical results were obtained with the exception of the ORF9 sgRNA where start sites differed by one nucleotide (**Table 3**). All the mapped 5′-terminal nucleotides were purines and were conserved between the two isolates. The 5′UTRs of the characterized sgRNAs were variable in size with no detectable conserved sequences surrounding the TSS (Jarugula et al., 2010; Maree et al., 2010). This is in contrast to CTV and BYV, where conserved secondary structure elements were proposed to occur in the sgRNA promoters (Peremyslov and Dolja, 2002; Ayllón et al., 2004; Vitushkina et al., 2007). There also appears to be no correlation between the length of 5′UTR and the accumulation levels of the GLRaV-3 sgRNAs, suggesting that transcriptional regulation of the genus *Ampelovirus* is likely distinct from that of the genus *Closterovirus* (Jarugula et al., 2010).

GENETIC VARIANTS OF GLRaV-3

The genetic variability of GLRaV-3 has been studied extensively in recent years and research worldwide showed the existence of several genetic variants of GLRaV-3. Earlier studies on the genetic variability used single-stranded conformation polymorphism (SSCP) combined with sequence analysis of different genomic regions (Jooste and Goszczynski, 2005; Turturo et al., 2005). Turturo et al. (2005) investigated the genetic variability of three genomic regions; RdRp, HSP70h, and CP genes, for 45 GLRaV-3 isolates from 14 different countries. Their results for the RdRp and HSP70h regions showed that 10% of the isolates

Table 3 | Position of transcription start sites of GLRaV-3 sgRNAs.

ORF	ATG	Maree et al. (2010)		Jarugula et al. (2010)	
		Transcription start site[a]	Predicted sgRNA	Transcription start site[b]	Predicted sgRNA
2	9287				
3	10509				
4	10665	G-10477	sgRNA(ORF3/4)		sgRNA (HSP70h)*
5	12307	G-12185	sgRNA(ORF5)		sgRNA (p55)*
6	13848	A-13800	sgRNA(ORF6)	A-13800	sgRNA (CP)
7	14852	G-14815	sgRNA(ORF7)		sgRNA (CPm)*
8	16296	A-16273	sgRNA(ORF8)	A-16273	sgRNA (p21)
9	16850	G-16754	sgRNA(ORF9)	A-16755	sgRNA (p20A)
10	17390	A-17265	sgRNA(ORF10-12)	A-17265	sgRNA (p20B)
11	17932				sgRNA (p4)
12	18039				sgRNA (p5)

[a] *5′ end determined by RLM-RACE (Ambion) and mapped on the genome of isolate GP18 (GenBank: EU259806).*

[b] *5′ end determined by 5′ RACE (Invitrogen) and mapped on the genome of isolate WA-MR (GenBank: GU983863).*

**Putative sgRNAs.*

analyzed, had mixed variant infections, whilst 15% of the isolates had mixed infections when the CP region was analyzed (Turturo et al., 2005). Multiple alignment of sequences deposited in GenBank revealed that the sequences used in the Turturo study had nucleotide identities of above 90% between isolates in the regions studied. Using SSCP analysis, Jooste and Goszczynski (2005) classified two divergent GLRaV-3 variant groups, I and II, represented by isolates 621 and 623.

Sequence comparisons between isolates using different genome regions confirmed the genetic variation shown by earlier studies and indicated a greater diversity than originally estimated. Diversity studies using a portion of the RdRp or the HSP70h revealed isolates clustering into three groups. These groups had a higher than 95% similarity, between 90 and 95% similarity or approximately 70% similarity at the nucleotide level (Angelini et al., 2006; Soule et al., 2006; Prosser et al., 2007; Engel et al., 2008). Sequence data from a survey of GLD-associated viruses, using the HSP70h, showed a range of identity between 74.1 and 100% at the nucleotide level and 85.9–100% at the amino acid level between GLRaV-3 isolates from different geographic regions (Fuchs et al., 2009). Phylogenetic analysis of the HSP70h gene showed at least five possible variant groups (Fuchs et al., 2009). Subsequent studies using phylogenetic analysis of various genome regions, predominantly the CP but also HSP70h, CPm, p55, and RdRp, also confirmed five variant groups as well as identified diverse isolates currently grouped in group VI and more distantly related isolates suggesting a group VII (Chooi et al., 2009, 2013a; Gouveia et al., 2011; Sharma et al., 2011; Wang et al., 2011; Bester et al., 2012a; Seah et al., 2012). Due to limited sequence information and for the purpose of this review all isolates related to group VI, but divergent, will be referred to as group VI-like. Genetic variant groups I, II, and III were shown to be consistent between these studies but direct comparison of groups IV and V was not possible since these studies did not use the same genome regions or isolate sequences. Full-length genome comparisons of isolates from different variant groups indicated significant sequence variation in some genomic regions compared to others, as well as length variation in the 5′UTR, highlighting the risk of phylogenetic analysis using partial genome sequences. Although the biological relevance of the current genetic variant group classifications, based on partial sequences, remains to be determined, it will be beneficial for future studies to use a unified system to be able to draw direct comparisons between studies.

DISTRIBUTION OF GLRaV-3 VARIANTS IN GLD AFFECTED VINEYARDS

Several studies investigated the distribution of specific GLRaV-3 variants in vineyards. The distribution can be influenced by many factors such as specific virus-vector interactions, prevailing wind direction, combinations of GLRaV-3 variants, use of virus-infected planting material, and viticultural practices. In a South African study, group II variants occurred predominantly in the vineyards surveyed; suggesting that group II variants are most widespread (Jooste et al., 2011). In the same study, the natural spread of GLRaV-3 variants along the rows of a vineyard as well as the distribution patterns was documented (Jooste et al., 2011). Recently, group VI variants were identified in South African vineyards (Bester et al., 2012a) and their prevalence in some regions was shown (Jooste et al., 2012). Group I genetic variants were found to be dominant in a Chinese survey (Farooq et al., 2012), while in Portugal groups I and II were the most common (Gouveia et al., 2009). In a New Zealand study, group I and VI-like (similar to NZ2) variants occurred predominantly in a germplasm and commercial vineyard block, while the group VI variant was only found in high numbers in the germplasm block (Chooi et al., 2013b).

In a study of Napa Valley vineyards (Sharma et al., 2011), 27% of the GLRaV-3 isolates characterized were group I variants, while 13 and 31% were group II and III variants, respectively. The study reported that mixed variant infections occurred in 21% of infected samples and that single variant infections with group I and III were the most prevalent (Sharma et al., 2011). The transmission dynamics of variants I and VI in Napa Valley was tested (Blaisdell et al.,

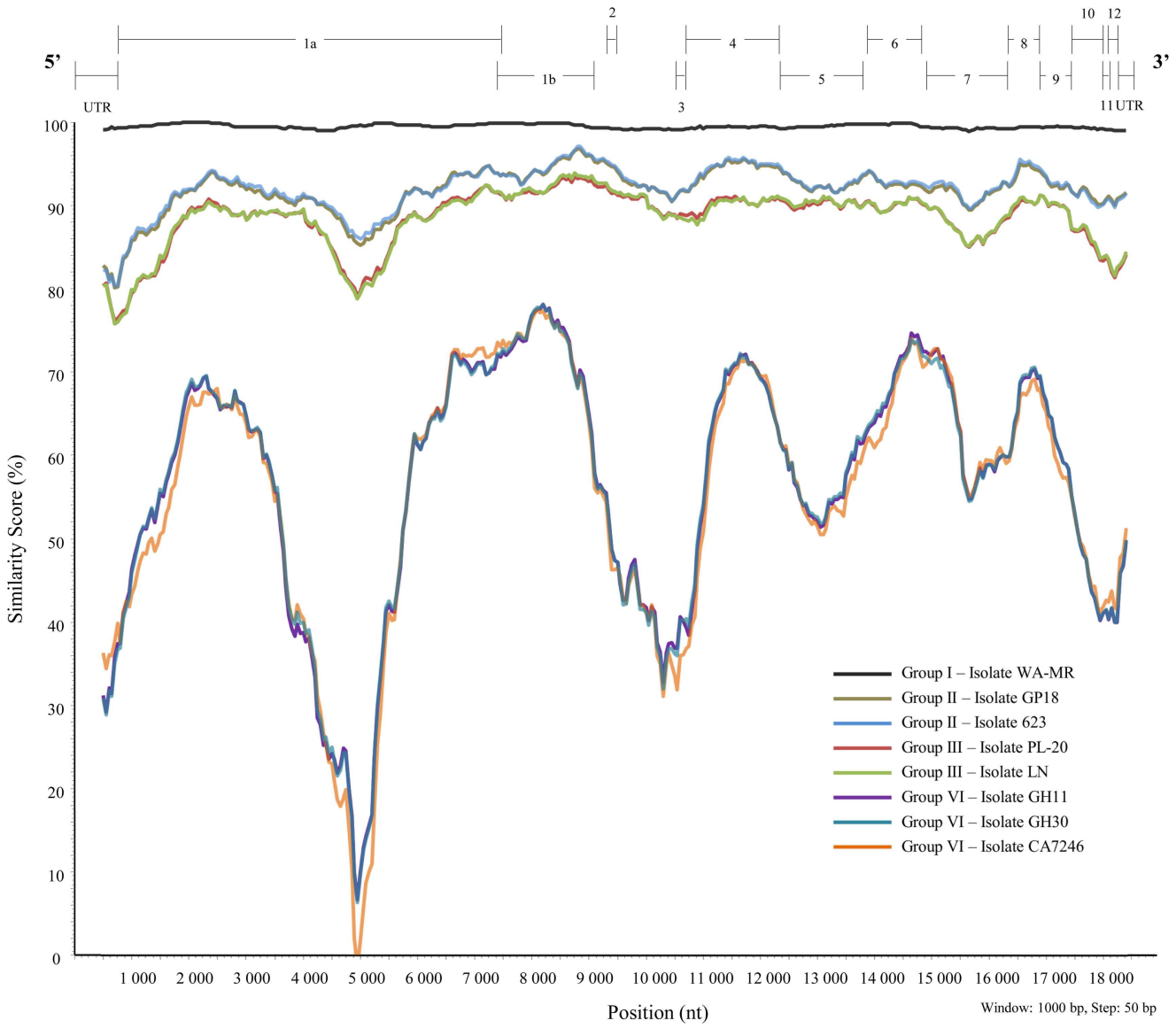

FIGURE 5 | Similarity plot constructed from a multiple alignment of nine full-length sequences representing six well-defined variant groups of GLRaV-3 using SimPlot, PHYLIP (Pylogeny Inference Package) v3.5.1 (Lole et al., 1999).

2012). The study found that vector transmission of the group VI variant alone was more frequent, followed by transmission with mixed infections of the two, while transmission with the group I variant alone was the least common. It should be highlighted that this is the first evidence that GLRaV-3 variants are biologically distinct. We expect that future work will be able to identify biological differences among the various variants within this species, if they exist.

GENOMIC VARIABILITY BETWEEN GLRaV-3 ISOLATES

A similarity plot (**Figure 5**) was constructed using isolate 621 from group I as reference sequence with a multiple sequence alignment constructed with BioEdit 7.0.5.3 (Hall, 1999) of full genome sequences of representatives of the different variant groups (I, II, III, and VI). Currently, there are no full-length sequences for representatives of groups IV and V. Genomic regions with high variability, or major variation between variant groups are discussed below.

5′ UTR

Variability in the 5′UTR was first described between isolates of groups I, II, and III after full-length genome sequences were generated for four South African GLRaV-3 isolates (Maree et al., 2008; Jooste et al., 2010). Isolate 621, representing group I, and isolates GP18 and 623, representing group II, all had a 5′UTR of 737 nt. The third variant, represented by isolate PL-20, contained a shorter 5′UTR of 672 nt, resulting in a genome that is 65 nt shorter than the sequences of group I and II variants (Jooste et al.,

2010). The WA-MR sequence from Washington showed a similar 5′UTR sequence and length of 737 nt (Jarugula et al., 2010) to that of the other group I isolate 621. Another full-length sequence of a representative of group III, isolate LN (GenBank: JQ423939), contained a much longer 5′UTR compared to PL-20. The recent full-length sequences of group VI variants from South Africa (Isolates: GH11 and GH30), and the USA (Isolate: CA7246) revealed 5′UTRs of 737 nt for isolates GH11 and CA7246, and a shorter length 5′UTR for isolate GH30 (Bester et al., 2012a; Seah et al., 2012). To determine if there are sequence or structural conservation within the 5′UTR it will be important to also have sequence information from groups IV and V.

ORF2

Sequence data showed no ORF homologous to the GLRaV-3 ORF2 in isolates from variant group VI. This was verified in sequence data of isolates GH11 and GH30 from South Africa (Bester et al., 2012a), partial sequence of NZ-1 from New Zealand and the Californian isolate CA7246 (Seah et al., 2012). The function of ORF2 in variants I–V remains unknown.

ORF11 and 12

The position and size of ORF11 is common to all GLRaV-3 variants from groups I–IV and VI. However, GLRaV-3 variants from group IV require an alternative start codon (ACG) (Wang et al., 2011). Moreover, based on sequence alignments, group VI isolates have frameshifts within ORF11 when compared to other GLRaV-3 variants from groups I–IV. This leads to changes in the amino acid sequence from amino acid 5 onward (Bester et al., 2012a; Chooi et al., 2013a). For the NZ2 isolate, the ORF11 is 18 nt longer than groups I–IV and VI resulting in polypeptide that is six amino acids longer. Compared to other GLRaV-3 variants, translation of the NZ2 ORF11 would occur in the same frame, however translation is predicted to start 3 nt upstream (1 nt overlap of ORF10) and terminate 15 nt downstream (14 nt overlap of ORF12) from the predicted start and stop sites of other GLRaV-3 variants (Chooi et al., 2013a).

The predicted start position of ORF12 is common to all known GLRaV-3 variants. However, a frameshift within the ORF12 of group VI variants and isolate NZ2 leads to a premature stop codon and in turn a reduction in size (Bester et al., 2012a; Chooi et al., 2013a). The ORF12 of GLRaV-3 variants from groups I to IV is 183 nt in size, which corresponds to a 61 amino acid polypeptide. In contrast, the ORF12 of group VI variants and isolate NZ2 is 18 nt and 12 nt shorter than groups I–IV, and as a result produces smaller 55 and 57 amino acid polypeptides, respectively.

Sequence variation along the GLRaV-3 genomic RNA is unevenly distributed. In particular, high sequence variation is evident for ORFs 11 and 12. Nineteen complete ORF11 sequences from isolates representative of groups I–IV and VI, and the corresponding trimmed sequence for the group VI-like NZ2 isolate were compared. High ORF11 amino acid variation between phylogenetic groups was also observed, as the average amino acid inter-group variations for groups I–IV ranged between 14.6 and 38.0% and group VI showed 68.1–74.2% divergence when compared to groups I–IV isolates. Isolate NZ2 showed an average 81.9 and 65.3% amino acid divergence compared to isolates from group III and group VI, respectively, while 86.1% compared to groups I, II, and IV isolates. This particularly high genetic variation observed in ORF11 supports the premise that this ORF is under neutral evolution (Wang et al., 2011), and that the predicted ORF is either not translated or is non-essential for virus infection (Seah et al., 2012).

In contrast to ORF11, less sequence variation was observed for ORF12. The average nucleotide inter-group variation for groups I–IV ranged between 6.0 and 17.4%, while isolates from group VI and group VI-like (Isolate NZ2) showed an average of 34.5–38.7% inter-group variation when compared to groups I–IV isolates. The average variation between isolates of groups VI and VI-like (Isolate NZ2) was 28.3%. It is evident that, like ORF2, genetic diversity studies indicate that functional research needs to be performed to better understand the role, if any, of ORF11 and 12 in GLRaV-3 biology.

3′ UTR

The GLRaV-3 3′UTR length varies; group I–III isolates are 277 nt, except for isolate 139 which is 250 nt; while the 3′UTR of isolates from group VI are 264 nt, except for CA7246 which is 274 nt. The 3′UTR of isolate NZ2 (group VI-like) is 289 nt in length (Chooi et al., 2013a). Based on the 277 nt 3′UTR sequence, the average nucleotide identity between isolates from groups I–III is 96.4%, while isolate NZ2 (group VI-like) only shares on average 78.7% nucleotide identity to isolates from groups I–III. Based on the shorter 264 nt group VI 3′UTR sequence, group VI isolates have on average 20.8 and 12.3% nucleotide variation to isolates from groups I–III, and the group VI-like (Isolate NZ2) respectively. The overall average nucleotide identity between all isolates from phylogenetic groups I–III, VI, and VI-like is 88.7%. The observed variability in the 3′UTR length and nucleotide identity is similar to BYV, where the 3′UTR of isolates U and Cal are 166 and 182 nt, respectively, and share 89.6% nucleotide identity (Agranovsky et al., 1994; Peremyslov et al., 1998).

Potential *cis*-acting elements that are critical for virus replication have likely conserved primary sequence and/or secondary structures, similar to conserved replication signals found in the 3′UTR of CTV. Sequence variation along the GLRaV-3 3′UTR is unevenly distributed. The highest sequence variation occurs at the 5′ end of the 3′UTR (region closest to ORF12) as nucleotide identities between isolates from phylogenetic groups I to III, VI and VI-like (Isolate NZ2) decrease to as low as 50%. While two regions from nucleotides 18,320–18,382, and 18,430–18,498 (based on GP18 numbering) have low sequence variation, as the average nucleotide identities between group I–III, VI, and VI-like (Isolate NZ2) isolates increase to 90% or greater. Thus, these areas of high conservation may represent possible cis-acting elements important for controlling GLRaV-3 replication.

RECOMMENDATION FOR NAMING AND DISTINGUISHING VARIANT GROUPS OF GLRaV-3

The diversity in the CP gene was examined based on the assumption that viral CPs evolved more rapidly than proteins involved in replication and expression of virus genomes, providing better phylogenetic resolution (Callaway et al., 2001). A total of 196 full-length CP sequences from Brazil, China, Chile, India, New Zealand, Poland, Portugal, South Africa, and the

USA that are deposited in GenBank were aligned. From this alignment, sequences representing different genetic groups of GLRaV-3 were arbitrarily selected to construct a phylogenetic tree (**Figure 6**). Six well-supported phylogenetic groups were detected in the analysis of full-length CP gene sequences of 53 isolates. GLRaV-3 variant groups I–VI were confirmed as previously identified along with group VI-like isolates that might be classified into new variant groups when more supporting data is available.

Nucleotide sequences of the CP region were analyzed and high homology, with variation of less than 2.2% within variant groups I–V, were found. Nine partial CP nucleotide sequences (Isolates: 7-1006, 7-1010, 21-9, 22-2, 43-12, 43-15, 44-2, 22-15, 21-12) from the Sharma et al. (2011) study were compared to other isolates in variant group VI. All these isolates were similar to the Californian isolate, CA7246, except isolate 43-15 (GenBank: JF421951). The Californian isolates (excluding 43-15) have 99.9–100% homology. The CP sequences of isolates 43-15 (partial), CB19 (partial, GenBank: EF445655) and 139 (GenBank: JX266782), grouped together most related to group VI but separate from isolates with the same geographical origin (Group VI-like). The New Zealand isolates, NZ-1 and NZ1-B, and South African isolates, GH11 and GH30, showed nucleotide divergence of 7.1% and 7.9–8.3% to the Californian isolates, respectively. The New Zealand and South African isolates from group VI differed by 7.1%. This illustrates that within group VI, genetic variation is greater than for the other variant groups, especially when partial nucleotide sequences from the group VI-like isolates CB19, 43-15, and 139 are included. The newly described isolate NZ2 showed a 17.7–18.7% nucleotide divergence to the group VI isolates.

It is clear from **Figure 6** that two main phylogenetic clades exist. Firstly, the clade that include isolates from groups I–V, and secondly, the clade containing the group VI and group VI-like isolates The classification of the GLRaV-3 phylogenetic groupings should be reevaluated when more sequence data are available, considering the significantly higher genetic variability within group VI clade compared to the other groups (Chooi et al., 2013a).

The availability of sequence data is crucial to classify GLRaV-3 variant groups. Analysis of the available data suggests that there are at least six well-supported phylogenetic groups within GLRaV-3 populations worldwide (**Figure 6**). We propose that these phylogenetic groups be named using a Roman numeral classification system, i.e., groups I–VI, to provide harmonization. In two studies (Sharma et al., 2011; Wang et al., 2011) letters were used for naming groups with groups 3a, 3b, 3c, 3d, 3e, and 3g being synonyms to groups I, II, III, IV, VI, and V, respectively while other studies have also used isolate names to designate variant groups. We further propose that full-length sequences, underlined in **Figure 6**, be included for each genetic group in future phylogenetic studies.

We suggest that ascending, consecutive Roman numerals be used for maintenance of shared terminology by the community. Based on partial sequences and the number of new variants recently found throughout the world, we expect that more genetic variation will be revealed in the future and that more GLRaV-3 phylogenetic groups will be identified.

DETECTION

The identification of disease-associated viruses has proven to be challenging since most diseased grapevines are infected with more than one virus. This is further complicated as unrelated viruses can cause similar disease symptoms, new infections typically have a low virus titer, viruses are often unevenly distributed in infected vines, and symptoms in some white cultivars and rootstocks are less noticeable. To date several techniques have been applied to detect viruses associated with GLD in plant material, including biological indexing, serology, nucleic acid-based methods, and next-generation sequencing.

BIOLOGICAL INDEXING

Until the late 1980s, the only reliable method of testing for GLD was hardwood indexing on biological indicators. A small chip bud from the selection to be tested (the candidate vine) is grafted to an indicator grapevine cultivar (Rowhani and Golino, 1995; Constable et al., 2010). The indicator plants with the newly grafted material are planted in the field and observed for at least two seasons for the development of virus disease symptoms (Weber et al., 2002). *V. vinifera* cvs Cabernet Franc, Pinot noir, Cabernet Sauvignon or Barbera may be used as indicator host, depending upon personal preferences and/or climatic conditions under which the indicator is grown. Green-grafting is another biological indexing technique used to screen grapevine material for viruses including GLRaV-3 (Taylor et al., 1966; Walker and Golino, 1999; Pathirana and McKenzie, 2005). Green scions or buds are grafted onto green shoots and has a higher success rate and is capable of overcoming the graft incompatibility sometimes experienced between distantly related *Vitis* species (Walker and Golino, 1999; Walter et al., 2008). Biological indexing onto woody indicators is labor intensive, time-consuming, and dependent on the successful inoculation of associated viruses (Weber et al., 2002). Uneven distribution of the virus, strain variation within the associated virus species, low virus titer, and the lack of symptom expression can also affect the results obtained with indexing (Rowhani et al., 1997; Constable et al., 2010). Biological indexing detects the disease rather than the associated viruses and although this technique can be a successful detection method, it requires a skilled virologist for disease confirmation and sometimes relies on subjective visual observations.

SEROLOGY

Many different formats of serological diagnostic techniques have been developed; these include enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and immuno-strip tests (Schaad et al., 2003). For a historical overview of GLD related antisera and monoclonal antibodies, see Gugerli (2009). Although, ELISA is not as sensitive as nucleic acid-based techniques its robustness and scalability makes it popular for routine testing by industry for the detection of GLD associated viruses in grapevines used for propagation. It is a robust, simple, and cost-effective detection method that is scalable for high-throughput processing (O'Donnell, 1999; Ward et al., 2004). Disadvantages of the technique are that it has a high developmental cost and is not as sensitive as nucleic acid-based methods (O'Donnell, 1999). Since

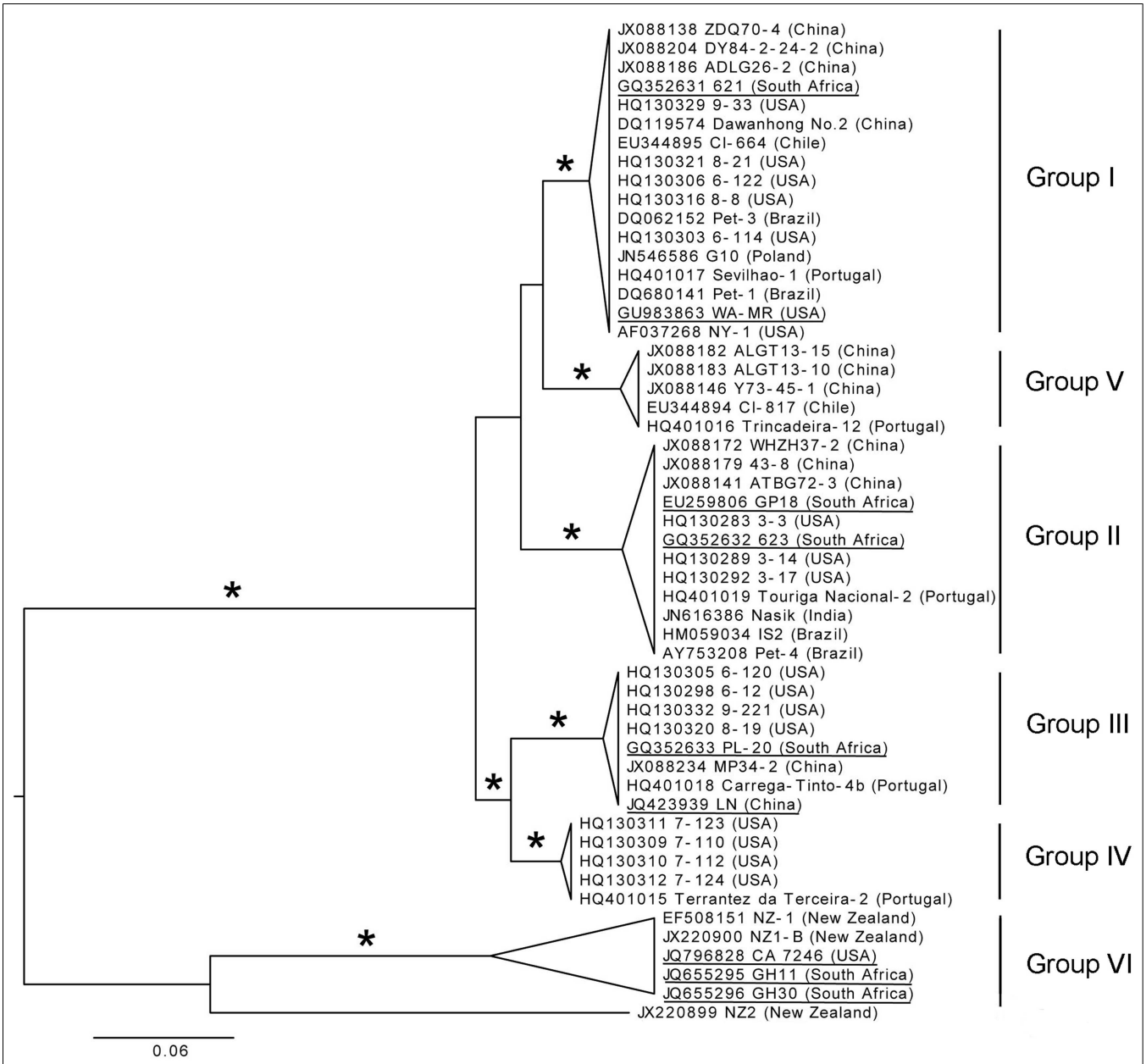

FIGURE 6 | Phylogenetic tree of full-length CP gene from representative GLRaV-3 isolates (Isolate NZ1, GenBank: EF508151, is a partial sequence). Proposed GLRaV-3 variant groups are shown with roman numerals. Maximum likelihood tree is shown, but analyses with distance and maximum parsimony methods provide similar topology. The tree is midpoint rooted for presentation and asterisks indicate ≥75% branch support with all three methods. Accession number, isolate name, and country where samples were collected are shown; fully sequenced genomes are underlined for reference. The phylogenetic analysis was performed with PAUP* (Swofford, 2003) and image generated with FigTree (Rambaut, 2006).

the first antiserum was produced against closterovirus-like particles (Gugerli et al., 1984) several groups have produced their own polyclonal antisera or monoclonal antibodies to develop ELISAs to detect GLRaV-3 specifically (Teliz, 1987; Zee et al., 1987; Gugerli et al., 1990; Goszczynski et al., 1995; Ling et al., 2000, 2001). Currently, it is unknown if all industry recommended ELISA kits can detect all the newly reported genetic variants. The robustness of ELISA makes it likely that all genetic variants can be detected. In South Africa, the industry standard kit (locally produced) could detect GLRaV-3 from genetic variant groups I, II, III, and VI with equal efficiency (Bester, 2012). However, in New Zealand it was found that genetic variants from group VI and those related to isolate NZ2 were weakly detectable and required modifications of protocols (Cohen et al., 2012).

NUCLEIC ACID-BASED METHODS

Nucleic acid-based methods have increasingly been used in recent years to develop diagnostic assays for plant pathogens. Reverse

transcription-PCR (RT-PCR) was developed for pathogens with RNA genomes (Ward et al., 2004) such as most of the known viruses in grapevine, including GLRaV-3. The genomic RNA of GLRaV-3 is found to be heterogeneous and up to date six genetic variants of the virus have been reported (Jooste et al., 2010; Gouveia et al., 2011; Wang et al., 2011; Kumar et al., 2012). Due to this genomic variability, two different multiplex PCRs were described for the detection and differentiation of four and five of the genetic variant groups of GLRaV-3, respectively (Bester et al., 2012b; Chooi et al., 2012). Another approach to PCR is called immunocapture PCR (IC-PCR). It is used for the detection of GLRaV-3 by utilizing antibodies, produced against the recombinant major CP, to immobilize the virus on the surface of a microfuge tube and continue with RT-PCR amplification (Nolasco et al., 1997; Ward et al., 2004; Engel et al., 2008). Spot-PCR has also been successfully applied for the detection of pathogens in woody plants, where a small drop of unbuffered sap from grapevine leaf petioles is placed on filter paper and used as the template for PCR (La Notte et al., 1997; Dovas and Katis, 2003; Osman and Rowhani, 2006). Another alternative to conventional PCR is the Loop-mediated amplification of nucleic acid (LAMP) technique. The LAMP method relies on the isothermal amplification of a target sequence by a strand displacing DNA polymerase and four primers with six target areas. This method has been applied for the detection of viruses including GLRaV-3 by adding reverse transcriptase to the LAMP protocol (RT-LAMP) (Nolasco, 2010; Pietersen and Walsh, 2012).

The quantification of target DNA has been simplified with the introduction of real-time PCR where unknown samples are quantified absolutely or relatively by comparing it to a standard DNA sample or to a reference gene (Feng et al., 2008). Different fluorescent probe-based chemistries have been developed of which TaqMan probes are more commonly used for grapevine virus detection. A real-time TaqMan RT-PCR assay was developed for the simultaneous detection of GLRaV-1, -2, -3, and -4 and some of the related GLRaV-4 strains and shown to be more sensitive than conventional one-step RT-PCR (Osman et al., 2007). TaqMan low-density arrays have also been introduced as a modified method of real-time TaqMan PCR. This method uses microtiter plates with dried TaqMan PCR primers/probes complexes added to the wells. It was developed for the detection of 13 different grapevine viruses (Osman et al., 2008). Recently, real-time RT-PCR high-resolution melting (HRM) curve analysis has been applied to detect and differentiate the genetic variant groups of GLRaV-3 utilizing the DNA binding dye, SYTO 9, as an alternative to TaqMan probes (Bester et al., 2012b). Other methods used to differentiate between genetic variants of GLRaV-3 include single-strand conformation polymorphism (SSCP) profiles and asymmetric PCR-ELISA (APET) (Jooste and Goszczynski, 2005; Turturo et al., 2005; Gouveia et al., 2009, 2011; Jooste et al., 2010).

More recently, oligonucleotide microarray analysis has been developed and used to detect several viruses or genes at the same time. A grapevine microarray, containing 570 unique probes designed against highly conserved and species-specific regions of 44 plant viral genomes could accurately detect 10 grapevine viruses (Engel et al., 2010). Three members of the family *Closteroviridae*, e.g., GLRaV-4, -7, and -9 were detected for the first time in Chilean grapevines using this microarray (Engel et al., 2010). This approach provides a powerful tool for high-throughput screening that can be useful for plant certification purposes. As more viral sequences become available, additional probes can be designed, raising the possibility of detecting divergent virus isolates. However, microarray technologies in general are still expensive and require extensive data analysis. Recently, the successful application of macro-array methodology was demonstrated as an alternative to microarray technology. Thompson et al. (2012) provided an unbiased multiplex detection system using a single robust macro-array platform for grapevine viruses. The relative simplicity and robustness of this methodology will be accessible to most molecular biology laboratories due to the only major equipment required being a thermocycler and a hybridization oven. This platform can differ in detection sensitivity in comparison to RT-PCR, but can complement other molecular detection methods by providing a multiplexing component (Thompson et al., 2012).

NEXT-GENERATION SEQUENCING

Present grapevine disease diagnostics rely on ELISA or nucleic acid-based methods to target viruses that have in the past been associated with diseases (Adams et al., 2009). Although these techniques can be very specific and reliable, they do not take into account the contribution of other known or unknown viruses that may be involved in the disease etiology. Different virus variants can also exist that may go undetected if highly specific RT-PCR protocols are used. The use of metagenomic sequencing to establish the total viral complement of a sample has been shown to avoid these limitations of current plant virus diagnostics (Adams et al., 2009; Al Rwahnih et al., 2009; Kreuze et al., 2009; Coetzee et al., 2010). Second generation or next generation sequencing (NGS) instruments have been developed, avoiding the limitations associated with Sanger sequencing (Hall, 2007; Mardis, 2008). The use of universal adaptors, rather than sequence specific primers, makes NGS specifically suitable to sequence all the genetic material present in a sample without prior knowledge of the organisms present (Hall, 2007; Mardis, 2008; Tucker et al., 2009). Although NGS is currently not used for GLRaV-3 diagnostics, two studies have applied NGS successfully to identify known and novel viruses from diseased plant material. Coetzee et al. (2010) established the viral profile of a severely diseased vineyard and identified a new GLRaV-3 variant that was not previously detected in South Africa. A Canadian research group also used Illumina NGS reads to assemble a complete genome sequence of GLRaV-3 (GenBank: JX559645). These studies indicate the usefulness of NGS technologies as a diagnostic tool to identify a plant virus when no prior knowledge of the virus is available. Next-generation sequencing is still relatively expensive to be used for routine diagnostics. However, data generated can be used to develop more accurate diagnostic assays since NGS can provide information regarding disease complexes, dominant variants of viral species and an indication of the frequency of viruses found in infected material.

HOST-PATHOGEN INTERACTIONS

TRANSMISSION OF GLRaV-3

The vector transmission biology of GLRaV-3 has been poorly characterized despite its obvious importance to disease spread under

natural conditions. Spread of GLRaV-3 through contaminated plant material is still widespread and of significant economic and quarantine importance. Strategies to limit such virus dissemination are based on the production of clean propagative material through certification programs and educational efforts promoting the planting of certified accessions (Rowhani et al., 2005). In addition, vine-to-vine transmission of leafroll via dodder (*Cuscutacampestris*) is also possible for experimental purposes (Woodham and Krake, 1983). There is no evidence of GLRaV-3 mechanical transmission through pruning or other plant management practices. Here we focus on the vector transmission of GLRaV-3, which is expected to be the only means of pathogen spread after establishment of a new healthy vineyard. A review on the biology of grape-colonizing mealybugs is available elsewhere (Daane et al., 2012).

Work on the vector transmission of GLRaV-3 was initiated by Rosciglione and Gugerli (1987) and Engelbrecht and Kasdorf (1990), who demonstrated that the vine mealybug (Hemiptera, Pseudococcidae), *Planococcus ficus,* was a vector of GLD. This work had two important impacts on the academic and viticulture communities; it promoted new studies that led to the identification of several new insect vectors of GLRaV-3 and further work on disease spread in the field (reviewed by Charles et al., 2006b). Transmission of GLRaV-3 has been demonstrated for various species of mealybugs (Pseudococcidae) and a few species of soft scale insects (Coccidae) (list of experimental vectors can be found in Tsai et al., 2010), but little is known about parameters that affect the transmission efficiency of this virus. Although soft scale insects are experimental vectors of GLRaV-3, they are not considered to be epidemiologically important and are not discussed in detail here. Despite the limited amount of work characterizing GLRaV-3 transmission by mealybugs, important insights have been gained through experimental research. It appears that first instar nymphs are more efficient vectors of GLRaV-3 than older nymphs or adults (Petersen and Charles, 1997; Tsai et al., 2008). These finding may be influenced by the difficulty associated with handling adult mealybugs. The removal of adults from feeding sites may result in breakage of their long stylets that are still inserted into plants rendering them unable to feed. On the other hand, differences in probing behavior between adults and nymphs may also explain these observations (Cid and Ferres, 2010; Sandanayaka et al., 2012). Regardless, because adults are largely immobile and small nymphs may be easily dispersed, including via wind (Barrass et al., 1994), the young life stages are expected to be responsible for disease spread in the field. The role of adult mealybugs in disease spread is not well understood but the subterranean survival of viruliferous mealybugs on root remnants has significant implications for disease management especially where vineyards are replanted (Bell et al., 2009). Because mealybugs have feeding tissue preferences that vary based on species and season, efforts have been made to compare the transmission efficiency of insect vectors feeding on different plant tissues. However, no effect was found when insects were confined on different tissues for virus acquisition and inoculation (Tsai et al., 2011).

Insect-borne plant viruses have a myriad of interactions with their respective vectors (Nault, 1997). There is no knowledge on the molecular interactions between GLRaV-3 and any of its vectors. However, temporal aspects of transmission such as the time required for virus acquisition or inoculation, as well as retention, allow general inferences on the mode of pathogen transmission. Cabaleiro and Segura (1997) tested the effect of time on mealybug virus acquisition and inoculation, with acquisition only occurring after 3 days of plant access, while inoculation by mealybugs reared on infected plants did not occur after 24 h. The loss of infectivity after 24 h is representative of a non-persistently or semi-persistently transmitted virus, although a 3-day minimum acquisition access period is not. Further studies include the detection of GLRaV-3 using IC-RT-PCR of dissected organs of *P. citri* and immunogold labeling and transmission electron microscopy to identify the location of the virus in the primary salivary glands (Cid et al., 2007). On the other hand, Douglas and Krüger (2008) reported that 1 h and 30 min were enough for acquisition of GLRaV-3 by *P. longispinus*. More recent work with the same vector and virus species suggested that 24 h were necessary for pathogen acquisition (Sandanayaka et al., 2012). Such contrast in results is not unexpected for poorly studied systems with low transmission rates that are difficult to manipulate experimentally, primarily due to small sample sizes. Furthermore, differences in experimental conditions may explain some of these discrepancies. The small amount of work on the transmission biology of GLRaV-3 represents a significant gap in knowledge.

The first study aimed at addressing several temporal aspects of GLRaV-3 transmission simultaneously used *P. ficus* as an experimental vector (Tsai et al., 2008). In that study transmission efficiency peaked with acquisition and inoculation access periods of 24 h. In addition, the virus was retained and transmitted by insects up to 4 days after acquisition; molting; and/or loss of virus over time may have resulted in loss of infectivity. These are characteristic hallmarks of semi-persistently transmitted viruses, where transmission efficiency increases with hours of plant access period, and viruses are retained in vectors over a limited number of hours or days (Ng and Falk, 2006). For *Lettuce infectious yellows virus*, another member of the family *Closteroviridae*, the cibarium of its whitefly vector was identified as the likely virus retention site (Chen et al., 2011). The foregut of mealybug vectors is expected to be the retention site for GLRaV-3, but semi-persistently transmitted viruses may also bind to the tip of stylets (Uzest et al., 2007).

Altogether, several mealybugs and at least one soft scale transmit GLRaV-3. This suggests a lack of transmission specificity, which also appears to apply to the other ampelovirus species causing GLD (Tsai et al., 2010; Le Maguet et al., 2012). First instar nymphs appear to be more efficient vectors than adult mealybugs, and transmission likely occurs in a semi-persistent manner. However, these conclusions are based on a limited number of studies, and more research needs to focus on the transmission of GLRaV-3 so that robust knowledge is obtained for the development of science-based disease management strategies that incorporate all aspects of this disease.

CYTOPATHOLOGY

GLRaV-3 is restricted to the phloem of infected hosts (*V. vinifera*, interspecific hybrids and American rootstocks) in whose organs

and tissues it is unevenly distributed (Boscia et al., 1991; Credi and Santucci, 1991; Rowhani et al., 1997). Cytopathological modifications, which are prominent in differentiating sieve tubes, companion cells and phloem parenchyma cells, are characterized by the presence of: (i) inclusion bodies made up of membranous vesicles 50–100 nm in diameter, derived from proliferation of the bounding membrane of mitochondria (Kim et al., 1989). These vesicles, which are released in the cytoplasm following disruption of mitochondria (Faoro et al., 1992), contain a network of fine fibrils identified as RNA, and are thought to be sites of replication (Faoro and Carzaniga, 1995); (ii) loose bundles to compact aggregates of virus particles that often fill the lumen of sieve tubes and may also be localized in the nuclei. Virus clusters can be surrounded by a bounding membrane, giving rise to characteristic intra-cytoplasmic enclaves (Faoro et al., 1992).

ECONOMIC IMPACT OF GLRaV-3 AND EFFECT ON CROP AND VINE HEALTH

GLRaV-3 incurs substantial economic losses to the wine, table, raisin, and nursery industries. Yield losses of 20–40% are not uncommon (Habili and Nutter, 1997). The annual cost of GLD is estimated to $1,600–2,350 per hectare of *V. vinifera* cvs. Cabernet Sauvignon and Merlot in New Zealand (Nimmo-Bell, 2006), $300–2,400 per hectare of *V. vinifera* cv. Cabernet Sauvignon in South Africa (Freeborough and Burger, 2008), and $1,000–1,600 per hectare of *V. vinifera* cv. Cabernet Franc in the Finger Lakes region of New York (Atallah et al., 2012).

More specifically, GLRaV-3 reduces yield, cluster size, delays fruit ripening, alters berry color by lowering anthocyanin content, increases titratable acidity, in particular malic and tartaric acids, and changes fruit juice chemistry by reducing soluble solids and modifying aromatic profiles, as shown in *V. vinifera* cvs. Cabernet Franc, Cabernet Sauvignon, Merlot (Borgo et al., 2003), Albariño (Cabaleiro et al., 1999), Chardonnay (Komar et al., 2007), and Dolcetto (Mannini et al., 2012). Wines made from fruits harvested on GLRaV-3-infected cvs. Nebbiolo (Mannini et al., 1998), Tempranillo (Legorburu et al., 2009), and Merlot (Alabi et al., 2012a) have less pigments, phenolics, tannins, and alcohol compared to wines made from healthy vines. In interspecific hybrids Vidal blanc and St Vincent, although GLRaV-3 infection is latent, berry weight is reduced, and titratable acidity is increased in fruit juice (Kovacs et al., 2001).

GLRaV-3 causes a drastic reduction in leaf photosynthesis during post-veraison (Gutha et al., 2012; Mannini et al., 2012) and in free amino acids such as valine and methionine, or glutamic acid in berries of *V. vinifera* cv. Pinot noir (Lee et al., 2009) but an increased skin and pulp weight (Lee and Martin, 2009). Transcriptome analysis showed alteration of the berry maturation process, in particular of genes involved in the anthocyanin biosynthesis and sugar metabolism, in GLRaV-3-infected *V. vinifera* cv. Cabernet Sauvignon (Vega et al., 2011). Similarly, a 2- to 10-fold increase in key genes involved in the flavonoid biosynthetic pathway is measured in leaves of GLRaV-3-infected *V. vinifera* cv. Merlot compared to healthy vines, leading to *de novo* synthesis of anthocyanins such as quercetin and myricetin (Gutha et al., 2010).

A wealth of information is available on the detrimental effects of GLRaV-3 on vine health and crop from field trials with different scion/rootstock combinations or own-rooted vines in Australia, Africa, Europe, and the USA. Though the magnitude of detrimental effects depend on factors such as cultivar, clone, rootstock genotype, vine age, and environmental conditions (Mannini, 2003), data are consistent with GLRaV-3-infected vines being stressed, producing poorly, and substantially reducing vineyard profitability.

SMALL RNA PROFILING IN GLRaV-3-INFECTED GRAPEVINES

The availability of the grapevine genome sequence allows gene expression profiling, which provides a method to analyze the response of grapevine to various biotic and abiotic stresses at the genetic level. Gene expression in plants is a highly regulated process; one key factor in this regulation are microRNAs (miRNAs), which have been shown to be involved in plant development and plant response to biotic and abiotic stresses. MicroRNAs are a class of small, 21–24-mer, non-coding sRNAs, which are conserved and play a role as "master regulators" of gene expression. In a recent study, Alabi et al. (2012b) profiled endogenous host and viral sRNAs (vsRNAs) in GLRaV-3-infected grapevines by NGS. Altered expression levels of several known *V. vinifera* (vvi)-miRNAs involved in organ and plant development were observed in infected grapes compared to virus-free plants. Particularly vvi-miRNA 156 and 167, which in *Arabidopsis thaliana* target "Squamosa promoter binding protein-like" and "Auxin Response Factor (ARF)" transcription factors, respectively, are both down-regulated, whereas the reverse occurs with vvi-miR166, whose increased levels in *Arabidopsis thaliana* inhibits the expression of its HD-ZIPIII target, thus causing extensive developmental alterations. Surprisingly, a lower expression level of vvi-miR168, which translationally regulates Argonaute 1 (AGO1) expression in *A. thaliana* and *N. benthamiana*, was induced by virus infection. A possible explanation of this unexpected finding may be the condition of the analyzed tissues, which were collected from symptomatic leaves during mid-September, when replicating virus titers are low. Indeed these vines had a low viral RNA concentration, as demonstrated by the small number of vsRNAs detected in the library (0.07% of the total reads). In line with a similar high-throughput analysis performed on citrus plants affected by *Citrus tristeza virus* (CTV) (Ruiz-Ruiz et al., 2011), GLRaV-3-derived sRNAs seem to more densely cover the 3′-terminal region of the viral genome, thus likely originating from the nested set of subgenomic RNAs produced by this virus. The most abundant vsRNAs size class was 21 nucleotides, suggesting that the majority of vsRNAs are processed by a grapevine DCL4-homolog, as previously found for viruses belonging to different taxonomic groups (Pantaleo et al., 2010). Moreover, the involvement of a viral double-stranded RNA as substrate in producing these sRNAs is suggested by the finding of an equal number of vsRNAs of positive and negative polarities. From these investigations, inferences can be drawn which confirm the effects of virus replication on the different small RNA classes observed in annual plants (Chapman et al., 2004; Bazzini et al., 2007). More research in this exciting new branch of disease etiology will shed light on the precise interaction of host plant and virus pathogen.

CONCLUSION

Grapevine leafroll disease is considered to be one of the most economically destructive virus diseases of grapevine and a major constraint to the production of premium wine grapes. GLRaV-3 has been more closely associated with GLD than any other GLRaV, supporting the view that it is the "main etiological agent." Even though the genetic variation observed in the GLRaV-3 genome has been studied more intensively ever since the publishing of the first near complete genome sequence, research on GLRaV-3 lags behind that of other economically important plant viruses. Due to its narrow host range (infecting only *Vitis* species) and being phloem-limited, research on GLRaV-3 has largely focused on epidemiology and the development of reliable detection assays. Phylogenetic studies showed the existence of six genetic variant groups and, with the advances in sequencing technologies, more sequence data will be generated that will indubitably lead to the identification of additional genetic variants. It is therefore necessary to have sensitive and rapid diagnostic methods to test material for GLRaV-3 infection that are able to detect all variants that may influence disease etiology. This implies that newly developed and old diagnostic assays, especially PCR based assays, be verified to be able to detect all genetic variants and continuously be reevaluated to ensure that the assay remains valid as new sequence information becomes available. The role of the different genetic variants of GLRaV-3 in GLD etiology is still largely unknown and elucidating this role is an essential next-step. It is also important to investigate the interactions between the different GLRaV-3 variants, and in combination with the mealybug vectors, to potentially explain the dominant occurrence of some of the genetic variants. The successful construction of an infectious clone of GLRaV-3 provides a platform to study viral replication and gene expression, and determine the function of the GLRaV-3 genes that are currently unknown and also the function of the highly variable extended 5′UTR. GLRaV-3 is one of the most important grapevine viruses and with the use of the latest tools in molecular biology a complete understanding of its role in GLD etiology and host-pathogen interaction is attainable.

ACKNOWLEDGMENTS

The following authors would like to thank their respective funding bodies as listed. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the any of the funding bodies. Hans J. Maree, Rachelle Bester, Anna E. C. Jooste, and Johan T. Burger: Winetech and the South African National Research Foundation (NRF). Rodrigo P. P. Almeida: American Vineyard Foundation, USDA-NIFA SCRI, Oregon Wine Board. Marc F. Fuchs: New York State Wine and Grape Foundation, Lake Erie Regional Grape Program, Kaplan Funds, and Specialty Crop Block Grant Program. Rayapati A. Naidu: Washington State University's New Faculty Seed Grant Program, Agricultural Research Center and Agricultural Program in Extension in the College of Agricultural, Human, and Natural Resource Sciences (CAHNRS), and Washington Wine Commission's Wine Advisory Committee. Pasquale Saldarelli: National Research Council for the project "Conoscenze Integrate per la Sostenibilità ed Innovazione del made in Italy Agroalimentare."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 January 2013; accepted: 22 March 2013; published online: 16 April 2013.

Citation: Maree HJ, Almeida RPP, Bester R, Chooi KM, Cohen D, Dolja VV, Fuchs MF, Golino DA, Jooste AEC, Martelli GP, Naidu RA, Rowhani A, Saldarelli P and Burger JT (2013) Grapevine leafroll-associated virus 3. Front. Microbiol. ***4****:82. doi: 10.3389/fmicb.2013.00082*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 24 April 2013
doi: 10.3389/fmicb.2013.00094

Ecology and management of grapevine leafroll disease

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Grapevine leafroll disease (GLD) is caused by a complex of vector-borne virus species in the family Closteroviridae. GLD is present in all grape-growing regions of the world, primarily affecting wine grape varieties. The disease has emerged in the last two decades as one of the major factors affecting grape fruit quality, leading to research efforts aimed at reducing its economic impact. Most research has focused on the pathogens themselves, such as improved detection protocols, with limited work directed toward disease ecology and the development of management practices. Here we discuss the ecology and management of GLD, focusing primarily on *Grapevine leafroll-associated virus 3*, the most important virus species within the complex. We contextualize research done on this system within an ecological framework that forms the backbone of the discussion regarding current and potential GLD management strategies. To reach this goal, we introduce various aspects of GLD biology and ecology, followed by disease management case studies from four different countries and continents (South Africa, New Zealand, California-USA, and France). We review ongoing regional efforts that serve as models for improved strategies to control this economically important and worldwide disease, highlighting scientific gaps that must be filled for the development of knowledge-based sustainable GLD management practices.

Keywords: grapevine disease, Closteroviridae, vector, mealybug, integrated pest management

INTRODUCTION

Emerging plant diseases are a global threat to the food supply, environmental sustainability, and economic stability of regions and nations. In this paper, we discuss the ecology and management of grapevine leafroll disease (GLD), a worldwide disease that is caused by a complex of virus species in the family Closteroviridae, which contains emerging and re-emerging plant pathogens of economic importance. GLD is present in virtually all commercial grape (*Vitis vinifera*) growing regions; its distribution is thought to be due to regional, continental, and intercontinental transport of virus-infected plant material. While GLD has long been present in the major grape-growing regions, it has only recently been recognized as a disease of economic importance. Various hypotheses have been proposed to explain this (e.g., Golino et al., 2008), but none have been well supported. For example, there is no evidence of the emergence of a new virus species or strain (Wang et al., 2011), or introduction of a rapidly moving or efficient insect vector species associated with the increased incidence of GLD. The only common factors are the observation of vector-mediated pathogen spread in vineyards and an increased GLD awareness by academics, farmers, and other stakeholders. Regardless of the driving forces, GLD is now considered a disease of importance in viticulture, especially to wine grape growers who aim for a high quality uniform crop.

Herein, we will not focus on factors that have made GLD such a pre-eminent disease, although studies are needed to address this. We propose that the integration of disciplines is necessary to address GLD, and to devise disease management practices that are practical, sustainable, financially viable, and environmentally sound. Within this interdisciplinary context, our goal is to discuss various components of GLD that are relevant to its ecology, epidemiology, and management. Much of this review focuses on the mealybug-transmitted ampeloviruses, more specifically *Grapevine leafroll-associated virus 3* (GLRaV-3), which is the most widespread species in the virus complex causing GLD. We highlight notable gaps in the current body of knowledge that need to be addressed for the development of sustainable disease control practices. Then we discuss management strategies being implemented in each of four countries in four continents by presenting case studies.

GRAPEVINE LEAFROLL DISEASE

Grapevine leafroll disease has been described from different regions in Europe and elsewhere for over a century (Hoefert and Gifford, 1967), and was first shown to be transmissible to vines in 1936 (Scheu, 1936). The demonstration of graft transmissibility opened early avenues of GLD research, including the search for etiological agents and the impact of abiotic factors on symptom

development. Even today the etiology and symptomatology of GLD is not completely clear, as multiple virus species cause GLD, and symptoms result from complex biotic and abiotic interactions. Furthermore, there is no infectious clone for any agent associated with GLD.

Grapevine leafroll disease is most obvious and problematic in cool-climate regions, where fruit on infected vines has delayed ripening that results in lowered brix, which in turn affects wine quality (Over de Linden and Chamberlain, 1970; Goheen, 1988). The most obvious GLD symptoms appear in the fall, when red cultivars display leaf reddening with green venation (**Figure 1**). While symptoms are not as apparent in white cultivars, there is a slight leaf chlorosis. Both red and white cultivars develop downward rolling of leaf margins and phloem disruption. Significant losses result from a combination of factors including yield reductions of up to 40%, increased management costs, shortened vineyard life spans, and adverse impacts on wine quality resulting from decreased fruit quality and delayed maturation (Woodrum et al., 1984; Goheen, 1988; Credi and Babini, 1997; Martelli et al., 2002). The economic impact of GLD is still poorly understood, as are the implications of various control strategies. A recent study by Atallah et al. (2012) estimated the economic impact of GLD to range from US$25,000 to US$40,000 per hectare for vineyards with a 25-year lifespan. The authors analyzed various scenarios, incorporating disease prevalence, yield reduction and fruit quality; at low levels of disease incidence (1–25%), roguing can significantly decrease economic losses, which was identified as an economically important practice together with planting of virus-free plant material. The economic impact of vector management has not been explored.

Grapevine leafroll disease has three essential biological components: (1) a complex of viruses in the Closteroviridae, (2) grapevine host plants, and (3) species of mealybugs (Pseudococcidae) and soft scales (Coccidae) that transmit GLRaVs. Much of this review will focus on GLRaV-3, which is the best studied species worldwide and has been implicated in a majority of GLD spread that has been mediated by known insect vectors. While GLRaV-2 is of economic importance, this *Closterovirus* species has no known vectors (Martelli et al., 2002). In addition, GLRaV-7, a member of the proposed genus Velarivirus (Al Rwahnih et al., 2012), does not appear to cause GLD and also has no known vectors (Tsai et al., 2010).

GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES

Virus species causing GLD are sequentially named *Grapevine leafroll-associated virus 1*, *Grapevine leafroll-associated virus 2*, and so on (GLRaV-1, GLRaV-2, GLRaV-n). All GLRaVs are in the genus *Ampelovirus*, except for GLRaV-2 and GLRaV-7, as previously discussed. GLRaVs in the genus *Ampelovirus* are divided into two phylogenetic groups, one that includes GLRaV-4, -5, -6, -9, and others, and another comprising GLRaV-1 and -3 (Maliogka et al., 2009). The taxonomy of GLRaVs is undergoing significant changes with recent proposals awaiting International Committee on Taxonomy of Viruses (ICTV) approval; the most relevant proposal is a change in sequence similarity thresholds for delineating species that would collapse GLRaV-4, -5, -6, -9, and other proposed species and divergent variants into one species, GLRaV-4 (Martelli et al., 2012; Thompson et al., 2012).

Both groups of GLRaV ampeloviruses, like other species in the Closteroviridae, are filamentous virions with a large (13–18 kb) positive-sense single-stranded RNA genome (Fuchs et al., 2009; Martelli et al., 2012). However, there are important differences in genome structure between the groups. The genomes of GLRaV-4-like species are ∼5 kb smaller and lack several open reading frames on their 3′ ends that are present in GLRaV-1 and -3 (Thompson et al., 2012). Despite the large genetic diversity among GLRaV species, little is known about the phenotypic variability in disease symptoms among or within species. One careful study of GLRaV-2 demonstrated that disease symptoms were associated with the phylogenetic clustering of variants (Bertazzon et al., 2010), but similar work has not been performed with other viruses. Despite this gap in knowledge, GLRaV-3 has emerged as the key species causing GLD worldwide. The reasons behind the prominence of GLRaV-3 are poorly understood, especially because other GLD-causing species also co-exist with GLRaV-3, often within one vineyard or plant (Sharma et al., 2011), and some can be transmitted by the same vector species (Le Maguet et al., 2012). Notably, GLRaV-3 has been identified as the main species

FIGURE 1 | Leaf symptoms of grapevine leafroll disease include inter-veinal reddening and leafrolling in red-fruited varieties. Symptoms are most pronounced around the harvest period. These photographs were taken in the fall (September) in Napa, CA, USA. Photographs show symptomatic leaf **(A)**, group of leaves **(B)**, and whole plant **(C)**.

being transmitted by vectors throughout the world (see case studies).

The importance of GLRaV-3 genetic diversity is not understood from a phenotypic or ecological perspective. However, some important insights into GLRaV-3 ecology have been gained from genetic diversity studies. First, it appears that most variants are present in major grape-growing regions worldwide (Gouveia et al., 2011; Jooste et al., 2011; Sharma et al., 2011). Second, it is likely that much of the diversity within the species has yet to be discovered, given the increasing number of well-supported phylogenetic clades (e.g., Sharma et al., 2011; Seah et al., 2012). Lastly, there is no evidence of positive selection in GLRaV-3 field populations (Wang et al., 2011), suggesting that the virus is not undergoing novel selective pressures.

HOST PLANTS

Although ampeloviruses colonize a wide range of plant taxa, GLRaVs appear to be limited to grapevines (*Vitis*). To our knowledge, GLRaVs have only been isolated from *Vitis* spp. Focus on the commercially widespread *Vitis vinifera* may have limited our knowledge of potential host range, although a recent survey in Napa Valley, California, which included 41 plant species in 12 families in addition to wild grapes (*Vitis californica* and *Vitis californica* × *Vitis vinifera* hybrids), showed that wild *Vitis* can be infected with GLRaV-2 and GLRaV-3 (Klaassen et al., 2011). Because of extensive exchange of easily propagated plant material that has occurred worldwide (Rowhani et al., 2005), transport of virus-infected plant material has been identified as a major factor responsible for the global spread of GLD and its etiological agents. Quarantine regulations and national programs aimed at reducing the import of pathogens have been established in several countries, and are responsible for providing virus-free plant material to farmers. The integration of these practices into management of GLD is discussed below.

INSECT VECTORS

Plant to plant transmission of GLRaV-3 by the mealybug *Planococcus ficus* (Signoret) was the first demonstration of an insect vector of a GLD pathogen (Engelbrecht and Kasdorf, 1990). Since then, several species of mealybugs have been shown to transmit GLRaV species, including *Pseudococcus maritimus* (Ehrhorn), *Pseudococcus viburni* (Signoret), *Pseudococcus longispinus* (Targioni-Tozzetti), *Pseudococcus calceolariae* (Maskell), *Pseudococcus comstocki* (Kuwana), *Planococcus citri* (Risso), *Phenacoccus aceris* (Signoret), and *Heliococcus bohemicus* Sulc (reviewed in Daane et al., 2012; Herrbach et al., 2013). Additionally, the soft scales *Pulvinaria vitis* (L.), *Parthenolecanium corni* (Bouché), *Ceroplastes rusci* (L.), *Neopulvinaria innumerabilis* (Rathvon), *Coccus longulus* (Douglas), *Parasaissetia nigra* (Nietner), and *Saissetia* sp. are also vectors (Belli et al., 1994; Mahfoudhi et al., 2009; Le Maguet, 2012; Herrbach et al., 2013; Krüger and Douglas, 2013). Impressive here is the breadth of vector species, which is essentially inclusive of all common mealybugs and soft scales found worldwide where GLD is of concern.

Recognition of insect vectors is essential for the development of disease management practices, including control of the correct vector species. However, the ecological relevance of different mealybug or soft scale species to GLD spread has yet to be properly addressed. Tsai et al. (2010) found no evidence of strict vector–virus species specificity for transmission and, to date, it appears that all GLRaV species can be transmitted by the different grape-associated mealybug species tested. This hypothesis was further supported with the demonstration that *Ph. aceris* transmits six *Ampelovirus* species (Le Maguet et al., 2012). Therefore, all mealybugs colonizing grapevines should be considered potential GLRaV vectors until proven otherwise, and vector biology rather than species becomes most important.

Vineyard mealybugs generally have four larval instars for the female and five for the male (Ben-Dov, 1995). The small (∼0.5 mm), unsettled first instar, or crawler, is considered to be the dispersal stage, and can be easily moved on personnel, equipment, infested nursery stock (Daane et al., 2012), and carried by the wind (Barrass et al., 1994). Whereas all mealybug and soft scale life stages may be capable of transmitting GLRaV-3, the younger nymphs appear to be more efficient (Petersen and Charles, 1997; Tsai et al., 2008). Vector species with more annual generations or higher fecundity would pose a greater threat. Variability in annual number of generations and fecundity exists. For example, in coastal California wine grape vineyards there are approximately one, two, three, and four annual generations of *Pa. corni*, *Ps. maritimus*, *Ps. viburni*, and *Pl. ficus*, respectively (Geiger and Daane, 2001; Gutierrez et al., 2008; Varela et al., 2013).

Mealybugs and soft scales are phloem feeders that use long, slender mouthparts to suck plant fluids (Daane et al., 2012). Most vineyard mealybug species can feed on the trunk, canes, leaves, and berries; however, there is variation in seasonal feeding location and movement on the vine among and within species, as described for *Ps. maritimus* (Geiger and Daane, 2001; Grasswitz and James, 2008), *Pl. citri* (Cid et al., 2010), and *Pl. ficus* (Becerra et al., 2006). Some mealybug species commonly maintain a portion of their population on vine roots, such as *Ps. calceolariae* (Bell et al., 2009) and *Pl. ficus* (Walton and Pringle, 2004). This presents a considerable replant problem as after the vine is removed, remnant roots can remain viable for years, supporting GLRaVs and mealybugs that bridge the old infested vineyard to the new replants (Pietersen, 2006).

Control of different vector species can vary considerably. Monitoring insect populations is an essential component of pest control; however, visually monitoring for mealybugs, especially at low densities, is too labor intensive to be cost effective. Sex pheromones for numerous species have recently been identified, including *Pl. ficus*, *Ps. viburni*, *Ps. maritimus*, *Ps. longispinus*, and *Ps. calceolariae* (reviewed in Daane et al., 2012), and trap counts can be used to predict berry damage (Walton and Pringle, 2004); however, there are no economic injury levels determined for these insects as GLD vectors. To control GLD spread, most vineyard managers have adopted a zero tolerance for vectors, and monitoring manifests as presence/absence scores. Efficient insecticides for mealybugs and soft scales exist, particularly some neonicotinoids and biosynthesis inhibitors (Daane et al., 2012). However, *Pl. ficus* first instar

nymphs can both acquire and inoculate GLRaV-3 in less than 1 h (Tsai et al., 2008). Because the more effective insecticides are systemic, and the vector must feed on the plant to be killed, the applications may reduce mealybug densities in the treated vineyard but not necessarily protect it from virus spread by dispersing mealybugs. For some mealybug species, insecticides alone do not provide complete control, and additional control is provided by natural enemies. In New Zealand, for example, *Ps. viburni* was brought under exceptional control by release of the parasitoid *Acerophagus* (*Pseudaphycus*) *maculipennis* Signoret (Charles et al., 2010). In contrast, *Anagyrus pseudococci* Signoret is the primary parasitoid of *Pl. citri* and *Pl. ficus* around the world (Daane et al., 2012), but parasitism alone does not deliver control sufficient to reduce the spread of GLD. Mating disruption, which works best at lower pest densities, is being investigated for *Pl. ficus* (Walton et al., 2006) and may become an integral part of future control measures.

Of those countries reported in this review, *Pl. ficus* and *Ps. calceolariae* appear to be of greatest concern in most regions, but all mealybugs and soft scales should be viewed as potential vectors. The role played by different vector species in GLD epidemiology and vector ecology is still poorly understood. Regardless of the species, for GLD management through vector control, it is likely that multiple monitoring and control techniques must be employed to maintain the exceptionally low pest densities needed to suppress and control GLD.

VIRUS TRANSMISSION BIOLOGY

Among insect-borne plant viruses, those transmitted by mealybugs and soft scales are among the least understood. These insects transmit other viruses of economic importance to a range of crops such as cassava, banana, pineapple, and cocoa (Herrbach et al., 2013). The characterization of transmission parameters has rarely been performed, severely limiting our understanding of disease epidemiology. Nevertheless, the importance in understanding the transmission of ampeloviruses infecting grapevines has recently become apparent, and several insect vectors species are now being studied for their efficiency to transmit different GLRaV species. Most work did not go beyond the identification of new insect species as vectors. In many cases virus source plants were infected with multiple virus species, which presents a challenge because multiple infections may lead to cases of virus facilitation or competition. While a better picture of GLRaV transmission by vectors is emerging, much remains undone. Nevertheless, trends can be inferred and used to generate testable hypotheses. The transmission of GLRaV-3 appears to be more efficient than that of other GLRaV species; based on inferences from studies designed to identify new vector–virus combinations rather than compare transmission efficiency. Competing hypotheses may explain these observations. First, viruses that are transmitted less efficiently may reach lower populations within plants than GLRaV-3. Therefore they may be acquired less frequently from the phloem, resulting in lower transmission rates. Alternatively, molecular interactions between virus and vector may affect transmission efficiency. Lastly, GLRaVs may be transmitted with similar efficiency, but those with observed lower transmission may require a higher number of virions inoculated to generate a successful infection.

In the Closteroviridae, all vector-borne viruses studied so far are transmitted in a semi-persistent manner (Karasev, 2000), but in this regard GLRaVs are poorly characterized. Cabaleiro and Segura (1997b) provide important insights into the biology of GLRaV-3 transmission by *Pl. citri*; however, they mentioned that their results were not conclusive to characterize transmission as semi-persistent. Conclusive evidence of semi-persistent transmission of GLRaV-3 was only obtained by Tsai et al. (2008). Transmission efficiency of GLRaV-3 by *Pl. ficus* first instars peaked with 24-h acquisition and inoculation access periods, with a leveling-off after 48 h (Tsai et al., 2008). *Pl. ficus* mealybugs lost the ability to transmit GLRaV-3 four days after acquisition (Tsai et al., 2008). It is imperative that similar experiments with more virus and vector species be performed, although given the phylogenetics of the group (Tsai et al., 2010), it is expected that all GLRaV ampeloviruses will be transmitted in a semi-persistent manner.

Reported transmission rates are difficult to compare given the varied experimental methods and generally low number of replicates used. For example, with semi-persistent transmission, a vector can lose the ability to transmit a virus upon molting to the next life stage, and longer experimental acquisition access periods used may have resulted in insects being moved to a new plant immediately after molting (and losing acquired virus). Such a protocol would effectively result in a shorter acquisition access period. Here, we report calculated *P*s values, following Swallow (1985), which provide an estimate of infection rate or probability of transmission by a single insect derived from experiments that used insect groups (**Figure 2**), based on existing transmission studies. When any one particular published study included multiple experiments, we combined those experiments to report one *P*s per published study, only including those experiments relevant to the question (e.g., mealybug life stage).

Earlier life stages of mealybugs have higher reported transmission efficiency than more mature life stages. *Pl. ficus* first and second instar nymphs have reported $Ps = 0.04–0.2$ (Tsai et al., 2008; Mahfoudhi et al., 2009), and adults have $Ps = 0.009–0.02$, about 10-fold lower than the nymphs. *Ph. aceris* first and second instars have $Ps = 0.05$ and 0.02, respectively (Le Maguet et al., 2012). *Ps. longispinus* first instar nymphs transmit GLRaV-3 at $Ps = 0.08$, and *Ps. calceolariae* first instar nymphs have $Ps = 0.02$, while no transmission was found by third instars of either mealybug species (Petersen and Charles, 1997). Because nymphs settle and feed more quickly than adults (Sandanayaka et al., 2012), it is possible that transmission by adult mealybugs would increase with longer access periods, although most studies appeared to use sufficiently long periods that this should not have confounded the results.

There also appears to be variation in transmission efficiency of GLRaV-3 among mealybug species (**Figure 2**). Three different research groups found similar *P*s values for *Pl. ficus* nymphs, ranging from 0.04 to 0.2 (Douglas and Krüger, 2008; Tsai et al., 2008, 2010, 2011; Mahfoudhi et al., 2009). Estimated *P*s for *Ph. aceris* was 0.004–0.03, for *Ps. calceolariae* 0.02, and for *H. bohemicus* 0.002–0.003 (Petersen and Charles, 1997; Sforza et al., 2003; Zorloni et al., 2006; Le Maguet et al., 2012); but different life stages

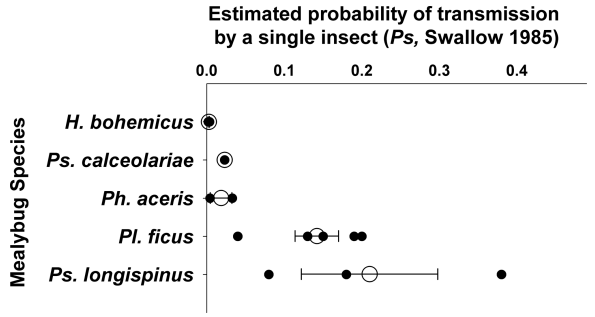

FIGURE 2 | Estimated transmission efficiency of GLRaV-3 per individual per day (*Ps*; per Swallow, 1985) by different mealybug species, including studies that tested transmission by first and/or second instars, nymphs, adults, or mixed life stages, and used access periods of 1 day or longer. When available, only earliest life stages tested are included. From individual publications including multiple experiments, those experiments are combined to produce one estimate of *P*s for each mealybug species. Results are based on a limited number of peer-reviewed publications per species; *Pl. ficus* – 5, *Ps. longispinus* – 3, *H. bohemicus* and *Ph. aceris* – 2, and *Ps. calceolariae* – 1. Figure shows *P*s for individual publications (dark circles), mean (open circles), and standard error.

were used and the results are probably not directly comparable. Widely variable results were obtained within *Ps. longispinus*, with transmission ranging from $Ps = 0.08–0.38$ (Petersen and Charles, 1997; Kuniyuki et al., 2005; Douglas and Krüger, 2008). The variation found within *Ps. longispinus* and among studies in general could be due to varied experimental techniques, to differences in transmission efficiency among insect populations or species, or to differences in GLRaV-3 variants that were tested.

It is not known whether GLRaV-3 populations within a donor plant affects transmission by mealybugs, but many viruses are transmitted at higher rates when the donor plant has higher viral infection (Froissart et al., 2010). GLRaV-3 populations vary seasonally in magnitude and distribution within a host plant, but the general trends are not well understood; virus population in leaves may increase during the growing season before dropping as leaves senesce (Tsai et al., 2012). Differences in transmission efficiency when mealybugs either acquire from, or inoculate to different plant tissues have not been found, although there is evidence that acquisition from stems may lead to lower transmission than from petioles or leaves (Tsai et al., 2011). Transmission by *Ps. longispinus* and *Ps. calceolariae* nymphs, for example, was tested early and late in the growing season from known infected vines in a vineyard, and no difference was found between the two time points (Petersen and Charles, 1997). While a change in transmission with viral population, plant tissue, or season has not been found, this possibility should not be ignored.

DISEASE ECOLOGY

Evidence of GLD spread in vineyards was first found in South Africa (Engelbrecht and Kasdorf, 1985), and confirmed there using an interplant study with healthy vines among established infected vines (Engelbrecht and Kasdorf, 1990). A similar interplant study in Spain also provided evidence of GLD spread (Cabaleiro and Segura, 1997a; Cabaleiro et al., 2008) following observations that older vineyards tended to have higher GLD incidence. In both cases mealybugs were recorded present at the interplant study sites. Controlled greenhouse tests of GLRaV-3 transmission by *Pl. ficus* (Engelbrecht and Kasdorf, 1990) and *Pl. citri* (Cabaleiro and Segura, 1997b) linked mealybugs to the observed vineyard spread. GLD spread in established vineyards, 8–10 years after the initial planting, has been documented in Australia (Habili et al., 1995; Habili and Nutter, 1997), California-USA (Golino et al., 2008), and France (Le Maguet et al., 2013). The rate of spread was similar in these studies, close to 10% increase per year once GLD infections were identified as being present, and newly infected vines were spatially aggregated, indicating vine-to-vine spread.

Leafroll spread through newly planted blocks adjacent to highly infected blocks has been documented in South Africa

FIGURE 3 | (A) Vineyards with high GLD incidence (dark red) serve as source of inoculum for adjacent blocks, in which disease spatial distribution is patchy, suggesting initial introduction of virus into uninfected blocks followed by within-block spread. **(B)** Example of secondary spread within rows, where an initial infection spread to neighboring plants. Both photographs were taken from the wine-producing region of Western Cape, South Africa.

(Pietersen, 2006; **Figure 3**), New Zealand (Charles et al., 2009), and Italy (Gribaudo et al., 2009). In New Zealand, populations of *Ps. longispinus* were monitored in nearby older leafroll-infected blocks, and the number of newly infected vines tended to increase more dramatically one growing season after large mealybug populations were found in neighboring blocks with 100% GLD incidence. Spatial analysis indicated that infected vines were randomly distributed throughout the blocks in early years, but aggregated toward the end of the study, indicating that long distance dispersal, such as wind-borne crawlers, as well as vine-to-vine movement of mealybugs was contributing to leafroll spread. In Italy, 20% virus prevalence was found 10 years after planting, indicating notably less apparent spread than in other regions (Engelbrecht and Kasdorf, 1990; Habili et al., 1995; Pietersen, 2006; Cabaleiro et al., 2008; Charles et al., 2009).

Grapevine leafroll disease is caused by a number of virus species, and within those species, there are genetically distinct variants. Within a growing region, for example, the geographical distribution differs among genetically distinct GLRaV-3 variants (Jooste et al., 2011; Sharma et al., 2011), yet little is known about what processes have led to this variation, or its impact on GLD. Furthermore, mixed variant infections within one plant are common and differential transmission of the variants may occur. In this complex system, interactions need to be considered among multiple virus and vector species. Potential virus and vector exchange with neighboring unmanaged communities needs to be evaluated. The effects of abiotic factors such as climate and nutrient availability need to be considered. Finally, a holistic view of the effects of various management practices is needed.

GLRaVs and their variants may vary in severity and may interact with each other during transmission and establishment in the host (Jooste et al., 2011). Some studies have also implicated GLRaV-1, -3, -4, and -9 in facilitating transmission of *Grapevine virus A* (GVA, *Vitivirus*; Zorloni et al., 2006; Hommay et al., 2008; Tsai et al., 2010; Le Maguet et al., 2012; Herrbach et al., 2013) but the evidence is inconclusive. These and other potential interactions could lead to changes in symptomatic disease prevalence and spread in vineyards. Some plant viruses can actually be beneficial to plants (Roossinck, 2011), and environmental conditions can alter the nature of effects a virus has on its host. The responses of GLD severity to varied environmental conditions, some of which can be controlled by changing management practices, remain largely unknown. Specific horticultural practices that are expected to affect the impact of GLD on yield and fruit quality should be studied. For example, partial defoliation of vines, which is expected to improve ripening, has been shown to improve the quality of must (freshly pressed fruit juice) from grapes infected with GLRaV-3 (Pereira-Crespo et al., 2012).

Pathogen-vector specificity can affect regional patterns of disease caused by vector-borne pathogens. Different genetic variants of a pathogen can differ in transmission efficiency by one vector species (Power, 1996; Tsetsarkin et al., 2011). Alternatively, one virus can be transmitted more or less efficiently by different vector species. GLRaV-3 is transmitted by many vector species and can be regarded as a "vector generalist" (Tsai et al., 2010), but GLRaV-3 transmission efficiency can differ among vector species (Douglas and Krüger, 2008). Adaptation to a vector that is already present, or introduction of a new vector into an area, can lead to dramatic changes in the prevalence of a vector-borne pathogen (Purcell and Feil, 2001). Furthermore, introduction of a new vector with a higher transmission efficiency of one pathogen variant than another can lead to changes in the relative prevalence of pathogen variants in a region, which can be as devastating as the introduction of a new pathogen. More knowledge is needed about the interactions of GLRaVs with their vectors.

CURRENT DISEASE MANAGEMENT OPTIONS

Despite the economic impact of GLD on the world's wine industry, efforts to manage this disease are still being developed or have only recently been implemented over large agricultural areas (e.g., Pietersen et al., 2013). Here we provide a summary of current management strategies being utilized or tested in four countries – South Africa, New Zealand, California-USA, and France. Our goal is to highlight management options that have been used to address both shared and unique challenges associated with this disease, with the expectation that each case study provides novel insights into the complexities of controlling GLD in the field.

A CASE STUDY OF DISEASE MANAGEMENT OPTIONS – SOUTH AFRICA

In South Africa, GLRaV-3 is the most important virus causing GLD (Pietersen and Kasdorf, 1993) and is transmitted predominantly by *Pl. ficus* and to a lesser extent by *Ps. longispinus* and multiple soft scale insect species (Walton and Pringle, 2004; Douglas and Krüger, 2008; Krüger and Douglas, 2013). Management of GLD is primarily through the provision of healthy planting material via the South African Vine Improvement Association (VIA). The VIA supplies the majority of planted vines utilized in the industry, and all VIA wine grape cultivars or clones are subjected to virus elimination via heat therapy and *in vitro* meristem tip propagation (Engelbrecht and Schwerdtfeger, 1979). Hardened off plantlets are established and maintained in insect-free greenhouses as *nuclear plants* (i.e., plant material of the highest level of sanitation in the certification scheme). On establishment, and every 5 years thereafter, these plants are subjected to compulsory tests for GLRaV-1, -2, and -3 (Goszczynski et al., 1995), *Grapevine fanleaf virus*, GVA and GVB by ELISA and by immunoelectron microscopy (Pietersen and Kasdorf, 1993) for GLRaV-4 and -5 in addition to the previously listed viruses. Furthermore, these plants are subjected to hardwood indexing on seven *Vitis* indicators (for 2 or 3 years depending on the disease). For plants to be certified as nuclear material, they must be negative for all viruses tested as well as GLD, grapevine stem grooving disease, grapevine corky bark disease, Shiraz disease, grapevine fleck disease, grapevine vein necrosis, and grapevine vein mosaic disease.

Planting material from nuclear blocks is propagated to establish *foundation blocks*, either in greenhouses or open field plantings. Open field foundation block vineyards must be on virgin soil (i.e., not previously planted in grapevines) that must test free of *Xiphinema index* (California dagger nematode), and must be at

least 25 m from other vineyards. The vines from these blocks are tested every year by ELISA for GLRaV-1, -2, and -3 if they are located in high risk areas (less than 25 m from other grapevines if mealybugs are recorded in the vicinity) or every 3 years if in low risk areas (no mealybugs trapped or observed, and the block is at least 25 m from vineyards of lower phytosanitary status). Plants testing negative for GLD in foundation blocks may be used to establish *mother-blocks*. Mother-blocks are typically commercial grape-growing vineyards and only need to be 3 m away from other vineyards. They can be planted in untested virgin soil or on soil that has previously been planted to *Vitis* but tests free of *X. index*. Visual inspection for GLD symptoms is conducted annually in autumn on red cultivars, Chardonnay, Cape Riesling, and Semillon.

The use of certified planting material and the above plan, however, do not rid South African vineyards of GLD. Mother-blocks in traditional grape production areas become infected rapidly with GLD. For example, during a 2001–2006 spatio-temporal study of 55 red cultivar mother-block vineyards in which no specific GLD control was applied, once GLD infections were initially found there was an average annual GLD increase of 1.94 times (Pietersen, 2006). Because of this, South African mother-blocks are only utilized for planting material if GLD infection levels of less than 5% exist in the vineyard. At infection levels below 5%, the producer may permit the removal of infected vines, or canes from infected and single adjacent vines within the row may be cut and dropped annually before planting material is collected. In spite of these measures, GLD-infected planting material can still be found within the certified material, with randomly occurring GLD-infected vines in newly established vineyards observed in 3% of all the mother-blocks (Pietersen, 2006). Based on the average rate of infection amongst the 55 mother-blocks monitored, it was estimated that the initial GLD incidence in the planting material was less than 1%. Since the mid-2000s many local plant improvement organizations have been propagating mother-block material in areas in which grapevines have not been grown previously. Certified material is therefore now differentiated as mother-blocks in low risk areas (three-star rated material) and in areas at risk to GLD re-infection (one-star material). At this time, three-star material is still relatively scarce; therefore responsible producers apply systemic insecticides at planting, and rogue GLD-infected vines in the newly established vineyards.

Secondary spread from a GLD-infected vine to adjacent vines in a row is the major cause of new GLD infections in the industry and occurred in all mother-blocks monitored (Pietersen, 2006; **Figure 3**). Roguing of infected vines is feasible and effective on an experimental scale (Pietersen et al., 2003). Removal of infected vines, combined with mealybug control, is extremely effective at controlling GLD in commercial vineyards, and this practice is becoming more widely applied (Pietersen and Walsh, 2012). Pietersen (2006) also presented circumstantial evidence of GLD spread in a replanted vineyard from a preceding vineyard, either through the presence of viruliferous mealybugs on remnant root material, or on volunteer hosts. The persistence of GLRaV-3 in remnant roots and potential of transmission by mealybugs from these has subsequently been demonstrated (Bell et al., 2009). Fallow periods of up to two seasons, during which remnant roots are removed, have been utilized in a number of commercial vineyards locally (Pietersen and Walsh, 2012). A clear demonstration of the efficacy of this strategy on its own must still be shown.

Gradients of GLD infection from the edges of a vineyard are commonly observed. Pietersen (2006) recorded gradients of various slopes from 70% of the 55 mother-blocks analyzed. These gradients reflect initial introduction of the virus from a source external to the vineyard, and in 32% of the blocks monitored the gradient could clearly be ascribed to an adjacent GLD-infected vineyard. These gradients are likely due to immigrating first instar mealybugs, either by their own motility over short distances, or on farm workers' clothing, on implements, by wind, ants, or possibly even by birds. A number of strategies have been employed to reduce the introduction of the disease from external sources (Pietersen and Walsh, 2012), including stringent control of mealybugs in all vineyards within the region, planting new vineyards far from heavily infected vineyards, avoiding traffic (implements and workers) from infected to healthy vineyards, or if unavoidable, washing implements with soapy water when moving between vineyards, and conducting work in healthy vineyards before moving into an infected vineyard. Following such a program, the near-eradication of GLD has been achieved at a commercial wine estate in the Somerset West district, from 100% infection on 41.26 ha in 2002 to 0.027% infection on 77.84 ha in 2012 (Pietersen et al., 2013). This result provided strong evidence that by using the full suite of GLD and mealybug control strategies available, disease incidence and its progression can be reversed. Further studies are required to determine the relative efficiency of individual components of the integrated control strategy.

A CASE STUDY OF DISEASE MANAGEMENT OPTIONS – NEW ZEALAND

Grapevine leafroll disease was first described in New Zealand in the early twentieth century (Bragato, 1902), but it was not until the 1960s that research to quantify its impact on vine performance and fruit quality started (Chamberlain et al., 1970; Over de Linden and Chamberlain, 1970). Today, GLRaV-3 is the most widespread and economically damaging disease affecting grapevines (Bonfiglioli and Hoskins, 2006). Concerned with the long-term impact of GLRaV-3 on wine quality, the national sector body, New Zealand Winegrowers (NZW), developed the grafted grapevine standard, with one of its aims being to minimize the probability of plant material with diseases such as GLRaV-3 being released to the industry.

A grower survey in 2005 revealed few respondents were well informed about the threats posed by GLRaV-3 or the options available for limiting its spread. Furthermore, a review of local and international literature, aimed to identify GLRaV-3 research priorities and knowledge gaps, was prepared (Charles et al., 2006). Of the numerous recommendations generated by these NZW initiatives, a plan for grower education and communication was prioritized. A collaborative program was established in which viticulturists, winemakers, and vine nursery groups collaborated with plant virologists, vine physiologists, and entomologists in

a multi-disciplinary integrated approach to establish a GLRaV-3 control program.

A GLRaV-3 control pilot project began in 2009 in two North Island winegrowing regions: the Gimblett Gravels, a winegrowing sub-region in Hawke's Bay, and Martinborough. The project focused on controlling GLRaV-3 in red grape varieties because symptomatic vines are relatively easily identified visually by the dark red downward curling leaves with green veins. The project had three aims: (1) to visually identify and map the presence of GLRaV-3 in vines in both regions; (2) to control GLRaV-3 through a combination of vine removal, hygiene practices, and improved vector management; and (3) to enable eventual vine replacement, whilst incorporating the new knowledge into "best practice" guidelines for nationwide dissemination (Hoskins et al., 2011). Here, we summarize the process and some of the achievements of the first 3 years of a 6-year project.

The control of GLRaV-3 in the field has focused on two strategies. The first was the removal (or roguing) of individual symptomatic vines (or small clusters of symptomatic vines), with most vineyard owners roguing symptomatic vines only. The second strategy, whole block removal, was adopted in blocks where roguing individual vines was considered unlikely to contain or control the disease. In New Zealand, the economic threshold of GLD incidence beyond which roguing was thought to be practical was ∼20% of vines (Hoskins et al., 2011).

In the Gimblett Gravels and Martinborough regions, participating vineyards supplying ∼40 individual wineries encompassed an area of ∼1,100 ha. Training of vineyard personnel to identify GLRaV-3 symptomatic vines accurately was initiated. Once trained, vineyard personnel systematically moved through every red grape variety block late in the season identifying symptomatic vines, plotting their position with GPS and marking vines to guide the roguing done in winter. While the regional mapping of symptomatic vines is ongoing and the data have yet to be fully interpreted, individual vineyard owners are provided annually with preliminary block-specific results. The provision of this information has substantially aided the profile of the project and its educational goals, particularly in measuring the incidence and changes to the spread of GLRaV-3 (Hoskins et al., 2011).

Augmenting the regional mapping of GLRaV-3 were block-specific studies focused on GLRaV-3 identification together with monitoring the disease vectors, mealybugs. Data were analyzed from nine blocks in the Gimblett Gravels planted in various red grape varieties (∼21,000 vines). The objective was to determine if a combined approach of GLRaV-3 visual identification and roguing, supported by good vector control could reduce disease incidence to a point where less than 1.0% of vines per block were rogued annually. While this study is still underway, preliminary results are presented here (V. A. Bell, unpublished results).

In the nine study blocks, the percentage of symptomatic vines identified and rogued per year steadily declined from an average of 11.8% in 2009 to 2.7% in 2012. Over this period, a total of 4,902 symptomatic vines were rogued across the nine study blocks (23.8% of the original plantings). After 3 years, the evidence suggests roguing can successfully control GLRaV-3, although as discussed, good vector management was integral to a successful outcome.

In 2011 and 2012, mapping the positions of symptomatic vines in each block revealed 82.4 and 88.6%, respectively, were in close proximity to a vine rogued since 2009, supporting similar findings in earlier studies (Habili and Nutter, 1997; Cabaleiro and Segura, 2006; Pietersen, 2006). Of these neighboring vines, most at risk of acquiring GLRaV-3 were the "first" vines, the within-row immediate neighbors of a vine rogued at least 12 months earlier. This pattern of GLRaV-3 spread suggested the infection pathway, mediated by vector dispersal, was from the vine rogued at least 12 months earlier. In 2010, an average of 78% of all "first" vines had no visual symptoms of GLRaV-3, indicating they were either healthy or if infected, the visual symptoms were yet to express. By 2012, "first" vines relative to other "nearest neighbors" remained most at risk of GLRaV-3, although on average, 92% of "first" vines were symptomless. Based on the results of this study, the risk of a "first" vine acquiring GLRaV-3 was low, especially as the benefits of roguing and effective vector management accumulated over time. Consequently, good control of GLRaV-3 was achieved under almost all circumstances by roguing symptomatic vines only.

A further important aspect of the project was to determine the extent to which vector populations influenced GLRaV-3 control outcomes. Throughout this study, the vector most commonly encountered was the mealybug *Ps. calceolariae*, which colonizes all aerial parts and the roots of grapevines. Monitoring indicated mealybug numbers declined in most blocks over time as vineyard managers heeded warnings to improve vector control and to adopt better hygiene practices, such as removing the remnant roots of rogued vines. Being long-term reservoirs of GLRaV-3 (Bell et al., 2009), remnant roots colonized by *Ps. calceolariae* provide a likely pathway for the disease to infect young replacement vines.

In 2012, GLRaV-3 incidence in three of the nine study blocks was reduced to less than 1.0%, and in these blocks since 2010, mealybug counts from the third and final generation in late summer (March) were consistently low, ranging from two to eight mealybugs per 100 vine leaves inspected. Significantly, in two of these blocks (identified as A and B), disease incidence in 2009 was relatively high at 10.1 and 16.0%, respectively, so to have effectively controlled GLRaV-3 in just 3 years was an encouraging result. Given the known economic impacts of GLRaV-3, it was not possible to include an "unmanaged control" component in any of the study blocks. Despite this position, the finding of significant mealybug populations (78–175 mealybugs per 100 vine leaves inspected) in another two study blocks (C and D) provided useful comparisons with blocks A and B. In 2009, GLRaV-3 incidence in blocks C and D was 9.9 and 15.1%, respectively, but by 2012 cumulative vine losses due to GLRaV-3 were ca. 40%, culminating in the removal of all residual vines in both blocks.

With symptomatic vines identified and rogued each year in all nine study blocks, what most distinguished blocks C and D from the other seven was the high number of mealybugs. In this instance, poor mealybug control was probably due to non-adherence to insecticide (i.e., buprofezin) best practice with water

volumes about one-third the label recommendations, thus compromising coverage and vine wetting. These contrasts in vector abundance demonstrated that roguing symptomatic vines alone provided relatively unsuccessful control of GLRaV-3 when it was not supported by effective mealybug management. In other words, while total eradication of *Ps. calceolariae* was not a prerequisite for controlling GLRaV-3, containing this disease was only achieved in those blocks where mealybug numbers were consistently low.

A CASE STUDY OF DISEASE MANAGEMENT OPTIONS – CALIFORNIA-USA

California accounts for 89.5% of domestic U.S. wine grape production – a total of 3.6 million tons in 2010 – with a farm gate value of US$2.06 billion. In a survey conducted by the American Vineyard Foundation in 2009, grape growers considered mealybug control and GLD one of their top priorities, solidifying this as a high priority research issue that threatens the sustainability of the industry. California grape growers have begun implementing multiple tactics in an effort to minimize current and future losses attributed to GLD. Although various GLRaV species are present in California, GLRaV-3 has been identified as the most important in the premiere wine-producing region of Napa Valley (Sharma et al., 2011).

California growers aim to minimize incidence of GLD and other grapevine diseases by planting material certified through the California Grapevine Certification and Registration (CGC&R) Program. Established in 1956, the CGC&R Program is administered by the California Department of Food and Agriculture (CDFA; Alley and Golino, 2000). It targets the elimination of grapevine diseases that spread from vine-to-vine by grafting and/or vegetative propagation. Under the auspices of the CGC&R Program, correctly named grape materials that pass specific disease tests are identified and/or created, and maintained as Foundation materials by Foundation Plant Services (FPS) at the University of California, Davis, for use by California commercial nurseries.

The CGC&R Program includes provisions for three levels of planting stock: California Foundation stock, California Registered stock, and California Certified grapevines. FPS at University of California, Davis maintains vines in the FPS Foundation block; materials derived from FPS Foundation vines are California Foundation stock. Vineyards planted by participants in the CGC&R Program using California Foundation stock material are known as California Registered increase blocks. They are inspected annually and tested for pathogens as needed by inspectors from CDFA. Material derived from the California Registered increase blocks is California Registered stock. When California Registered cuttings are rooted, or California Registered scion cuttings are grafted to California Registered rootstock cuttings, the resulting vines are classified as California Certified grapevines and are sold to growers for commercial planting. Nursery participation in the CGC&R Program is strongly encouraged but not mandatory. Other limitations to the CGC&R Program include the use of traditional screening methods (ELISA, RT-PCR, qPCR), which require prior knowledge of pathogens and are incapable of detecting unknown variants or agents. The variable population of GLRaV species in plant tissue, including rootstock and scion, is also a limitation to the production of reliable laboratory test results, and therefore material that is free of known viruses.

To manage GLD spread, California wine grape growers identify symptomatic vines, document annual changes in disease incidence in vineyard blocks, and remove diseased vines. Vine removal occurs only in blocks where disease incidence is below a threshold determined by each grower. Thresholds are typically generated by an economic analysis based on vineyard age, cost of replanting versus redevelopment, grape purchasing contracts, the wine program for which the grapes are destined, and other considerations. Generally, growers identify vineyards with greater than 20–30% disease incidence for redevelopment of the entire block, whereas vine removal occurs in vineyards with less than 20% disease incidence. However, the threshold for roguing versus redevelopment varies considerably among growers, especially when grapes are destined for a high price point wine, or when redevelopment is particularly costly or challenging, such as in hillside blocks. Timely removal of diseased vines is limited by the cost associated with routine and reliable identification of these vines. It is not common practice in California to regularly identify and rogue symptomatic vines, although some growers have made it a regular practice in recent years. Dedicating resources to this effort can be complicated because peak symptom development overlaps with harvest period. There has also been a general lack of awareness of the importance of this practice. Both concerns are being addressed through research and educational programs directed by researchers at the University of California, with the goal of increasing awareness of the importance of this practice while identifying faster and easier ways to do it. In particular, infected vines may be identified using hyperspectral imaging technology that measures differences in leaf spectral reflectance between GLRaV-3 infected and uninfected grapevines (Naidu et al., 2009).

Mealybug management is a major component of GLD control programs in California. Currently five mealybug species cause direct damage and are potential vectors of GLRaV in vineyards: *Pl. ficus*, *Ps. maritimus*, *Ps. viburni*, *Ps. longispinus*, and *Ferrisia gilli* Gullan (Daane et al., 2012). Recently, a multiplex PCR procedure was developed to identify seven species of mealybug typically found in California vineyards (Daane et al., 2011). The ability to identify young mealybug nymphs to species using rapid and sensitive detection techniques helps growers make informed decisions about mealybug management. Trapping programs using pheromone-loaded lures also provide important information on mealybug species presence.

Growers rely on a combination of tactics including insecticides, mating disruption, biological control, and management of some ant species to minimize populations of *Pl. ficus* (Daane et al., 2008). Unfortunately, the Argentine ant (*Linepithema humile*) in particular, which "farms" mealybugs, is very aggressive in California vineyards and growers therefore struggle to maintain the extremely low mealybug populations required to minimize virus transmission. Results of recent investigations suggest that regional management programs for *Pl. ficus*, utilizing a combination of these tools, may provide better long-term control than individual efforts by isolated growers. Efforts are therefore underway to develop and implement similar regional management programs

for other vineyard mealybug species. Populations of *Ps. maritimus* are of particular interest in coastal northern California vineyards, where they are commonly associated with spread of GLRaV-3, the most prevalent virus species that is spreading in the area.

A CASE STUDY OF DISEASE MANAGEMENT OPTIONS – FRANCE

In France, GLD is believed to be present since at least the early 1900s, as "rougeau" or "rougeot," and later as "enroulement foliaire," suspected then to be the same as the "*Rollkrankheit*" and "Leafroll" already described in Germany and the United States, respectively (Goheen et al., 1958; Vuittenez, 1958). However, GLD has long been seen as an unimportant problem for French viticulture, at least less crucial than fungal diseases and even *Grapevine fanleaf virus*. One reason may be that GLD symptoms were, and still are, often confused with other diseases or deficiencies, especially on white-berried cultivars. However, management of GLD was soon seen as a matter of sanitary selection (Vuittenez, 1958). At present, three species, GLRaV-1, -2, and -3, are detected in French commercial vineyards. GLRaV-1 and -3 are more frequent in north-eastern (Alsace, Beaujolais, Bourgogne, Champagne) and in southern (Mediterranean regions and Bordelais) vineyards, respectively, whereas GLRaV-2 is more common in the south-west. Over the last decades, infections by GLRaV-1 were recorded from many areas in Burgundy, Beaujolais, and Champagne. At the same time, wider infestations of mealybugs and soft scales were reported from these regions, probably related to the decreasing use of insecticides against the European grapevine moth (*Lobesia botrana*).

In France, sanitary selection was set up in the 1940s with the aim of producing healthy plant material to initially combat the spread of *Grapevine fanleaf virus* (Valat, 1972; Walter and Martelli, 1997). This process was greatly improved since then, due to progress in virological knowledge and detection methods, and is still seen today as the primary way to control GLD, which was incorporated into the system at a later date, among other viral and phytoplasmal diseases. According to French regulations (see www.legifrance.com), which follow a European Commission Directive, all planting material is classified in one of four categories: *initial*, *base*, *certified*, or *standard* (FranceAgriMer, 2013). The first three are produced only by specific institutions (initial, base) or nurseries (certified) and are subjected to detection tests to demonstrate the absence of viruses. Indexing is performed for any new clone prior to registration. During the pre-multiplication and multiplication processes, ELISA tests are used for all certified material. So far, only GLRaV-1 and -3 are taken into account among the GLRaVs, and all vines found infected at these steps have to be removed. The production of initial, base, and certified material is under the control of FranceAgriMer, a government agency. Growers can choose between certified and standard planting material, the first being more expensive but tested free of certain viruses (Walter and Martelli, 1997). Standard material is produced either in nurseries, where only visual inspections of symptoms are performed, or by growers who practice mass selection. Therefore, the use of standard material increases the risk of spreading GLD.

The use of insecticides against GLRaV vectors is allowed in France. However, few active ingredients, mainly organophosphates, are specifically registered for controlling scale and mealybug insects on grapevine. Moreover, insecticide implementation is not regulated nationally and will vary according to regional practices and viticultural advisers. Deeper biological and epidemiological knowledge is urgently required in order to adjust the use of insecticides to specific disease risk levels, depending on disease incidence and vector density. While natural enemies of vectors are known and experiments (e.g., using lacewings) are underway, there is no biological control program established in France. The development of vector monitoring by lure traps and mating disruption will first require the identification of the sex pheromones, still unknown, of common species like *Ph. aceris*, *H. bohemicus*, and *Pa. corni*.

In the course of certification schemes, thermotherapy- or meristem culture-based sanitation methods are sometimes used, especially for high value clones or cultivars. GLD management in France currently relies mainly on the sanitary selection of planting material, so in the long-term, healthy planting material seems the key to controlling GLD. More effort is to be devoted to improve sanitary selection, requiring deeper knowledge of the diversity of viruses and their effect on grapevine. Virological knowledge will also improve both specificity and sensitivity of detection methods used. Moreover, it should be desirable in the future to coordinate the certification schemes among countries producing planting material. In France, growers need to be better informed about GLD symptomatology and the detrimental effects of GLD, especially in case of co-infection of vines with two or more distinct viruses, a common phenomenon for grapevine. In addition, better information should assist nurseries and others producing plant material, particularly those using mass selection, to adopt procedures aimed at producing virus-clean grapevines. Moreover, a recent French study showed the risk to neighboring vineyards posed by leafroll-infected and scale insect-infested plots (Le Maguet et al., 2013). Therefore, a new plantation should take into account the sanitary status of neighbors and the possible spread of vectors from older to younger plots. Better guidelines (such as planting of hedges, vine-free strips between plots, insecticide treatments) should be tested for their efficacy, particularly for isolating mother plant blocks.

Finally, the wide variety of GLD epidemiological scenarios in France (e.g., Le Maguet et al., 2013) and the difficulty to define any damage thresholds hamper determining recommendations on how best to manage virus-infected and/or vector-infested vineyards. There is a crucial need for deeper knowledge of epidemiology, including determinant factors such as infection intensity, sensitivity of cultivars, virus and vector species present, and landscape structure. GLD management is more than a single grower's concern, instead requiring a collective approach across whole communities of growers, advisors, and scientists.

MANAGEMENT STRATEGIES – SUMMARY

The preceding case studies from grape-growing regions worldwide share remarkable similarities, and illustrate a combination of

approaches required for GLD control. Importantly, they illustrate that GLD must be managed at a large scale and that a long-term management strategy is needed. Especially in the case of GLRaV-3, infected vines or blocks will continually act as sources of inoculum, perpetuating disease spread. For this reason, a coordinated area-wide approach is required, with education of growers as the first step. A second component of control is access to uninfected propagation material. A centralized service that includes a stringent certification program is needed to provide disease-free planting material for a growing region, as is the case in the countries reported here. In some regions where vectors may live on infected roots from previous crops, extra care is needed to assure that the planting area does not contain a GLRaV-3 source in the remaining live roots of infected vines that were removed. In both newly planted blocks and those with mature vines, roguing of symptomatic vines, and possibly vines immediately adjacent to those symptomatic vines, appears to be effective in preventing future disease spread. A third aspect of GLD management is control of insect vectors. Because mealybug nymphs are the most infective life stage and could travel long distances in air currents, insect control is often needed before large mealybug populations are detected. Therefore, knowledge of the life cycle of vectors can inform decisions regarding implementation of insect control. Finally, effective control of an existing GLD problem cannot be achieved within one growing season. Instead, favorable results are found after multiple years of regional management practices that incorporate eliminating infected plant material and controlling vector populations.

Still lacking in GLD management is fundamental knowledge about disease spread. While a majority of management has focused on red varieties, largely because identification of symptoms is easier, GLD also affects white varieties. As long as white varieties continue to be overlooked, they may continue to be a source of virus thus hampering management efforts.

ACKNOWLEDGMENTS

Based in New Zealand but of the world, Dr Rod Bonfiglioli was a talented and widely respected plant virologist. His passing in May 2009 in New Zealand sadly coincided with the start of his long anticipated GLRaV-3 control project. It will remain an enduring legacy to a man unlikely to be forgotten by his many friends and colleagues. Rodrigo P. P. Almeida, Kent M. Daane, G. K. Blaisdell, and Monica L. Cooper acknowledge funding from the American Vineyard Foundation, Viticulture Consortium West, Oregon Wine Board, California Fruit Tree, Nut Tree and Grapevine IAB, California Department of Food and Agriculture, and United States Department of Agriculture (SCRI). Monica L. Cooper gratefully acknowledges the support of Napa wine grape growers and their staff. Vaughn A. Bell gratefully acknowledges funding provided by New Zealand Winegrowers, the Ministry for Primary Industries Sustainable Farming Fund, the Gimblett Gravel Winegrowers' Association, and the support of the vineyard owners and their staff. Etienne Herrbach acknowledges funding from INRA and France AgriMer. Gerhard Pietersen acknowledges funding from Winetech, South Africa.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 January 2013; accepted: 03 April 2013; published online: 24 April 2013.

Citation: Almeida RPP, Daane KM, Bell VA, Blaisdell GK, Cooper ML, Herrbach E and Pietersen G (2013) Ecology and management of grapevine leafroll disease. Front. Microbiol. ***4****:94. doi: 10.3389/fmicb.2013.00094*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 20 May 2013
doi: 10.3389/fmicb.2013.00099

Crinivirus replication and host interactions

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Criniviruses comprise one of the genera within the family *Closteroviridae*. Members in this family are restricted to the phloem and rely on whitefly vectors of the genera *Bemisia* and/or *Trialeurodes* for plant-to-plant transmission. All criniviruses have bipartite, positive-sense single-stranded RNA genomes, although there is an unconfirmed report of one having a tripartite genome. *Lettuce infectious yellows virus* (LIYV) is the type species of the genus, the best studied so far of the criniviruses and the first for which a reverse genetics system was developed. LIYV RNA 1 encodes for proteins predicted to be involved in replication, and alone is competent for replication in protoplasts. Replication results in accumulation of cytoplasmic vesiculated membranous structures which are characteristic of most studied members of the *Closteroviridae*. These membranous structures, often referred to as *Beet yellows virus* (BYV)-type vesicles, are likely sites of RNA replication. LIYV RNA 2 is replicated *in trans* when co-infecting cells with RNA 1, but is temporally delayed relative to RNA 1. Efficient RNA 2 replication also is dependent on the RNA 1-encoded RNA-binding protein, P34. No LIYV RNA 2-encoded proteins have been shown to affect RNA replication, but at least four, CP (major coat protein), CPm (minor coat protein), Hsp70h, and P59 are virion structural components and CPm is a determinant of whitefly transmissibility. Roles of other LIYV RNA 2-encoded proteins are largely as yet unknown, but P26 is a non-virion protein that accumulates in cells as characteristic plasmalemma deposits which in plants are localized within phloem parenchyma and companion cells over plasmodesmata connections to sieve elements. The two remaining crinivirus-conserved RNA 2-encoded proteins are P5 and P9. P5 is 39 amino acid protein and is encoded at the 5′ end of RNA 2 as ORF 1 and is part of the hallmark closterovirus gene array. The orthologous gene in BYV has been shown to play a role in cell-to-cell movement and indicated to be localized to the endoplasmic reticulum as a Type III integral membrane protein. The other small protein, P9, is encoded by ORF 4 overlaps with ORF 3 that encodes the structural protein, P59. P9 seems to be unique to viruses in the genus *Crinivirus*, as no similar protein has been detected in viruses of the other two genera of the *Closteroviridae*.

Keywords: phloem-limited, plasmalemma deposit, whitefly vector, *Crinivirus*, quintuple gene block

INTRODUCTION

Most plant viruses have positive-sense single-stranded RNA (ssRNA) genomes that vary in size among viruses in different taxa. Members in the family *Closteroviridae* possess the largest and most complex ssRNA genomes which vary in size from ca. 15–20 kb (Martelli et al., 2012a). Closteroviruses (the generic name for viruses in the family) are currently placed within three approved and one proposed genera (Martelli et al., 2012a). The genus *Closterovirus* contains viruses whose genomes are monopartite, and that are transmitted to plants by various aphid vectors. The genus *Crinivirus* encompasses viruses whose genomes are bipartite (although one member has a proposed tripartite genome). Criniviruses are exclusively transmitted by whiteflies of two genera: *Bemisia* and *Trialeurodes*. The genus *Ampelovirus* has members with monopartite genomes, and the viruses are transmitted by mealybugs. The newly proposed genus, *Velarivirus*, contains members formerly within the genus *Ampelovirus*, but which represent a different phylogenetic clade (Martelli et al., 2012b). However, despite these genomic and biological differences all closteroviruses possess many commonalities. All members have characteristic long, flexuous rod-shaped virions, which range in size from ca. 750–2000 nm, depending on the specific virus. All closteroviruses share two conserved gene modules including one encoding proteins associated with replication (ORFs 1A and 1B), and the quintuple gene block, or the "hallmark closterovirus gene array" encoding for proteins that are not associated with replication, but are virion components or are involved in other biological processes of closterovirus infections. For criniviruses, these two gene modules are separated onto the two distinct genomic RNAs, and at least for one crinivirus the separation of these gene modules likely plays a role in temporal regulation of genome replication and gene expression.

Lettuce infectious yellows virus (LIYV) is the type member of the genus *Crinivirus*. Studies on LIYV date back to the late 1970s when several crops in California and Arizona [including lettuce (*Lactuca sativa*; **Figure 1A**), melons (*Cucumis melo*), and sugar

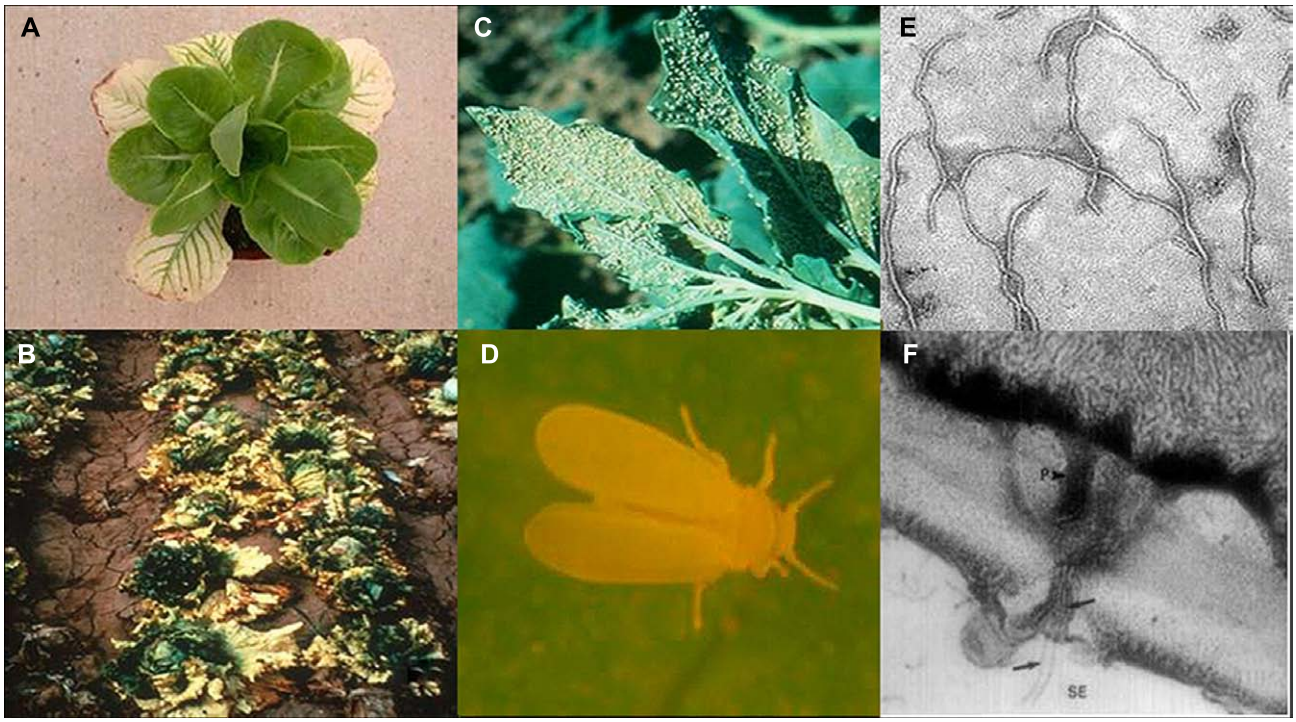

FIGURE 1 | *Lettuce infectious yellows virus* (LIYV) infected lettuce plants close up (A) and field shot (B). The sweetpotato whitefly, *Bemisia tabaci* New World (formerly called biotype A) colonizing a *Chenopodium* spp. plant in the field **(C)** and close up **(D)**. LIYV virions by transmission electron microscopy **(E)** and **(F)** a thin section showing cross section of pore-plasmodesma connecting sieve element and phloem parenchyma cell, showing flexuous rod virions (black arrows) within plasmodesma and in both cells, and plamalemma deposits on the phloem parenchyma cell membrane above plasmodesmatal pore (adapted from Hoefert et al., 1988 with permission from Elsevier).

beets (*Beta vulgaris*)], were severely affected by this newly discovered virus, resulting in losses exceeding $20 million in a single growing season (Flock and Duffus, 1982). Due to the severe economic losses caused by LIYV at that time, LIYV became a subject of intense investigations. By 1982, it was recognized as a distinct and emerging "new" virus and was found to be associated with the rapid expansion and spread of the sweet potato whitefly, *Bemisia tabaci* biotype A (now New World; Flock and Duffus, 1982; De Barro et al., 2011; **Figure 1D**). Primary work focused on characterizing LIYV whitefly transmission properties, host range, examination of virion morphology, and its effect on host cells (Duffus et al., 1986; Hoefert et al., 1988). Advances in DNA sequencing and molecular biology demonstrated the bipartite nature of the LIYV genome. LIYV was the first crinivirus whose genome was sequenced and was the first for which reverse genetics approaches were developed that further enabled studies of replication, gene expression, and protein functions (Klaassen et al., 1995, 1996). Although today LIYV is not agriculturally important due in part to displacement of the *Bemisia tabaci* biotype A (New World) by a more competitive, and more aggressive non-LIYV vector whitefly, *Bemisia tabaci* biotype B (now called Middle East/Asia Minor; De Barro et al., 2011), studies on LIYV continued and have proved to be critical in establishing a basic understanding of crinivirus–host and crinivirus–vector interactions. These efforts also aided further studies with other criniviruses, many of which are currently of great economic importance. Here, we intend to review these seminal studies that allowed the development of current understanding of LIYV/crinivirus replication and host plant interactions.

LIYV AS THE SEMINAL CRINIVIRUS

Lettuce infectious yellows virus was discovered coincident with the explosion of the *Bemisia tabaci* biotype A (New World) population in southern California and Arizona in the late 1970s. Although whiteflies, and particularly, *Bemisia tabaci* had been recognized as a plant virus vector for many years, LIYV was recognized as a novel type of virus at that time. *Bemisia tabaci*-mediated LIYV transmission was semi-persistent. Transmission electron microscopic studies on purified virions and LIYV-infected plants showed that LIYV virus like particles (virions) were similar to those of the closteroviruses known at that time. The virions were long, flexuous rods (**Figure 1C**; Duffus et al., 1986) and in plants the virions and cytopathologies of infection were limited to phloem cells (Hoefert et al., 1988). Although initial virion size estimates suggested lengths of ~2000 nm for LIYV (Duffus et al., 1986) similar to lengths of known aphid-transmitted closteroviruses including *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV), subsequent studies revealed that LIYV has shorter particle lengths of approximately 800 nm (Tian et al., 1999), and further studies on other later-discovered criniviruses showed similar virion lengths (Liu et al., 2000). We now know that these lengths reflect the sizes of the encapsidated genomic RNAs.

Virion purification and RNA extraction and analysis showed another unique feature; purified LIYV virion preparations contained two distinct ssRNA molecules of 8,118 nucleotides and 7,193 nucleotides, respectively, thus, suggesting that LIYV has a bipartite genome (Klaassen et al., 1994). This was in contrast to the other closteroviruses that were characterized at that time [e.g., BYV, CTV, and *Beet yellow stunt virus* (BYSV)] all of which had a single large, single-stranded genomic RNA (Bar-Joseph and Hull, 1974; Dodds and Bar Joseph, 1983; Reed and Falk, 1989). By 1995, both of the LIYV genomic RNAs were sequenced (Klaassen et al., 1995), which enabled comparisons of the LIYV genomes with those of BYV and CTV, the only other closteroviruses sequenced at that time. Comparison of deduced protein amino acid sequences with those of other filamentous plant viruses showed that the LIYV major coat protein (CP) sequence was most similar to the coat protein sequences of BYV and CTV (Klaassen et al., 1994) and allowed for a more precise taxonomic classification of LIYV, which led to the establishment of the genus *Crinivirus* within the family *Closteroviridae*. The genus name *Crinivirus*, comes from the latin "crinis" for "hair" (Martelli et al., 2002).

Sequencing the LIYV genomic RNAs showed that the LIYV RNAs 1 and 2 contained the gene modules that are characteristic of BYV and CTV, but also showed them to be separated between the two genomic RNAs, 1 and 2 (**Figure 2**). Later sequencing of other crinivirus genomes showed that they also have bipartite genomes with conservation of most of the gene content and order (**Figure 2**) with the possible exception of *Potato yellow vein virus* (PYVV), which is suggested to have a tripartite genome (Livieratos et al., 2004).

GENOME ORGANIZATION

Lettuce infectious yellows virus RNA 1 is 8,118 nt and contains a 5′ cap structure and the 3′ terminus is not polyadenylated. RNA 1 includes a 97 nucleotide 5′ untranslated region followed by two ORFs, 1A and 1B. ORF 1A encodes a potential protein of 1873 amino acids. Alignment of the ORF 1A protein amino acid sequences of LIYV with those of the BYV ORF 1A protein showed that the highest sequence similarity was in the methyl-transferase (MTR) and RNA helicase (HEL) motifs (Klaassen et al., 1995). Although amino acid sequences upstream of the MTR domain did not show statistically significant similarity, there were motifs identified for both LIYV and BYV that showed the characteristic signature of papain-like proteases (Agranovsky et al., 1994; Klaassen et al., 1995; Peremyslov et al., 1998). Agranovsky et al. (1994) performed site directed mutagenesis of this region for BYV and showed that the catalytic cysteine residue of the leader protease is contained in this motif. It is suggested that the homologous cysteine residue of LIYV has the same role (Peng et al., 2001). Further studies showed that the leader proteinase of BYV is important for genome amplification and required for long-distance transport of the virus within plants (Peng and Dolja, 2000; Peng et al., 2003). ORF 1B encodes a putative protein that shows characteristic motifs of a RNA-dependent RNA polymerase (RdRp). It is believed that the RdRp domains of BYV and CTV are expressed directly from the genomic RNA itself via a +1 ribosomal frameshift event (Agranovsky et al., 1994; Dolja et al., 1994). Genome structure analysis, and alignment of the amino acid sequences of LIYV and BYV around the overlap region of ORFs 1A and 1B showed that the C-terminal portion of BYV ORF 1A aligned with the N-terminal portion of LIYV ORF 1B, and thus the potential frameshift sites in these two viruses are not homologous. Translation frameshift events are found for several plant viruses, but these are typically a −1 frameshift and these have been well studied (Dreher and Miller, 2006). However, mechanisms have been described for +1 frameshifting in retrotransposons and some other viruses. These appear to be much simpler than those for a −1 frameshift and need not be associated with distinct structural features (Farabaugh et al., 1993). Studies have indicated that in viruses that are in the family *Closteroviridae* this +1 frameshifting may occur at a conserved GUU_stop_C motif at the ORF 1 stop codon and this +1 slippage is likely to occur at the P-site from GUU to UUU with a stop codon in the A-site. However, even to this there is exception as in the case of CTV where the frameshifting occurs upstream of the ORF 1 stop codon and it has been precisely shown that the frameshifting occurs at the GUU_CGG_C sequence which aligns with the GUU_stop_C motif in other closteroviruses (Firth and Brierley, 2012). Other criniviruses show very similar organization for ORF 1A and 1B. Downstream of ORF 1B, LIYV RNA 1 contains ORF 2. The protein encoded by this ORF, P34, shows no similarity to any proteins in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov), but shows analogy in respect to its size and location to ORF 2 of CTV and BYSV (Dolja et al., 1994; Karasev et al., 1994) that belong to the genus *Closterovirus*. However, other criniviruses also encode proteins on RNA 1 downstream of ORF 1B, but these vary in size and possible function (**Figure 2**).

For example, *Sweet potato chlorotic stunt virus* (SPCSV) has been shown to encode a protein, P22, from the 3′ terminal ORF on RNA 1. P22 exhibits RNase III endonuclease activity and *in vitro* has been shown to cleave double-stranded small interfering RNAs (Cuellar et al., 2009). In plants P22 functions as an RNA silencing suppressor protein, even suppressing resistance against the unrelated potyvirus, *Sweet potato feathery mottle virus* (Cuellar et al., 2009). Interestingly, not all isolates of SPCSV contain an ORF encoding for P22 (Cuellar et al., 2008). Multiple suppressors of RNA silencing have also been shown to be encoded by *Tomato chlorosis virus* (ToCV). One of these, P22, is encoded by the ToCV RNA 1 3′ ORF, similarly to that seen for SPCSV (Canizares et al., 2008). And although the 3′ end of RNA 1 does not show nucleotide sequence homology to other criniviruses the fact that other 3′ end proteins in other criniviruses show silencing suppressor activity motivated us to test if P34 could be a potential silencing suppressor. However, so far we have no evidence indicating in 16c *Nicotiana benthamiana* assays suggesting that P34 could be a potential silencing suppressor.

Lettuce infectious yellows virus P34 is likely to be translated from a highly abundant subgenomic RNA, which is the most abundant LIYV-specific RNA found within LIYV-infected cells (Yeh et al., 2000). Subsequent studies have shown that P34 is an RNA-binding protein and plays an important role in the replication of LIYV RNA 2 (Yeh et al., 2000; Wang et al., 2010). Further evidence suggesting that P34 might play a role in LIYV RNA replication was shown by its localization to the endoplasmic reticulum (ER) and to the perinuclear envelope (Wang et al., 2010; **Figure 3**).

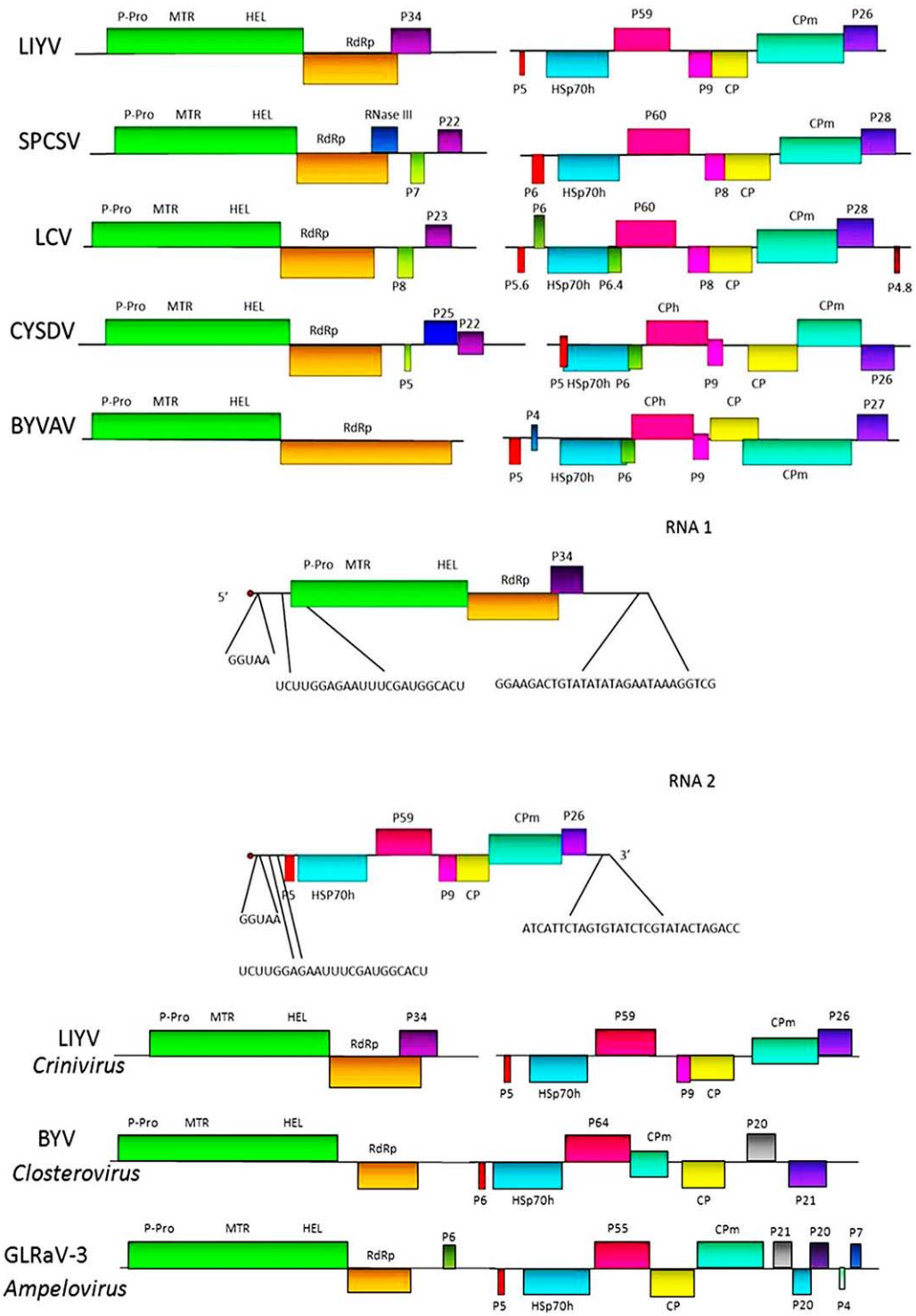

FIGURE 2 | Upper section shows genome maps for the bipartite genomic RNAs for five criniviruses. LIYV = *Lettuce infectious yellows virus*; SPCSV = *Sweet potato chlorotic stunt virus*; LCV = *Lettuce chlorosis virus*; CYSDV = *Cucurbit yellow stunting disorder virus*, and BYVAV = *Blackberry yellow vein associated virus*. Colored boxes indicate specific ORFs. P-Pro – papain-like protease; MTR = methyl-transferase; HEL = helicase; RdRp = RNA-dependent RNA polymerase; HSp70h = heat shock protein 70 homolog; CP and CPm = major and minor capsid proteins, respectively. Other ORFs labeled with P and a number indicate proteins and their approximate molecular mass (P26 = a 26 kDa protein). Middle section shows the comparative nucleotide sequences at the 5′ and 3′ terminal regions of the LIYV genomic RNAs 1 and 2. Lower section shows for comparison genomic maps of viruses in the two other genera of the family *Closteroviridae*: *Closterovirus* and *Ampelovirus*.

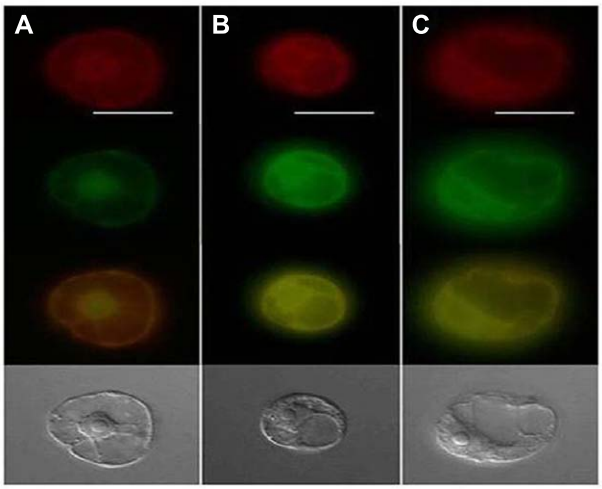

FIGURE 3 | Epifluorescence microscopy of endoplasmic reticulum (ER) and GFP localization where ER (red) on top panel, GFP (green) in second panel, merged green/red in third panel and the bottom panel displays the transmitted light images. Panel **(A)** shows TMV 30B-GFPc3 (GFPc3 is GFP where ER localization signal have been removed) inoculated cells while **(B)** shows cells that were inoculated with TMV GFP:P34, and **(C)** is cells inoculated with TMV P34:GFP. Image from Wang et al. (2010) with permission.

Lettuce infectious yellows virus RNA 2 is 7,193 nt, 5′ capped, not polyadenylated and does not encode proteins necessary for RNA replication. RNA 2 contains a 5′untranslated sequence of 326 nt. This sequence only shows limited homology with LIYV RNA 1 including the first five nucleotides (5′-GGUAA-3′) and a stretch of 23 nucleotides (5′-UCUUGGAGAAUUUCGAUGGCACU-3′). These 23 nucleotides in RNA 1 are from positions 83 to 105 and surround the first AUG which begins at nucleotide 99. However, in LIYV RNA 2 this 23 nt stretch is found upstream (at positions 121–143) of the first AUG start codon that is located at position 327(Klaassen et al., 1995; **Figure 2**). The significance of the common 23 nucleotide sequence is not known. It is important and worthwhile to note here, that LIYV RNA 1 and RNA 2 show no nucleotide sequence homology in their 3′-termini, unlike that found for most multipartite plant viruses and most of the other criniviruses sequenced so far. ORF 1 of LIYV RNA 2 encodes for a small protein of ∼5 kDa. This is the first ORF of the conserved closterovirus quintuple gene block, and the putative LIYV protein, as well as those for other closteroviruses is predicted to be highly hydrophobic and to form a transmembrane helix. ORF 2 encodes the Hsp70h. This is conserved among closteroviruses, and before many closterovirus genomes were sequenced, conservation among the specific motifs shared among heat shock 70 proteins was used to design degenerate oligonucleotide primers that proved to be very useful for generic closterovirus detection (Karasev et al., 1994; Tian et al., 1996). Interestingly, additional conservation between the Hsp70h-related proteins of closteroviruses was observed in the C-terminal regions indicating that these domains might be involved in protein–protein interactions, and may be important for chaperone activity (Klaassen et al., 1995). LIYV RNA 2 ORF 3 encodes a protein, P59, that shows significant similarities with the deduced amino acid sequences of CTV P61 (Pappu et al., 1994) and BYV P64 (Agranovsky et al., 1991; Klaassen et al., 1995). ORF 4 overlaps with ORF 3 and encodes a small protein, P9. LIYV ORF 4 is not part of the closterovirus quintuple gene block, but a similarly positioned ORF encoding for a similarly sized protein is conserved among criniviruses (Dolja et al., 2006; **Figure 2**), but no function has yet been determined for this protein. Amino acid alignment of crinivirus P9 homologs does not show significant amino acid similarity, but the predicted secondary structures of these proteins are very similar (Stewart et al., 2009a,b). ORF 5 encodes the ∼28 kDa LIYV coat protein (CP) which shows high similarity in sequence with coat protein sequences of BYV and CTV. The final quintuple gene block ORF, ORF 6 overlaps with ORF 5 and encodes a ∼52 kDa protein, the CPm (minor coat protein) that is predicted to be a diverged, duplicated copy of the CP. The C-terminal half of this protein contains the R, G, and D amino acid residues shown to be invariant in all filamentous virus coat proteins (Dolja et al., 1991). It is interesting to note that the order of the CP and CPm ORFs are the same for all criniviruses, but opposite to the order of the respective ORFs found for viruses in the genus *Closterovirus*. Furthermore, while the CPs are similar in size among most closteroviruses, the respective sizes of the CPm proteins differ among the viruses in some of the genera. For example, for viruses in the genus *Closterovirus* the CPm is ca. 24 kDa while in the genus *Crinivirus* the CPm is much larger, ranging in size from ca. 53 kDa for LIYV ro ca. 77 kDa for *Dioda vein chlorosis virus* (Tzanetakis et al., 2011). Within the genus *Ampelovirus* and proposed genus *Velarivirus*, the CPms vary in size (and possibly numbers) for various members. LIYV RNA 2 ORF 7 encodes a 26 kDa protein, P26. Similarly positioned ORFs encoding similarly sized proteins are found among all criniviruses, and while, like for P9, the amino acid sequences do not show significant similarity, their predicted secondary structures are similar (Stewart et al., 2009a). LIYV RNA 2 most likely serves as an mRNA only for P5. Subgenomic RNAs for LIYV RNA 2 ORFs 2–7 have been identified from infected plants and protoplasts (Rubio et al., 2002). The genomic RNA 2 components for other criniviruses are similar in overall organization to that of LIYV (**Figure 2**).

LIYV VIRIONS

Gaining the LIYV genome sequence information was a very important step for showing relationships of LIYV to other closteroviruses, and allowed for predicting potential roles of some LIYV-encoded proteins in LIYV infections. First, transmission electron microscopy (TEM) and immunogold labeling analyses confirmed that the LIYV virions, like those of BYV and CTV are morphologically polar (**Figure 4**). The CPm is localized to a short terminal region while the CP makes up the majority of the capsid. However, a surprising result was finding that the Hsp70h and P59 also are LIYV virion components suggesting even further complexity to LIYV and other closterovirus virions. This was first demonstrated when stringently purified LIYV virions were analyzed by SDS-PAGE and immunoblotting using antisera specific to four LIYV-encoded proteins: CP, CPm, P59, and to Hsp70h (Tian et al., 1999). However, TEM and immunogold labeling failed to allow for localizing the positions of P59 and Hsp70h on the virions (Tian et al., 1999). Later, elegant work with

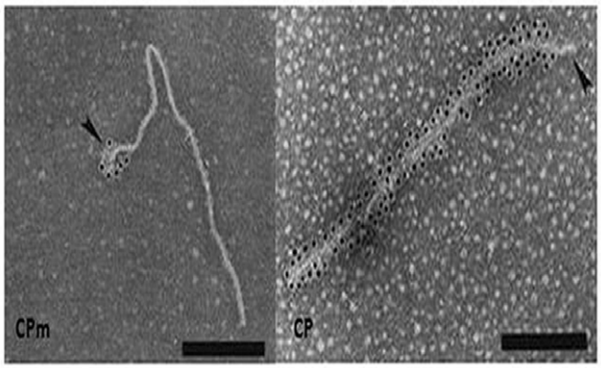

FIGURE 4 | Immunogold labeling of LIYV virions using antibody against CP and CPm. Gold labeling is indicated by the black dots, note that the CPm only encapsidates a short segment at one end of the virion while CP composes the remainder of the capsid. Image from Tian et al. (1999) with permission.

CTV and BYV suggested that the respective orthologous proteins form an interface on the capsid between the CP and CPm proteins (Peremyslov et al., 2004; Satyanarayana et al., 2004) This has not yet been demonstrated for any crinivirus.

Purified LIYV virions proved to be transmissible to plants by the whitefly, *Bemisia tabaci* biotype A (New World). This suggested that perhaps one or more of the four virion proteins might be a determinant of *Bemisia tabaci* transmissibility. Tian et al. (1999) used antisera to the four LIYV virion proteins to assess if they could interfere with, or neutralize, *in vitro* acquisition and subsequent transmission of LIYV by *Bemisia tabaci* biotype A to plants. Results from several experiments indicated that LIYV transmission efficiency was not affected by antisera to P59, CP, or Hsp70h, however, the CPm antiserum specifically and completely eliminated LIYV transmission by *Bemisia tabaci*. Only, when the CPm antiserum was diluted to 1% final concentrations was there incomplete neutralization of LIYV transmission by *Bemisia tabaci* biotype A (New World; Tian et al., 1999). This strongly suggested that the CPm had a primary role in LIYV transmissibility by *Bemisia tabaci* biotype A (New World). This has been supported by subsequent studies of Stewart et al. (2010), where they showed that deletion mutations in the LIYV CPm caused the loss of LIYV transmissibility by *Bemisia tabaci* biotype A (New World; Stewart et al., 2010), and elegant studies have recently demonstrated that the LIYV CPm specifically localizes and binds within the foregut of the vector whitefly, *Bemisia tabaci* biotype A (New World; Chen et al., 2011; and see Ng this volume). The studies of Stewart et al. (2010) also showed, however, that these same CPm deletions did not negatively affect systemic movement of the LIYV mutants in *N. benthamiana* plants. Interestingly, mutations in the three other virion proteins, CP, Hsp70h, and P59 resulted in the lack of the ability of these mutants to systemically infect *N. benthamiana* plants (Stewart et al., unpublished). These data suggest that LIYV virions lacking intact CPm can systemically invade *N. benthamiana* plants, but when any of the other three virion proteins are deleted, the ability to establish systemic infections was abolished.

LIYV REPLICATION

The LIYV virion RNA analysis and nucleotide sequence data strongly suggested that LIYV had a bipartite genome. Still, all other closteroviruses known at that time were monopartite. Thus infectivity data for the LIYV RNAs were needed in order to address how these RNAs are replicated, and how their replication/gene expression was regulated. Predictive analyses based on nucleotide and deduced amino acid sequences suggested that RNA 1 encoded replication proteins while RNA 2 encoded "other" proteins. The development of a reverse genetics system for LIYV (Klaassen et al., 1996) enabled the opportunity to answer these and other fundamental questions. Initial studies were done using protoplasts prepared from a *N. tabacum* suspension cell culture (Passmore et al., 1993; Sanger et al., 1994). These were inoculated with LIYV virions and virion RNAs but only showed accumulation of positive and negative-sense LIYV RNA 1. The failure to obtain RNA 2 replication was surprising but showed that LIYV RNA 1 alone was replication competent (Klaassen et al., 1995). Since *N. tabacum* is not a systemic host for LIYV, additional studies further assessed the replication of LIYV RNAs 1 and 2 in mesophyll protoplasts that were prepared from *N. benthamiana* plants, a plant which is known to serve as a systemic host for LIYV. These studies showed that LIYV RNAs 1 and 2 were replication competent in these protoplasts. Therefore, full-length cDNA copies of LIYV RNAs 1 and 2 were developed, cloned into plasmids and used to generate *in vitro* transcripts that very closely resembled authentic LIYV RNAs 1 and 2. The RNA 1 and 2 transcripts, separately and in combination, were then used to inoculate *N. benthamiana* protoplasts. Northern blot analysis of extracts from protoplasts confirmed that LIYV RNA 1 alone was replication competent, and when co-inoculated with RNA 1, RNA 2 also accumulated in protoplasts. The pattern and intensity of hybridization signals were indistinguishable from those obtained from protoplasts that were inoculated with purified LIYV virion RNAs. Furthermore, progeny LIYV virions were observed in protoplasts inoculated with both RNAs 1 and 2 (Klaassen et al., 1996). These data suggested that the replication of LIYV RNA 2 was dependent on RNA 1. However, there are several distinct features, which suggest that LIYV replication may differ from other viruses in the genus *Closterovirus*.

The separation of LIYV replication and non-replication associated genes onto the two LIYV genomic RNAs suggests that this could offer a means to regulate replication and gene expression. Indeed, subsequent careful, time course analyses showed that the LIYV genomic RNAs show asynchronous temporal accumulation and gene expression when both RNAs are simultaneously inoculated to protoplasts. LIYV RNA 1 genomic and subgenomic RNAs accumulate to high levels almost 24 h before significant accumulation of RNA 2 can be detected (Yeh et al., 2000). This suggested that there is a fundamental difference in the replication of the two LIYV genomic RNAs; LIYV RNA 1 is likely to be replicated in *cis* while RNA 2 replication is in *trans* (Yeh et al., 2000).

These results also raised the further questions as to how LIYV RNAs 1 and 2 interact and presumably utilize the same replication complex within infected cells. Unlike most multipartite ssRNA plant viruses, the LIYV genomic RNA 1 and RNA 2 have very little nucleotide sequence homology within their 3′ terminal regions. This also is in contrast to what has been found for other

criniviruses, which do show homology within their 3′ terminal regions. Could there be other proteins, besides ORF 1A and 1B that are required for LIYV RNA 2 replication and accumulation? Mutagenesis studies confirmed that LIYV RNA 2-encoded proteins do not affect RNA 1 and/or RNA 2 accumulation, but mutagenesis studies of the LIYV RNA 1 3′ end ORF encoding P34 gave an unexpected result. Although knockout mutations in this ORF did not affect the replication of LIYV RNA 1, they severely reduced the accumulation of LIYV RNA 2 (Yeh et al., 2000). These studies indicated that P34 is a *trans* enhancer of RNA 2 replication. Subsequent studies have shown additional properties for P34 further supporting a role in LIYV RNA 2 replication. Electrophoretic mobility shift competition assays using increasing concentrations of various unlabeled nucleic acids with fixed amounts of P34 and a ^{32}P-labeled LIYV RNA 2 defective RNA [M5, the smallest replication-competent LIYV defective RNA 2 (Yeh et al., 2000)] showed that while ssRNA unlabeled competitors efficiently displaced the labeled M5 RNA, double-stranded RNAs (dsRNA), ssDNA, and dsDNA competitors did not, indicating that P34 is a ssRNA-binding protein. Furthermore, topology algorithms predicted that P34 is a membrane-associated protein, and deletion analysis mapped the P34 RNA-binding domain to its C-terminal region (Wang et al., 2010). One hypothesis is that perhaps P34 may be involved in targeting RNA 2 to LIYV replication sites within cells, or somehow protects or helps facilitate RNA 2 replication. Intracellular localization studies using a P34: green fluorescent protein (GFP) fusion showed that P34 exhibits perinuclear localization and that it colocalizes with the ER (Wang et al., 2010; **Figure 3**). The RNA 1 3′ ORF complement of other criniviruses is extremely variable (Dolja et al., 2006; Martelli et al., 2012a) and P34 shows no significant sequence identity even to proteins that are encoded by similarly positioned ORFs of other criniviruses. Whole plant studies with *Sweet potato chlorotic stunt virus* suggested a possible temporal accumulation of the SPCSV genomic RNAs 1 and 2 (Kreuze et al., 2002) similar to that seen for LIYV, but recent studies with *Lettuce chlorosis virus* (LCV) did not show obvious temporal regulation/accumulation differences among LCV RNAs 1 and 2 (Salem et al., 2009). Thus additional studies are needed to understand how other crinivirus RNAs 1 and 2 are replicated and interact within cells.

Although it has been realized for many years that viruses utilize host membranes as scaffolds for replication (den Boon et al., 2010; Laliberte and Sanfacon, 2010; Diaz and Ahlquist, 2012), how closteroviruses use host membranes has received relatively limited study. It has long been observed that closterovirus infections in plants result in extensive proliferation of ER membranes giving rise to characteristic vesiculated membranous inclusion bodies. This was originally observed for BYV giving rise to the name of "BYV-type inclusion bodies" (Lesemann, 1988). Abundant vesiculated membranous BYV-type inclusion bodies also can be found in plants (primarily within companion cells) and protoplasts infected by LIYV, and interestingly these also form in protoplasts infected by only LIYV RNA 1. LIYV-infected cells also show accumulations of lipid droplets surrounding these inclusion bodies (Medina et al., 1998; **Figure 5**), and LIYV infection induces re-organization of the ER (Wang et al., 2010). Together, these observations suggest that LIYV requires intact membranes for RNA replication. This is further supported by our recent preliminary studies where the effects of two drugs, cerulenin and brefeldin A, were used to assess LIYV replication levels in *N. benthamiana* protoplasts (unpublished). Cerulenin inhibits *de novo* fatty acid and steroid biosynthesis. Cerulenin binds in equimolar ratios to β-keto-acyl-ACP synthase, thus blocking its interaction with malonyl-CoA thereby affecting fatty acid synthesis. In contrast, brefeldin A inhibits the transport of proteins from the ER to Golgi, and also induces retrograde protein transport from the Golgi to the ER (Klausner et al., 1992). We used fluorescence microscopy and northern hybridization analysis and found that while cerulenin greatly reduced LIYV infection and accumulation in protoplasts, we saw no detectable effects on TMV (*Tobacco mosaic virus*; **Table 1** and **Figure 6**-unpublished). In contrast, brefeldin A, although resulting in earlier cell death, showed no effects on LIYV, but showed a slight increase in TMV replication in protoplasts as measured by GFP fluorescence. The observations give additional support that LIYV replication depends on both ER-derived membrane recruitment and *de novo* biosynthesis of lipids.

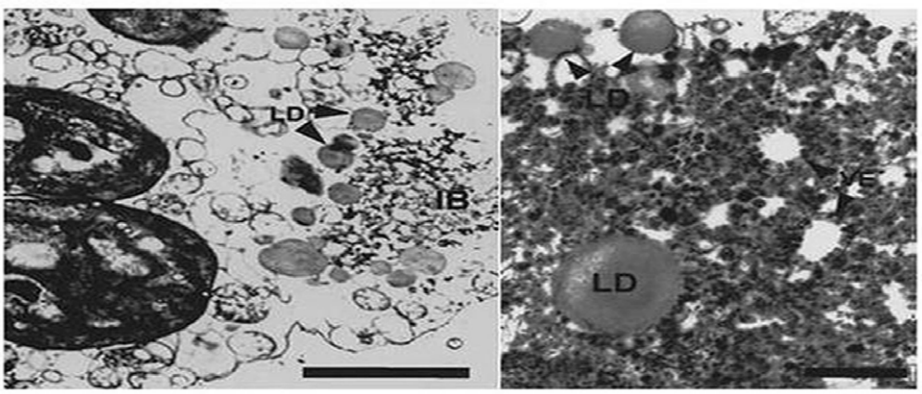

FIGURE 5 | Transmission electron microscopic (TEM) analysis shows lipid droplets (LD) that surround vesicles (VE) within the BYV-type inclusion bodies in LIYV RNA 1 and RNA2-infected *N. benthamiana* mesophyll protoplasts. Image from Medina et al., 1998 with permission.

Table 1 | Effects of cerulenin and brefeldin A on LIYV and TMV infectivity in *N. benthamiana* protoplasts.

	LIYV[1]	TMV[2]
Control	21.5 ± 8.7[3]	39.6 ± 1.3
Cerulenin 50 μM	0.43 ± 0.3	33.3 ± 9.6
Brefeldin A 10 μg	20.6 ± 8.7	53.5 ± 21.9

[1] *Cells were inoculated with transcripts for LIYV RNAs 1 and 2, plus the M5-GFP.*
[2] *Cells were inoculated with transcripts for TMV GFP 30B.*
[3] *Percentage of GFP fluorescent cells.*

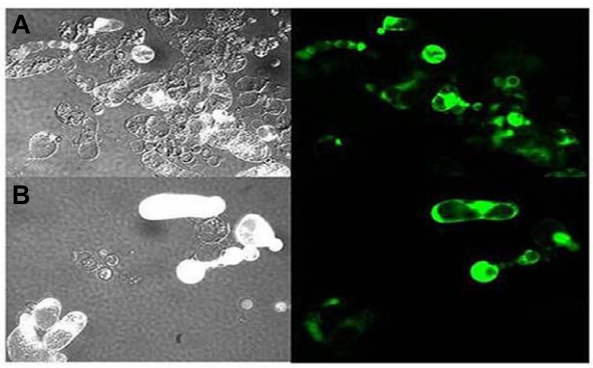

FIGURE 6 | Epifluorescent images on the morphology of protoplasts that are inoculated with transcripts for LIYV RNAs 1 and 2, and the M5 GFP defective RNA which is engineered to express the green fluorescent protein (GFP). Panel **(A)** shows control protoplasts (in bright field) inoculated with LIYV and M5 GFP and **(B)** shows fluorescence of protoplasts inoculated with LIYV and M5 GFP followed by cerulenin (50 uM) treatment. Cerulenin reduced LIYV infectivity (as assessed by protoplast fluorescence); however, in contrast, it did not have effect in TMV infectivity (see **Table 1**).

NON-VIRION PROTEINS

While much recent research has focused on studying the genomic RNA sequences, genome organization, and phylogenetic relationships for many newly discovered criniviruses, there are still many crinivirus-encoded proteins whose roles in infections have not been elucidated. For example, LIYV RNA 2 encodes for a protein at its 3′ terminus, P26. Similarly positioned genes encoding similarly sized proteins are found among all criniviruses sequenced to date. LIYV P26 is not a virion component (Tian et al., 1999) and microscopic studies have shown that P26 associates with, unique to LIYV, plasmalemma deposits (PLDs) (Medina et al., 2005; **Figures 7A,B**). Plasmalemma deposits were first described by Pinto et al. (1988) and Hoefert et al. (1988) in their extensive and beautiful electron microscopic studies of LIYV infection development in lettuce plants. They showed that the plasmalemma deposits were primarily found in companion cells, often in pit fields and adjacent to plasmodesmata connections to sieve elements. They noted that the plasmalemma deposits frequently had what appeared to be LIYV virus particles associated with them and these were oriented perpendicular to the plasmalemma. They also observed what appeared to be LIYV virions in adjacent sieve tube cells exiting plasmodesmata (**Figure 1F**) and speculated that the plasmalemma deposits might have roles in transporting LIYV virus particles from companion to sieve tube cells. Recent studies have shown that the plasmalemma deposits also are formed in LIYV-infected protoplasts, and they also contain aggregates of LIYV virions arranged perpendicular to the plasmalemma. "Sacks" of LIYV virions can also be found external to the plasmalemma of LIYV-infected protoplasts immediately adjacent to the plasmalemma deposits (**Figures 7A,B**). Moreover, further studies showed that P26 still associates to plasmalemma deposits when expressed from the heterologous TMV vector indicating that no other crinivirus protein is needed for this association. Hence, P26 association to the plasmalemma deposits is unique (Stewart et al., 2009b). Interestingly, interaction studies indicated that P26 is also capable of self-interaction (Stewart et al., 2009a). It is possible that P26 might be interacting with LIYV virion components due to its association with PLDs, or with other host factors to facilitate the movement of LIYV virions either within cells directing them to the cell periphery and/or through the plasmodesmata that connects the cells. To elucidate the functions of P26 has been quite challenging since it has shown to be non-essential for LIYV replication processes in protoplasts (Yeh et al., 2000). Whole plant

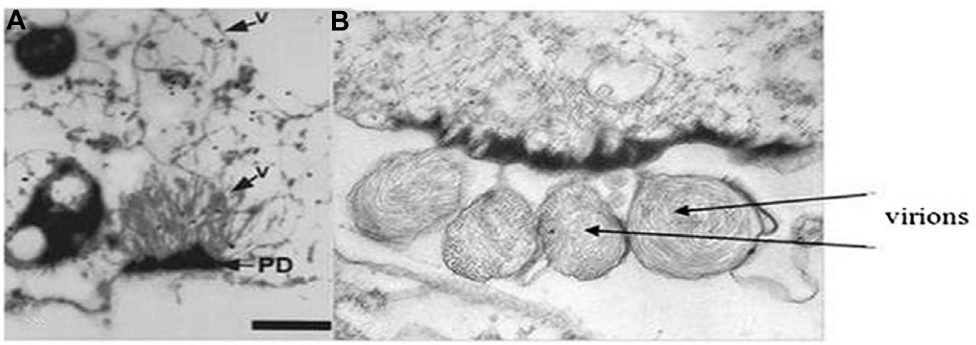

FIGURE 7 | Transmission electron micrographs showing (A) virions (see arrow and V) extending from the plasmalemma deposits (PD) and into the cytoplasm of a LIYV-infected *N. benthamiana* protoplast (image is from Medina et al., 1998 with permission). Panel **(B)** shows sacks of LIYV virus-like particles external to the plasmalemma, directly adjacent to abundant plasmalemma deposits in a single *N. benthamiana* protoplast.

infections of LIYV mutants derived from protoplasts has been difficult to achieve (Ng and Falk, 2006). More recent whole plant inoculations using *Agrobacterium tumefaciens* to deliver specific LIYV mutants to *N. benthamiana* plants (Grimsley et al., 1986; Wang et al., 2009) have shown in a few experiments so far that P26 mutants do not systemically infect *N. benthamiana* plants (unpublished), further supporting a role for LIYV P26 in systemic plant infection.

It is worthwhile to note that LIYV RNA2 encodes two other proteins. ORF 1 encodes a small hydrophobic protein, P5, with a transmembrane helix (Klaassen et al., 1995). The orthologous gene (protein) in BYV has been shown to play an important role in the cell-to-cell movement of BYV, and it has been indicated to be localized to the ER as a type III integral membrane protein (Alzhanova et al., 2001; Peremyslov et al., 2004; **Figure 2**).

Another small protein, P9, is also encoded on LIYV RNA 2. Although no functions have yet been assigned to this protein, a P9-like protein is predicted to be encoded by a similarly positioned ORF in all of the members of the genus *Crinivirus* sequenced so far (**Figure 2**). However, this protein shows high sequence variability among these viruses. Yeast two hybrid studies of P5 and P9 with each other and with the other LIYV RNA 2-encoded proteins showed that P9 is self-interacting (Stewart et al., 2009a). Whether these P9 protein homologs in other criniviruses also show self-interaction remains to be determined. Further studies are underway to determine roles of P9, and/or P5 in LIYV infections. Reverse-genetics systems have currently been established to elucidate the possible functions of these proteins in both *in planta* as well as via protoplast inoculation to investigate possible roles in replication. The presence of ORFs encoding these proteins in all crinivirus genomes sequenced so far indicate important roles and possible interactions with host factors, and thus, playing important roles in the virus life cycle.

CONCLUSIONS AND PERSPECTIVES

We have attempted to give an overall picture of what is known, and some things that remain to be studied for an understanding of crinivirus replication and host interactions. Although more and more criniviruses are being identified and their genomes are being sequenced, we are in need of more fundamental studies on their biology and molecular biology. LIYV has served well as a model crinivirus, but it is interesting to note that phylogenetically, LIYV is not closely related with the majority of the criniviruses. Studies with other criniviruses might give different information, or validate LIYV as a good model crinivirus.

Presently, excellent progress is being made in gaining a better understanding of crinivirus:whitefly interactions (Stewart et al., 2010; Chen et al., 2011; and see Ng this volume). This is important, and no doubt will lead to new fundamental information on the complex biology of criniviruses, but also could lead to novel strategies for controlling diseases caused by various criniviruses. By contrast, we still know too little about crinivirus:host plant interactions, and in particular how criniviruses move within the phloem. We have alluded to some ideas for LIYV *in planta* movement such as possible roles of plasmalemma deposits of LIYV P26, and these are based on biological studies as well as excellent electron and more recently light microscopic analyses of plants and even protoplasts (Hoefert et al., 1988; Pinto et al., 1988; Medina et al., 2005; Stewart et al., 2009b). Novel and important recent studies with GFP-tagged CTV have given new insights into how CTV multiplies and spreads within phloem cells of different citrus types (Folimonova et al., 2010), and such accomplishments with criniviruses are needed.

Also needed is a greater understanding of crinivirus replication and host interactions. Clearly, the temporal regulation seen for LIYV is intriguing, and all criniviruses have the same dilemma when virions are inoculated into a cell: how do the two genomic RNAs get together at the same intracellular location so both RNAs can express their genetic information and be replicated, ultimately to yield progeny virions? The identification almost 50 years ago of the membranous vesiculated "BYV-type inclusion bodies" (Lesemann, 1988) suggests a role for cellular membranes in at least BYV replication, and our studies with LIYV suggest this as well. Our recent demonstration of the negative effects of cerulenin on LIYV replication further supports the role of the endomembrane system for supporting LIYV replication. These studies could potentially lead to the development of novel control strategies against criniviruses that are current threats in world agriculture.

Despite their economic importance and widespread incidence in various host plants almost worldwide, reverse genetics systems are now available for only two criniviruses: LIYV and LCV (Wang et al., 2009; Chen et al., 2012). Both can be delivered to at least *N. benthamiana* plants via agroinoculation, but it can still be a challenge to efficiently deliver these phloem limited viruses to plants, particularly to hosts other than *N. benthamiana*, and improvements here would be very important. Current studies, particularly in herbaceous plants, require the use of the appropriate crinivirus whitefly vector for virus studies. This can be difficult to impossible in some locations (e.g., if the virus or whitefly vector is an exotic pathogen/pest) and hinders even opportunities to rapidly screen germplasm for virus resistance. Effective delivery to natural hosts of specific criniviruses by agroinoculation, or some other means not relying on the whitefly vector would yield great practical as well as fundamental benefits.

Although many criniviruses presently cause important diseases in many crop plants, successful strategies for their control are limited. In areas where vector populations are high, insecticides are often used but generally are ineffective in preventing crinivirus inoculation to susceptible plant hosts. It is interesting that no successful genetically engineered approaches capable of inducing RNA interference-based immunity are known for criniviruses. This is despite efforts at least with SPCSV in sweet potatoes (Kreuze et al., 2008). However; there have been some improvements with other members of the family *Closteroviridae*, such with CTV (Soler et al., 2012). Genetically engineered resistance against many different plant viruses will be a part of future disease control strategies, and a greater understanding of crinivirus replication and host plant interactions might allow for opportunities to effectively use such strategies to control diseases caused by criniviruses.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 February 2013; paper pending published: 22 February 2013; accepted: 06 April 2013; published online: 20 May 2013.

*Citation: Kiss ZA, Medina V and Falk BW (2013) Crinivirus replication and host interactions. Front. Microbiol. **4**:99. doi: 10.3389/fmicb.2013.00099*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

METHODS ARTICLE
published: 04 April 2013
doi: 10.3389/fmicb.2013.00077

A quantum dot-immunofluorescent labeling method to investigate the interactions between a crinivirus and its whitefly vector

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Successful vector-mediated plant virus transmission entails an intricate but poorly understood interplay of interactions among virus, vector, and plant. The complexity of interactions requires continually improving/evaluating tools and methods for investigating the determinants that are central to mediating virus transmission. A recent study using an organic fluorophore (Alexa Fluor)-based immunofluorescent localization assay demonstrated that specific retention of *Lettuce infectious yellows virus* (LIYV) virions in the anterior foregut or cibarium of its whitefly vector is required for virus transmission. Continuous exposure of organic fluorophore to high excitation light intensity can result in diminished or loss of signals, potentially confounding the identification of important interactions associated with virus transmission. This limitation can be circumvented by incorporation of photostable fluorescent nanocrystals, such as quantum dots (QDs), into the assay. We have developed and evaluated a QD-immunofluorescent labeling method for the *in vitro* and *in situ* localization of LIYV virions based on the recognition specificity of streptavidin-conjugated QD_{605} (S-QD_{605}) for biotin-conjugated anti-LIYV IgG (B-αIgG). IgG biotinylation was verified in a blot overlay assay by probing SDS-PAGE separated B-αIgG with S-QD_{605}. Immunoblot analyses of LIYV using B-αIgG and S-QD_{605} resulted in a virus detection limit comparable to that of DAS-ELISA. In membrane feeding experiments, QD signals were observed in the anterior foregut or cibarium of virion-fed whitefly vectors but absent in those of virion-fed whitefly non-vectors. Specific virion retention in whitefly vectors corresponded with successful virus transmission. A fluorescence photobleaching assay of viruliferous whiteflies fed B-αIgG and S-QD_{605} vs. those fed anti-LIYV IgG and Alexa Fluor 488-conjugated IgG revealed that QD signal was stable and deteriorated approx. seven- to eight-fold slower than that of Alexa Fluor.

Keywords: fluorescence imaging, photostability, cibarium, foregut, quantum dot, *Lettuce infectious yellows virus*, *Bemisia tabaci*

INTRODUCTION

Affiliates of the genus *Crinivirus* (family *Closteroviridae*) infect diverse plant species (Wisler et al., 1998), and share common features, such as an exclusive tropism for phloem tissues and formation of filamentous virions that are transmitted in a semi-persistent manner by specific phloem-feeding whiteflies of the *Bemisia tabaci* species complex (Brown et al., 2000; Ng and Falk, 2006b; Dinsdale et al., 2010). The biology of semi-persistent transmission is described based on the classical observation that: (1) virions are acquired by the vector within minutes to hours; (2) acquired virions are retained in the vector from hours to days but are lost when the vector molts; (3) virions need not circulate through the vector or invade its salivary glands and other internal organs in order to be transmitted (Nault, 1997; Ng and Perry, 2004; Ng and Falk, 2006b). Current studies (as described below) have further advanced the concept that retention of virions in specific sites within the insect vector is critical in assuring virus transmission.

Our contributions to the understanding of the whitefly transmission of criniviruses have focused primarily on studies of *Lettuce infectious yellows virus* (LIYV), the type species of *Crinivirus*. These studies have benefited from the use of membrane feeding, a procedure that allows insects with piercing and sucking mouthparts to ingest virion-augmented artificial liquid diet sandwiched between a pair of stretched parafilm. Results from these studies provided concrete evidence that the whitefly *B. tabaci* biotype A can acquire and transmit LIYV virions purified from various sources, including cesium sulfate-sucrose density gradient-purified virions prepared from infected plants, and partially purified virions prepared from tobacco protoplasts inoculated with either virion RNAs or *in vitro* transcripts produced from cloned cDNAs corresponding to the viral genomic RNAs (Tian et al., 1999; Ng et al., 2004; Ng and Falk, 2006a). Results from these studies also suggested that transmission determinants of LIYV reside on the virion itself (Tian et al., 1999; Ng et al., 2004; Ng and Falk, 2006a), which contrasts with the aphid transmission of *Cauliflower mosaic virus* (CaMV) and viruses in the genus *Potyvirus*, where additional viral encoded

proteins are needed to mediate virus transmission (Leh et al., 1999; Blanc et al., 2001; Pirone and Perry, 2002).

In a recent study in which immunofluorescent localization was used to analyze whiteflies that were sequentially fed LIYV virions, anti-LIYV virion IgG, and an organic fluorophore (Alexa Fluor 488)-conjugated goat anti-rabbit IgG, we found that upon uptake, LIYV virions were retained within the anterior foregut or cibarium of its specific vector *B. tabaci* biotype A, but not within that of the non-vector *B. tabaci* biotype B (Chen et al., 2011). Our study also demonstrated that specific virion retention in *B. tabaci* biotype A corresponded with the vector's ability to successfully transmit LIYV (Chen et al., 2011). These observations are consistent with the notion that during acquisition feeding, the cibarium, a region in the alimentary tract posterior to the food canal, functions as a sucking pump to drive ingested plant sap (along with virions present in the sap) into the anterior foregut, the pharynx, and the esophagus of the insect; viruses that have established an intimate relationship with their whitefly vectors have the propensity to retain in these specific regions, whereupon they are eventually let go (egested) to be delivered into a plant during inoculation feeding (Harris, 1977).

During the course of our study, we observed that prolonged exposure of organic fluorophore to high intensity excitation light could result in diminished or loss of fluorescent signals in whitefly samples, particularly in situations where interactions were accompanied by weak signals. Indeed, photobleaching susceptibility is an inherent limitation associated with organic fluorophore-based analyses that can hamper observations requiring continuous exposure to blue light (Chan et al., 2002; Liu et al., 2005). In contrast, light-emitting semiconductor nanocrystals such as quantum dots (QDs) are less vulnerable to photobleaching because of their superior photostability (Alivisatos, 2004; Gao et al., 2005; Pinaud et al., 2006). Furthermore, they exhibit enhanced signal sensitivity due to a larger absorption cross section, larger stokes shift, and narrower fluorescence emission spectra when compared to organic fluorescent dyes and fluorescent proteins (Michalet et al., 2005; Pinaud et al., 2006). An additional advantage is that they are suitable for the detection of low copy numbers of biological molecules, or when these molecules are not densely concentrated in one location (Pinaud et al., 2006). As such, QDs are becoming a preferred label for the fluorescence imaging of biological samples. For example, they have been used extensively in mammalian cell biology studies and applications ranged from immunofluorescent localization of membrane receptors to the imaging of trafficking of cellular components by bio-conjugated QDs (Dahan et al., 2003; Wu et al., 2003; Chen and Gerion, 2004; Derfus et al., 2004; Lidke et al., 2004, 2007; Medintz et al., 2005; Bouzigues et al., 2007).

The work described in this paper pertains to the development of a QD-based strategy to examine LIYV-whitefly vector interactions occurring in a highly dynamic and turbulent region of the whitefly's alimentary tract, where interacting components associated with virus transmission are constantly awash with an inflow and outflow of fluid. This study represents an inaugural demonstration of the effects of fluorescence photobleaching and the feasibility of the QD-labeling method as an improved system in the study of crinivirus-whitefly interaction. This system should also be applicable to the localization of other foregut-borne viruses that exhibit a similar mode of transmission as LIYV.

MATERIALS AND METHODS

IgG PREPARATION, BIOTIN LABELING AND ANALYSIS

Polyclonal antibodies produced against LIYV virions were purified by ammonium sulfate precipitation and dialyzed using a 10 K molecular weight cut off (MWCO) membrane (Thermo-Pierce, Rockford, IL, USA), followed by DE52 cellulose (Whatman, England) anion exchange chromatography according to the methods of Harlow and Lane (1988). Fractions of IgG eluate were collected and quantified by UV spectrophotometry, assuming that an optical density of 1.35 corresponds to 1 mg/ml of IgG (Harlow and Lane, 1988). The IgG fractions with the highest OD readings were pooled and stored at 4˚C until they were ready to be biotinylated with Sulfo-NHS-LC Biotin according to the manufacturer's instructions (Thermo-Pierce, Rockford, IL, USA). Briefly, a 10 mg/ml of stock solution of Sulfo-NHS-LC Biotin was made immediately before use. Biotinylation was performed by incubating purified IgG with the stock solution of biotin in 1 × PBS (phosphate buffered saline; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) on ice for 2 h, at a IgG:Sulfo-NHS-LC-Biotin molar ratio of 1:20. Excess biotin was removed by gel filtration chromatography using D-Salt™Dextran columns according to the manufacturer's instructions (Thermo-Pierce, Rockford, IL, USA). The collection and quantification of biotinylated IgG eluate were as described above.

Labeling of biotinylated-anti-LIYV virion IgG by streptavidin-conjugated QD_{605} (S-QD_{605}) (Invitrogen) was performed in a blot overlay assay. Briefly, Biotinylated-LIYV IgG (B-αIgG) was separated by electrophoresis in a 12% SDS-PAGE at 100 V for 1.5 h, and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with 20 nM S-QD_{605} at room temperature for 1 h and then rinsed three times in wash buffer [1 × PBS with 0.3% (v/v) Tween 20]. Fluorescence imaging of the nitrocellulose membrane was performed using the Typhoon™9410 Variable Mode Imager (GE Healthcare, Sunnyvale, CA, USA) set at an excitation and emission wavelength of 457 and 610 nm, respectively, and a photomultiplier tube (PMT) voltage of 400 V.

DOUBLE ANTIBODY SANDWICHED ENZYME-LINKED IMMUNOSORBENT ASSAY AND IMMUNOBLOT ANALYSIS

Double antibody sandwiched-enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) was used to determine the recognition and detection sensitivity of B-αIgG for purified LIYV virions. Hundred microliter of anti-LIYV serum diluted 1/500-fold in carbonate coating buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6) was introduced into designated wells of a 96-well Polysorp™microtiter plate (Nunc, USA). Hundred microliter of LIYV virions purified according to the methods of Tian et al. (1999), and diluted in sample buffer [1 × PBS with 0.05% (v/v) Tween 20, 2% (w/v) polyvinylpyrrolidone (PVP), pH 6] to concentrations ranging from 12 to 0.00012 ng/μl was added to each of the anti-LIYV serum coated wells. Following this, 100 μl of B-αIgG (approx. 0.6 mg/ml) diluted 1/500-fold in conjugate buffer [1 × PBS with 0.05% (v/v) Tween 20, 2% (w/v) PVP, 0.2% (w/v) bovine albumin serum (BSA), pH 7.4] was introduced into

each of the designated wells. The final step involved the addition of NeutrAvidin-conjugated alkaline phosphatase (Thermo-Pierce, Rockford, IL, USA) at a 1/1000-fold dilution (2 μg/ml final concentration) in 1 × TBS (Tris buffered saline; 25 mM Trisbase, 1.3 mM KCl, 135 mM NaCl, pH 8) with 0.05% (w/v) Tween 20, 2% (w/v) PVP, and 0.2% (w/v) BSA to each of the designated wells. Plate incubation for each of the above steps was performed at 37˚C for 2–3 h in a humid chamber. Plates were rinsed three times in wash buffer [1 × PBS with 0.05% (v/v) Tween 20] at the end of each step. After the final rinse, plates were added with 100 μl of 1-Step™p-nitrophenyl phosphate substrate (Thermo Scientific, USA) at room temperature for 60 min for color development. The absorbance at 405 nm was measured in a Wallac Victor II Multilabel counter (Perkin Elmer, USA).

Immunoblot analysis of purified LIYV virions was as described previously (Tian et al., 1999), except that following the transfer of proteins to nitrocellulose membrane, the blot was incubated with B-αIgG at a 1/500-fold dilution in blocking buffer, followed by S-QD_{605} (10 nM), before it was analyzed by fluorescence imaging as described above.

WHITEFLY TRANSMISSION OF LIYV VIRIONS AND IMMUNOFLUORESCENT LOCALIZATION ASSAY

Three solutions were used for membrane feeding by whitefly vectors (*B. tabaci* biotype A) or non-vectors (*B. tabaci* biotype B) of LIYV. Solution 1: artificial diet [1 × TE (0.01 M Tris/HCl, 1 mM EDTA, pH 7.4) supplemented with 15% (w/v) sucrose and 1% (w/v) BSA] or artificial diet augmented with purified LIYV virions at a final concentration of 400 ng/μl (Klaassen et al., 1994; Tian et al., 1999; Ng et al., 2004). Solution 2: artificial diet augmented with B-αIgG at 1/500-fold dilution or anti-LIYV virion IgG at 1/362-fold dilution. Solution 3: artificial diet augmented with S-QD_{605} (20 nm final concentration) for detection of B-αIgG or with Alexa Fluor 488-conjugated goat anti-rabbit IgG (at 1/200-fold dilution; 10 μg/ml final concentration) for detection of anti-LIYV virion IgG. The experimental unit was a cage containing approx. 100 whiteflies (*B. tabaci* biotype A or B) taken randomly from the respective whitefly colony. In studies using biotype A (two independent experiments), there were altogether 11 and three replicates (cages) of virion-fed and diet-fed whiteflies, respectively (**Table 2**). In studies using biotype B (three independent experiments), there were altogether 18 and six cages of virion-fed and diet-fed whiteflies, respectively (**Table 2**). Following the ingestion of solution 1, 50 whiteflies from each cage were transferred to a lettuce plant for a 24 h inoculation feeding period (IAP). Plants were treated with an insecticide before being moved to an insect-proof greenhouse for symptom development. The remaining whiteflies in each cage (to be used for immunofluorescent localization) were fed solution 2, followed subsequently by solution 3. Whiteflies were all given a 10–12 h acquisition access period (AAP) for each of the three solutions. Whiteflies used for immunofluorescent localization were subjected to clearing after the first and the third solutions by allowing them to feed on artificial diet for 10–12 h to remove unbound components. These whiteflies were killed by freezing in −20˚C and stored in this temperature until their heads were ready to be dissected for analysis. Whiteflies were dissected in deionized water (containing two drops of Tween 20 per 50 ml) on a microscope slide. Afterward, a cover slip was placed over the samples and sealed on all sides with ordinary nail polish, and the samples were observed by widefield fluorescence microscopy as described previously (Chen et al., 2011). A two-tailed Fisher's exact test (JMP; SAS Institute) was used to evaluate the differences in percentage of: (1) virion-fed and diet-fed whiteflies (biotypes A and B) that contained fluorescence signals in their anterior foregut or cibarium, and (2) LIYV transmission by virion-fed biotype A and virion-fed biotype B.

FLUORESCENCE PHOTOBLEACHING ASSAY

Heads dissected from whiteflies that were found by widefield fluorescence microscopy to contain fluorescent signals of Alexa Fluor 488 or QD 605 in the anterior foregut or cibarium were subjected to fluorescence photobleaching on a Leica SP5 confocal microscope, using a 20×/0.75NA water objective. The argon laser was set at 20% (for imaging) or 80% (for imaging and fluorescence photobleaching). The fluorescein isothiocyanate (FITC; 488 nm) argon laser line was set at 15% (for imaging) or 100% (for excitation and fluorescence photobleaching), and emission was collected between 500 and 530 nm (for Alexa Fluor signals), and between 603 and 608 nm (for QD signals). Images were acquired in xyt mode for 100 frames [1232 s (approx. 20 min)]. Fluorescence intensity values from three different regions of interest (ROIs), where QD or Alexa Fluor signals were detected, were collected over time (between $t = 0$ and 1232 s), and used to estimate the average fluorescence photobleaching rate using LAS AF software (Leica Microsystems).

RESULTS

BIOTINYLATION OF ANTI-LIYV IgG AND LABELING WITH STREPTAVIDIN-QD CONJUGATE

To obtain biotinylated IgG produced against LIYV virions, anti-LIYV IgG was first purified from polyclonal anti-LIYV antiserum using ammonium sulfate precipitation and DE52 (Whatman, England) anion exchange chromatography (data not shown). Purified IgG from the most concentrated fraction (approx. 2.3 mg/ml) was biotinylated at an IgG:biotin molar ratio of 1:20, and purified by gel filtration chromatography. The biotinylated IgG eluate was collected and quantified, and fraction containing the highest IgG concentration (approx. 0.6 mg/ml) was used for all subsequent analyses and manipulations.

A blot overlay assay was used to evaluate qualitatively the extent of biotinylation in the anti-LIYV IgG (B-αIgG) by probing with streptavidin-conjugated QD605 (S-QD_{605}) (Invitrogen). B-αIgG was separated by electrophoresis in a 12% SDS-PAGE, transferred to nitrocellulose membrane, and overlaid with 20 nM S-QD_{605}. Fluorescence analysis of the probed membrane revealed the presence of QD-labeled proteins of approx. 50 and 25 kDa, which corresponded with the molecular masses of the heavy- and light-chain polypeptides of IgG molecules, respectively (Harlow and Lane, 1988) (**Figure 1**).

EVALUATION OF THE RECOGNITION BETWEEN BIOTINYLATED-ANTI-LIYV IgG AND LIYV VIRIONS

IgG produced against LIYV virions routinely detects >1 ng of the LIYV major coat protein (CP) in immunoblot analysis (Ng et al., 2004). Thus, it was necessary and of interest to evaluate the virion detection sensitivity of B-αIgG to ensure that it

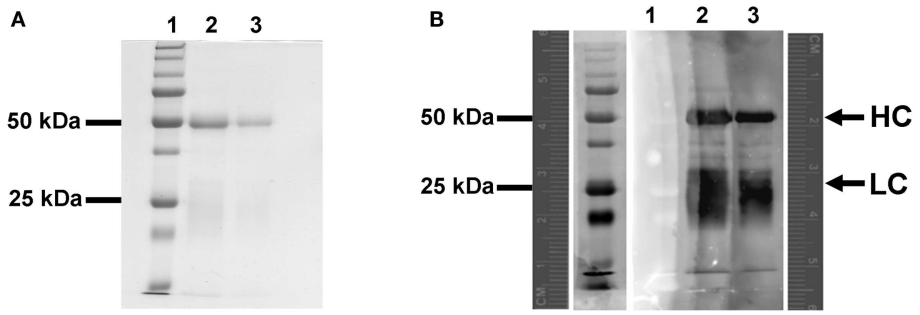

FIGURE 1 | Blot overlay assay of the interaction between biotinylated-anti-LIYV IgG and streptavidin quantum dot 605. (A) SDS-polyacrylamide gel electrophoresis separation and Colloidal Coomassie blue staining of biotinylated-anti-LIYV IgG (B-αIgG). **(B)** Fluorescent imaging of a nitrocellulose membrane blotted with SDS-PAGE separated B-αIgG and overlaid with 10 nM streptavidin-conjugated QD605 (S-QD_{605}). Lanes 1, low molecular weight prestained standards (the lane to the immediate left of lane 1 in **(B)** is an image of lane 1 captured under transmitted light); lanes 2 and 3, 3 and 1 μg of B-αIgG, respectively. A pair of fluorescence rulers is included in **(B)** to provide a reference for the migration distance of proteins detected under fluorescence and transmitted light. The positions of the 50 and 25 kDa prestained protein standards, and the heavy (HC) and light-chain (LC) polypeptides of B-αIgG are indicated.

Table 1 | Virion recognition specificity of biotinylated-anti-LIYV IgG in DAS-ELISA.

Virion concentration (ng/μl)	Absorbance values[a] at 405 nm		Signal/ noise
	Signal	Noise	
12	3.45	0.035	98.6
1.2	1.43	0.027	53.0
0.12	0.42	0.013	32.3
0.012	0.07	0.007	10.0
0.0012	0.02	0.008	2.5
0.00012	0.02	0.020	1.0

[a]*Average absorbance taken from wells incubated with LIYV virions (signal) and wells incubated with sample buffer (noise). Data are averages from three replicates. Reacting components in DAS-ELISA consisted of: coating antiserum (1:500 dilution), LIYV virions (at concentrations as indicated) or sample buffer, B-αIgG (1:500 dilution), and NeutrAvidin™alkaline phosphatase (1:1000 dilution). Absorbance readings were taken 60 min after addition of the substrate, p-nitro phenyl phosphate.*

was not affected by the biotinylation process. DAS-ELISA (see Materials and Methods for the details of the reacting components) was used to first ascertain the virion recognition specificity of B-αIgG (**Table 1**). The results revealed two findings: first, it further validated the success of IgG biotinylation, and provided clear evidence that B-αIgG was recognized by NeutrAvidin™by way of biotin-NeutrAvidin™non-covalent interaction, and that it also recognized and interacted with purified LIYV virions. Second, B-αIgG reacted positively with purified LIYV virions at concentrations ranging from 12 to 0.012 ng/μl (i.e., approx. 1200–1.2 ng per well in the microtiter plate) (**Table 1**), suggesting that its detection sensitivity was comparable to that of anti-LIYV IgG.

Immunoblot analysis was used to further characterize the specific recognition of purified LIYV virions by B-αIgG under denaturing conditions. Following separation of virion proteins by SDS-PAGE and transfer to nitrocellulose membrane, the blot was probed with B-αIgG, followed by incubation with 10 nM S-QD_{605} (**Figure 2**), and LIYV CP was detected by the direct fluorescence imaging of the blot (**Figure 2**). The results indicated that B-αIgG reacted positively with purified LIYV virions at concentrations ranging from 120 to 0.12 ng/μl (i.e., approx. 1200–12 ng) (**Figure 2**).

QD-IMMUNOFLUORESCENT LABELING AND WHITEFLY TRANSMISSION OF LIYV

An organic fluorophore (Alexa Fluor 488)-based protocol was developed recently for the immunofluorescent localization of LIYV virions within whitefly vectors (Chen et al., 2011). Results from that study revealed that a green fluorescent signal was seen in the anterior foregut or cibarium of the whitefly vector, *B. tabaci* biotype A, following virion acquisition, and this localized signal corresponded consistently with successful virus transmission (Chen et al., 2011). As our repertoire of analyzes using organic fluorophore-based imaging grew, it became apparent that the use of high intensity excitation light for the prolonged examination of interactions that accompanied low fluorescent signals, such as those involving the acquisition of low concentrations of virions or LIYV capsid proteins, accelerated the susceptibility of fluorescent signal decay. Therefore, the development of a high excitation light intensity tolerant method of analysis that could circumvent the drawback of rapid fluorescent signal decay was highly desirable. As such, we designed and conducted new experiments to test a QD-based strategy of virion localization using B-αIgG and S-QD_{605}. Here, we performed QD-immunofluorescent localization and whitefly transmission experiments to first determine if this approach gave reproducible results consistent with those observed using the Alexa Fluor 488-based protocol. Approx. 100 caged whitefly vectors (*B. tabaci* biotype A) or non-vectors (*B. tabaci* biotype B) were given two membrane feeding treatments–in the first treatment, whiteflies fed on a solution consisting of artificial diet augmented with 400 ng/μl of purified LIYV virions; in the second treatment, whiteflies fed on a solution consisting of artificial diet alone (i.e., no virions). Following acquisition feeding, half of the whiteflies in each treatment was transferred to non-infected

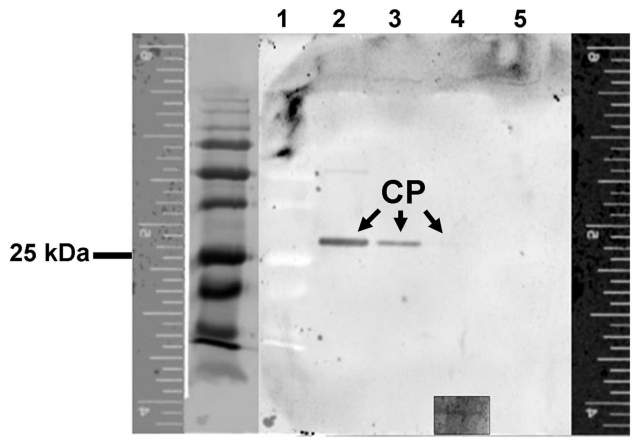

FIGURE 2 | Immunoblot and fluorescent imaging analysis to determine the recognition specificity between LIYV virions and biotinylated-anti-LIYV IgG *via* streptavidin quantum dot 605 binding. Lane 1, low molecular weight prestained standards (the lane to the immediate left of lane 1 is an image of lane 1 captured under transmitted light); lanes 2–5, LIYV virions (1200, 120, 12, and 1.2 ng, respectively). Fluorescence rulers are included to provide a reference for the migration distance of proteins detected under fluorescence and transmitted light. The positions of the major coat protein (CP) of LIYV are indicated. The inset at the bottom of lane 4 is an image of the position in the membrane at which the CP was detected at a higher PMT of 500 V. The position of a 25 kDa prestained protein standard is as indicated.

plants to determine virus transmissibility, while the remaining half of the whiteflies was fed artificial diet for several hours to flush out unbound virions (clearing). Afterward, the whiteflies were given sequential access to the following two solutions: artificial diet containing B-αIgG, and artificial diet containing S-QD_{605} (20 nM). After membrane feeding of the latter solution and clearing to flush out non-specifically bound antibodies, the heads of whiteflies were dissected and examined by widefield fluorescence microscopy (**Table 2**; **Figure 3**). In two independent experiments comparing vector whiteflies (Biotype A) fed on diet with or without virions, a red QD fluorescent signal was observed consistently in the anterior foregut or cibarium of 6–45% [at a combined total of 99 out of 376 (or approx. 26%)] of virion-fed vectors (**Table 2**; experiments 1 and 2; **Figure 3**), while signals were seen in 2–4% [at a combined total of 3 out of 131 (or approx. 2%)] of diet-fed vectors (**Table 2**; experiments 1 and 2). The difference in percentage of biotype A observed with QD fluorescence in the anterior foregut or cibarium in these two treatments was highly significant ($P < 0.0001$; Fisher's exact test). The corresponding LIYV transmission success by the other half of the virion-fed vectors and diet-fed vectors that were allowed inoculation feeding on lettuce plants was 7 out of 11 plants (or approx. 64%) and 0 out of 3 plants, respectively (**Table 2**). In contrast, QD fluorescent signal was seen in the anterior foregut or cibarium of only 8 out of 688 (or approx. 1%) of virion-fed non-vectors (biotype B) [i.e., 99% did not show QD signals (**Figure 3**)], and 1 out of 255 (or approx. 0.4%) of diet-fed non-vectors (**Table 2**). The difference in percentage of biotype B observed with QD fluorescence in the anterior foregut or cibarium in these two treatments was highly insignificant ($P = 0.4653$; Fisher's exact test). No corresponding LIYV transmission was observed in lettuce plants exposed to half of the virion-fed or diet-fed non-vector whiteflies (**Table 2**; experiments 3–5). The difference between the percentage of LIYV transmission by biotypes A and B was significant ($P = 0.002$; Fisher's exact test).

In all cases, no signal was observed in any other regions of the food canal (**Figure 3**) and mouthparts, including the distal end of the maxillary stylets (not shown). The red fluorescence seen in the eyes of both the virion-fed biotypes B (non-vector) and A (vector) was autofluorescence (**Figures 3A,B**), as similar fluorescence was also observed in the eyes of biotypes B (not shown) and A that did not feed on diet augmented with virions, B-αIgG, and S-QD_{605}. (**Figure 3C**).

These data demonstrated that the QD-based immunofluorescent localization approach is applicable to the study of LIYV-whitefly vector interaction. Consistent with our previous study (Chen et al., 2011), these results suggested that LIYV virions are retained in the anterior foregut or cibarium of the whitefly vector, *B. tabaci* biotype A, and that specific retention in these locations corresponded with *B. tabaci* biotype A-mediated transmission of the virus.

STABILITY OF QD-IMMUNOFLUORESCENT LABELING DETERMINED BY FLUORESCENCE PHOTOBLEACHING

We next determined if the QD signal observed in whitefly vectors could hold up to the rigors of continuous high intensity excitation light exposure better than Alexa Fluor signal. This objective was achieved by conducting fluorescence photobleaching experiments in parallel for viruliferous whiteflies fed B-αIgG, and S-QD_{605} vs. those fed anti-LIYV IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG. In these experiments, QD or Alexa Fluor fluorescent signal decay observed in the anterior foregut or cibarium of viruliferous whiteflies was measured for 1232 s (approx. 20 min), which facilitated the estimation of the photobleaching rate. We used the ROIs tool in the LAS AF software (Leica Microsystems) to define a 15 μm^2 circle, and used this defined area to mark three ROIs (not shown) in the anterior foregut or cibarium, where QD or Alexa Fluor fluorescent signals were observed. The signal intensity values (not shown) quantified in these ROIs were then used to estimate the rate of fluorescent signal decay over the 1232-s assay period. When the results were analyzed and compared, we noted a considerable difference in photobleaching rate between QD and Alexa Fluor fluorescence. In the representative analyses shown in **Figure 4A** and Movie S1A in Supplementary Material, QD fluorescent decay was not noticeable by visual inspection throughout the entire duration of the assay. In contrast, the decay of Alexa Fluor signal became visually discernible from $t = 350$ s to the end of the assay at $t = 1232$ s (**Figure 4B**; Movie S1B in Supplementary Material). An estimate of the average rate of photobleaching sampled from the marked ROIs was approx. 2×10^{-2}/s and 1.6×10^{-1}/s for QD and Alexa Fluor signals, respectively, i.e., about an eightfold difference. Taken together, these qualitative and quantitative data provided evidence that fluorescence emitted by QD within the anterior foregut or cibarium of viruliferous whitefly vectors was stable and deteriorated at a much slower rate compared to that of Alexa Fluor.

Table 2 | Correspondence between quantum dot signals in the anterior foregut or cibarium of *Bemisia tabaci* and successful LIYV transmission.

Experiment	*Bemisia tabaci* biotype A			Experiment	*Bemisia tabaci* biotype B		
	Virion-fed[a]	Diet-fed[b]	Transmission[c]		Virion-fed	Diet-fed	Transmission
1	4/28		−	3	1/25		−
	2/31		−		0/32		−
	10/36		+		0/28		−
	11/43		+		0/48		−
	10/42		+		1/42		−
		0/35	−		0/38		−
2	5/35		−			0/36	−
	13/31		−			1/34	−
	8/21		+	4	0/41		−
	13/33		+		0/30		−
	14/31		+		0/42		−
	9/45		+		1/40		−
		1/50	−		0/24		−
		2/46	−		0/32		−
						0/32	−
						0/43	−
				5	2/45		−
					2/45		−
					1/46		−
					0/43		−
					0/46		−
					0/41		−
						0/40	−
						0/40	−

[a] *LIYV virions were diluted to a concentration of approx. 400 ng/μl in artificial diet and presented to 100 whiteflies (B. tabaci biotypes A or B) for acquisition feeding, following which approx. 50 whiteflies were transferred to a target plant for inoculation feeding. The remaining (approx. 50) whiteflies were fed diet augmented with biotinylated-LIYV IgG and streptavidin-conjugated QD605. The heads of these whiteflies were excised and analyzed by widefield fluorescence microscopy. Fluorescence labeling was scored as the number of heads detected with QD signal in the foregut or cibarium over the total number of heads examined.*

[b] *The same treatment as above, except that whiteflies were fed artificial diet containing no virions.*

[c] *+ and − indicate infection and no infection, respectively, of target plants following inoculation feeding by whiteflies.*

DISCUSSION

For over 20 years, investigations on vector retention sites of foregut-borne, semi-persistently transmitted plant viruses have relied primarily on studies involving the transmission electron microscopy (TEM) analyses of serial thin sections of viruliferous insect vectors. For example, TEM analyses had shown that virions of several semi-persistently transmitted viruses (*Anthriscus yellows virus*, *Parsnip yellow fleck virus*, and *Maize chlorotic dwarf virus*), all unrelated to criniviruses, were bound to sites within the foregut of their respective insect vectors following virus acquisition (Murant et al., 1976; Childress and Harris, 1989; Ammar and Nault, 1991). One major disadvantage of the TEM approach is that it is tedious (involving long periods of labor), and requires a high level of skills and experience to perform, which could be the reasons underlying the hitherto limited number of reports in the literature. To overcome the challenges that are limiting our understanding of the processes occurring in the insect vector following virus uptake, we developed an Alexa Fluor-based immunofluorescent localization assay, the predecessor of the QD-immunofluorescent localization assay developed in the current study, and have used it to characterize the interactions between LIYV and its whitefly vector (Chen et al., 2011). Results obtained from the Alexa Fluor-protocol and the QD-protocol (discussed in detail below) have shown that both are innovative approaches and, as a whole, enabled us to test and prove the hypotheses that have already been alluded to in the preceding sections: i.e., that upon uptake, virions of LIYV are retained in specific sites in the anterior foregut or cibarium of the whitefly vector, and virions retained in these specific sites are positioned strategically to take advantage of the egestion process to facilitate their transmission into a plant during inoculation feeding. When higher concentrations (>100 ng/μl) of virions are used in membrane feeding experiments involving the Alexa Fluor-based protocol, continuous areas of strong fluorescent signals are typically found to occupy the anterior foregut or cibarium of whitefly vectors similar to the results shown in **Figure 4B**. However, in virion acquisition and transmission experiments that involve low virion concentrations (≤10 ng/μl), the signals may not be easily discernible. For example, we are currently conducting studies

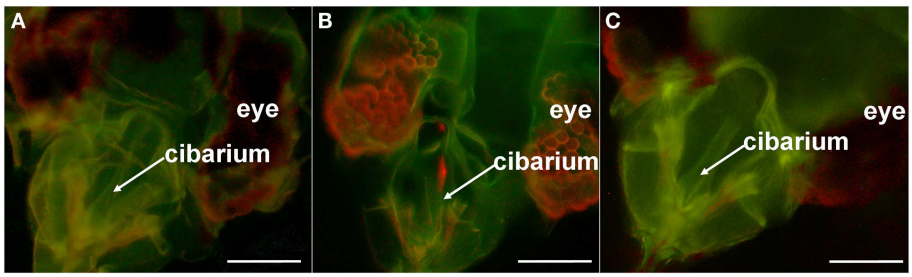

FIGURE 3 | Quantum dot (QD)-immunofluorescent localization of *Lettuce infectious yellows virus* in whitefly non-vector and whitefly vector by widefield fluorescence microscopy. The dissected heads of: **(A)** non-vector (*B. tabaci* biotype B) and **(B)** vector (*B. tabaci* biotype A) whiteflies following the sequential acquisition feeding of artificial diet containing LIYV virions, biotinylated-anti-LIYV IgG (B-αIgG), and streptavidin-conjugated QD605 (S-QD_{605}). **(C)** The dissected head of an unfed biotype A [i.e., it had not fed on artificial diet containing any of the components fed to the whiteflies in **(A,B)**]. Locations of the whitefly's anatomical features (eye and cibarium) are included as points of reference. Bars represent 45 μm.

to determine the range of virion concentrations that would support virion retention and transmission by whitefly vectors. At the lower end of the concentration range, weak signals prevail (Chen and Ng, unpublished data), which may require fluorophores to be exposed to excitation light for a longer duration and/or at a higher intensity in order for signals to be visualized unmistakably. Under such circumstances, fluorescent signals emitted from organic fluorophores would be susceptible to accelerated photobleaching, as has been demonstrated by our fluorescence photobleaching assay (**Figure 4B**; Movie S1B in Supplementary Material), thereby biasing the results in favor of no virion retention.

Our goal of the current study was to overcome the limitation of rapid fluorescence decay through the development of a QD-immunofluorescent localization system suitable for use with assays requiring continuous exposure of samples to high excitation light intensity. We began the study with preparative steps leading to the biotinylation of an anti-LIYV IgG. Successful biotinylation of this IgG, B-αIgG, was confirmed by testing its recognition of and affinity for virions, and streptavidin-conjugated QD605 (S-QD_{605}) or NeutrAvidin™-alkaline phosphatase using a combination of blot overlay assay (**Figure 1**), immunoblot detection (**Figure 2**), and functional detection by DAS-ELISA (**Table 1**). The virion detection limit of B-αIgG was between 12 and 1.2 ng, depending on the assay employed, and was comparable to that of non-biotinylated-LIYV IgG (Ng et al., 2004).

We then exploited the high affinity of biotin for streptavidin (Green, 1963) by using them as tools for the *in situ* visualization of virion retention within whitefly vectors (**Figures 3** and **4**). As discussed above, evidence has been presented here to demonstrate that the presence of QD signals in the anterior foregut or cibarium of viruliferous whitefly vectors corresponds to successful LIYV transmission (**Table 2**). The QD signals seen in these virion retention sites could not be due to the non-specific binding of B-αIgG and S-QD_{605}, especially since most vectors and non-vectors that fed on artificial diet alone (i.e., no virions) followed sequentially by artificial diet containing B-αIgG and S-QD_{605} did not contain signals in these locations (**Table 2**). Thus, data obtained using QD-immunofluorescent localization were consistent with those obtained using the Alexa Fluor-based protocol (Chen et al., 2011). As with the latter protocol, the QD-immunofluorescent localization assay also contains an inherent limitation. In order for a positive signal to be seen at the retention sites, the whitefly has to acquire all interacting components (i.e., virions, B-αIgG and S-QD_{605}) during membrane feeding. Thus, no signal will be observed in a whitefly that has acquired only one or two of the three components. Nonetheless, this approach has proven reliable in that a substantial number of virion-fed whiteflies clearly showed signals in their foreguts compared to fewer false positives seen in diet-fed vectors, as well as virion-fed or diet-fed non-vectors (**Table 2**). Given the inherent variability in the acquisition of the individual components, our estimate of 26% of biotype A that showed specific QD signals did not appear to deviate considerably from the 39% previously observed using the Alexa Fluor procedure (**Table 2**) (Chen et al., 2011). In the absence of virions (i.e., when whiteflies were fed only B-αIgG and S-QD_{605} in this study, or only anti-LIYV IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG in the previous study), the level of false positives, were also comparable, ranging from 2 to 4% and 0 to 2%, respectively (**Table 2**) (Chen et al., 2011). In the case of biotype B (the non-vector), the level of false positives remained consistent for both the QD and Alexa Fluor procedures whether whiteflies were fed all three components (false positive was 1% for both procedures), or only the second and third components (0.4 vs. 0%, respectively) (**Table 2**) (Chen et al., 2011). Recent studies aimed at understanding *Banana bunchy top virus* (BBTV) tropism within aphid vectors have used a biotin-streptavidin-Alexa Fluor-based immunofluorescent assay for the *in situ* localization of virus within the gut and salivary gland tissues of viruliferous aphids (Bressan and Watanabe, 2011; Watanabe and Bressan, 2013). The principle underlying this detection method and ours is similar in that both involve signal amplification. However, because the *in situ* BBTV localization approach is applied after insect tissues of viruliferous aphids have been dissected, and involves an extra reaction step (primary virus specific antibody, biotin-conjugated goat anti-rabbit antibody, followed by Alexa Fluor 488-conjugated streptavidin), it is unclear as to the utility of this approach in facilitating the *in situ* localization of LIYV and other foregut-borne viruses. Studies are currently being attempted to efficiently couple QD or other photostable nanocrystals and equivalents to virion specific IgG that could then be incorporated into the immunofluorescent localization assay to

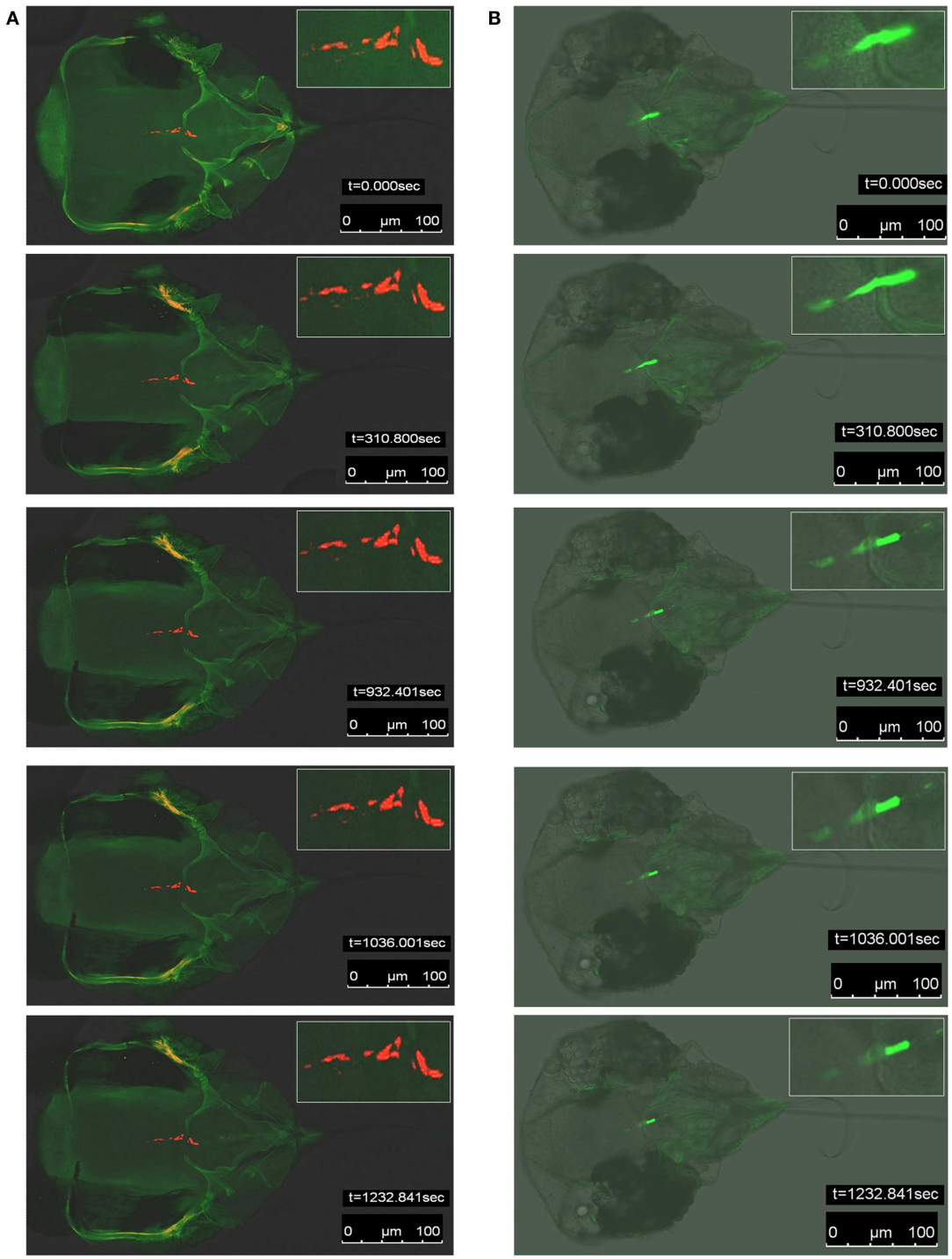

FIGURE 4 | Fluorescence photobleaching of specific quantum dot (QD) or Alexa Fluor signals in viruliferous whitefly (*B. tabaci* biotype A) vectors. **(A)** Snapshots of five representative confocal laser scanning microscopy (CLSM) images of fluorescence photobleaching in QD-immunofluorescent labeled (red signals) whitefly vectors fed artificial diet augmented with LIYV virions, biotinylated-anti-LIYV IgG (B-αIgG), and streptavidin-conjugated QD605 (S-QD_{605}). **(B)** Snapshots of five CLSM images of fluorescence photobleaching in Alexa Fluor 488-immunofluorescent labeled (green signals) whitefly vectors fed artificial diet augmented with LIYV virions, anti-LIYV virion IgG, and Alexa Fluor 488-conjugated goat anti-rabbit IgG. The Inset in each snapshot is an enlarged image of the region in which QD or Alexa Fluor signals were detected. The time intervals ($t = 0.000$, 310.800, 932.401, 1036.001, 1232.841 s) at which snapshots were taken and bars representing 100 µm are indicated in each snapshot.

cut down the number of steps in the assay to two, thereby streamlining and improving the versatility of the process. In addition, it would pave the way for the development of a system capable of labeling the virions of more than one virus concurrently; thus allowing us to address questions concerning the co-retention of multiple viruses.

One novelty of this work was the use of photobleaching to compare the stability of QD605 and Alexa Fluor 488 within the virion retention sites in the anterior foregut or cibarium of viruliferous whitefly vectors. Results from the study showed that the QD-immunofluorescent localization protocol provides sensitive labeling of LIYV virions in these retention sites, while exhibiting high fluorescence photostability over that of Alexa Fluor. A fundamental issue concerning the use of QD in immunostaining of tissues is the inherent size of the nanocrystals and hence potential difficulty of penetration and wash out. This does not apply in our case because the virion-antibody interaction sites are in an open lumen (the alimentary tract). Therefore, QD is as effective here as it is when used in surface labeling of cells for flow cytometry. Thus, due to the sensitivity of QD and its resistance to photobleaching, QD-based approaches should be particularly well-suited for localization studies of foregut-borne viruses involving the use of procedures that require prolonged exposure to high intensity excitation light, or when only low amounts of virions/viral encoded proteins are present.

ACKNOWLEDGMENTS

The author is grateful to Anita Pavitrin, David Carter, and Angel Chen for providing technical assistance. This work was supported by funds from the Los Alamos National Laboratory-University of California, Riverside Collaborative Program in Pathogen-Induced Plant Infectious Disease, and, in part, by a grant (1146797) from the National Science Foundation (to James C. K. Ng).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Virology/10.3389/fmicb.2013.00077/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 January 2013; paper pending published: 11 February 2013; accepted: 15 March 2013; published online: 04 April 2013.

Citation: Ng JCK (2013) A quantum dot-immunofluorescent labeling method to investigate the interactions between a crinivirus and its whitefly vector. Front. Microbiol. ***4****:77. doi: 10.3389/fmicb.2013.00077*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 16 May 2013
doi: 10.3389/fmicb.2013.00119

Epidemiology of criniviruses: an emerging problem in world agriculture

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The genus *Crinivirus* includes the whitefly-transmitted members of the family Closteroviridae. Whitefly-transmitted viruses have emerged as a major problem for world agriculture and are responsible for diseases that lead to losses measured in the billions of dollars annually. Criniviruses emerged as a major agricultural threat at the end of the twentieth century with the establishment and naturalization of their whitefly vectors, members of the genera *Trialeurodes* and *Bemisia*, in temperate climates around the globe. Several criniviruses cause significant diseases in single infections whereas others remain asymptomatic and only cause disease when found in mixed infections with other viruses. Characterization of the majority of criniviruses has been done in the last 20 years and this article provides a detailed review on the epidemiology of this important group of viruses.

Keywords: *Crinivirus*, Closteroviridae, whitefly, transmission, detection, control

INTRODUCTION

The genus *Crinivirus* is one of the three genera in the family Closteroviridae and includes viruses with segmented genomes, transmitted by whiteflies (Martelli et al., 2011). Details on the molecular biology of the criniviruses are presented in the Kiss et al.(2013) article and for the most part will not be duplicated in this communication. Instead this article will focus on virus epidemiology.

Criniviruses are emerging worldwide, with the first member of the genus, *Beet pseudo-yellows virus* (BPYV) identified in the 1960s (Duffus, 1965). Since then there has been a steady increase in the number of new species with most identified over the past 20 years (Winter et al., 1992; Celix et al., 1996; Duffus et al., 1996a,b; Liu et al., 1997; Wisler et al., 1998a; Salazar et al., 2000; Wisler and Duffus, 2001; Martin et al., 2004; Martín et al., 2008; Tzanetakis et al., 2004; Okuda et al., 2010).

Crinivirus genomic RNAs are encapsidated into long flexuous rods averaging between 650 and 1000 nm in length (Liu et al., 2000; Kreuze et al., 2002), and have large bipartite or tripartite genomes of positive-sense single-stranded RNA totaling approximately 15.3–17.7 kb. Genome organization is similar across the genus, but there are also apparent differences among species. RNA1 encodes proteins that are associated predominantly with replication, whereas RNA2 [or RNAs 2 and 3 for *Potato yellow vein virus* (PYVV)] encodes up to 10 proteins with a range of functions including but not limited to virus encapsidation, cell-to-cell movement, and vector transmission. Most genomic RNAs have common or highly conserved nucleotides at the 5′ end ranging from 4 to 11 nucleotides in length. The 3′ untranslated regions for each virus other than *Lettuce infectious yellows virus* (LIYV) share a region of approximately 150 nucleotides with a high degree of genetic conservation between the genomic RNAs.

Crinivirus transmission is species-specific and performed exclusively by whiteflies in the genera *Trialeurodes* and *Bemisia* in a semi-persistent manner; the reason they are identified with increasing frequency in tropical and subtropical climates where whitefly populations are present. They often cause symptoms that are readily mistaken for physiological or nutritional disorders or pesticide phytotoxicity. Typically, infection is associated with a loss of photosynthetic capability, often characterized by interveinal yellowing of leaves, leaf brittleness, reduced plant vigor, yield reductions, and early senescence, depending on the host plant affected. Some plants may exhibit an interveinal reddening rather than yellowing. Others may exhibit chlorotic mottle on some leaves, usually progressing into interveinal discoloration. Symptoms generally first appear 3–4 weeks after infection, and are most apparent on the older areas of the plant, whereas new

growth appears normal. For example, a tomato plant infected with a crinivirus may show extensive interveinal yellowing on leaves near the base, developing interveinal chlorosis on leaves in the middle of the plant, but no symptoms near the apex (**Figure 1**). Similarly, an infected cucumber plant may appear healthy near the growing point of the vines, but exhibit progressively more severe interveinal yellowing toward the crown (**Figure 1**). In both cases it is not uncommon for brittle, symptomatic leaves to snap when bent.

An interesting characteristic of many of the criniviruses studied to date is their ability to interact with other viruses in plants and alter symptoms. Studies have shown host-specific competition between crinivirus species that influence accumulation of other viruses present in the plant and consequently symptom severity (Karyeija et al., 2000; Susaimuthu et al., 2008; Wintermantel et al., 2008). Other viruses interact with distantly related or unrelated co-infecting viruses, resulting in increased disease severity whereas single crinivirus infections may remain asymptomatic (Karyeija et al., 2000; Tzanetakis et al., 2004, 2006b).

Management of criniviruses is predominantly through management of their whitefly vectors. Criniviruses routinely emerge in areas with regularly occurring or persistent whitefly populations, or as vector populations migrate or are moved to new regions. An effective vector control regimen can slow spread or reduce severity of infections; however, such methods will not prevent infection as most criniviruses can be transmitted within the relatively short acquisition and transmission periods of a few hours (Wisler and Duffus, 2001). Sources of host plant resistance have been identified to some criniviruses (McCreight, 1987, 2000; Lopez-Sesé and Gomez-Guillamon, 2000; Aguilar et al., 2006; Eid et al., 2006; Garcia-Cano et al., 2010; McCreight and Wintermantel, 2011) and efforts to identify additional sources are in progress. This may offer potential for effective control and reduced pesticide application as resistance is incorporated into commercial cultivars. Recent studies have also shown that deterrence may effectively reduce whitefly and subsequently virus pressure within fields. For example, acylsucrose expressed through type IV glandular trichomes on tomato have been shown to interfere with the ability of whiteflies to settle and feed steadily, and this can significantly reduce primary and secondary spread of the *Begomovirus*, *Tomato yellow leaf curl virus* (Rodriguez-Lopez et al., 2011, 2012). Although no conclusive studies have been completed with criniviruses, preliminary studies on tomatoes expressing acyl sugars demonstrated delayed *Tomato infectious chlorosis virus* (TICV) symptom development in the field by as much as a month compared with controls (Mutschler and Wintermantel, 2006).

In this communication we provide information on the recent advances in crinivirus epidemiology and associated diseases. Viruses will be presented according to their phylogenetic grouping (Wintermantel et al., 2009b; **Figure 2**) as members of each group tend to have similar vectors and host ranges (**Table 1**).

GROUP-1

ABUTILON YELLOWS VIRUS

Abutilon yellows virus (AYV) is a partially characterized crinivirus originally identified from the common weed velvetleaf (*Abutilon theophrasti* Medic.) collected from Illinois in 1977 (Liu et al., 1997). AYV has flexuous filamentous particles of 12 nm in diameter, approximately 850–900 nm in length (Liu et al., 1997, 2000) but the genome remains uncharacterized; with the exception of the

FIGURE 1 | (A) Symptoms of *Tomato infectious chlorosis virus* infection, showing interveinal yellowing on middle to lower portions of a tomato plant, while newer growth remains asymptomatic; **(B)** symptoms of mottling and interveinal chlorosis resulting from *Beet pseudo-yellows virus* infection of cucumber. Symptoms are prominent near the crown, less apparent near ends of vines.

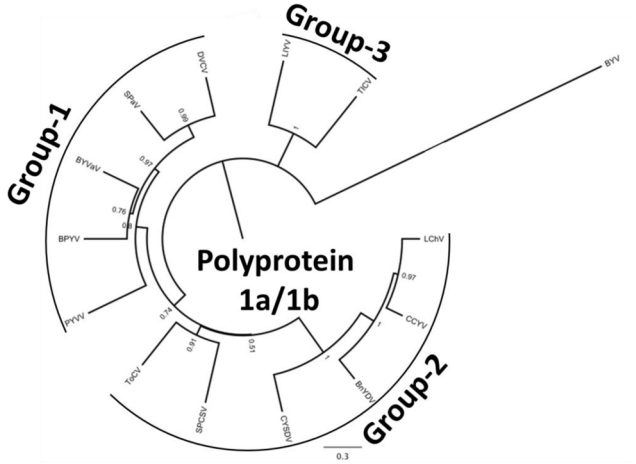

FIGURE 2 | Phylogenetic analysis of the genus *Crinivirus* based on the 1a/1b fusion polyprotein sequences. All protein sequences have been obtained from the GenBank genomic sequences of the respective virus. BnYDV, *Bean yellow disorder virus*; BYVaV, *Blackberry yellow vein associated virus*; BPYV, *Beet pseudo-yellows virus*; CCYV, *Cucurbit chlorotic yellows virus*; CYSDV, *Cucurbit yellow stunting disorder virus*; DVCV, *Diodia vein chlorosis virus*; LChV, *Lettuce infectious chlorosis virus*; LIYV, *Lettuce infectious yellows virus*; PYVV, *Potato yellow vein virus*; SPaV, *Strawberry pallidosis associated virus*; SPCSV, *Sweet potato chlorotic stunt virus*; ToCV, *Tomato chlorosis virus*; TICV, *Tomato infectious chlorosis virus*. *Beet yellows virus* (BYV) is used as the outgroup. The bar represents 0.3 amino acid changes/site.

Table 1 | *Crinivirus* species and their known vectors.

Virus	Whitefly vector					
	BtA	BtB	BtQ	Baf	Tvp	Tab
Abutilon yellows virus (AYV)						X
Beet pseudo-yellows virus (BPYV)					X	
Bean yellow disorder virus (BnYDV)			X			
Blackberry yellow vein associated virus (BYVaV)					X	X
Cucurbit chlorotic yellows virus (CCYV)		X	X			
Cucurbit yellow stunting disorder virus (CYSDV)	X	X	X			
Diodia vein chlorosis virus (DVCV)					X	X
Lettuce chlorosis virus (LCV)	X	X				
Lettuce infectious yellows virus (LIYV)	X					
Potato yellow vein virus (PYVV)					X	
Strawberry pallidosis associated virus (SPaV)					X	
Sweet potato chlorotic stunt virus (SPCSV)		X		X		
Tomato infectious chlorosis virus (TICV)					X	
Tomato chlorosis virus (ToCV)	X	X	X		X	X

BtA, Bemisia tabaci biotype A; BtB, B. tabaci biotype B; BtQ, B. tabaci biotype Q; Baf, Bemisia afer; Tab, T. abutilonea; Tvp, Trialeurodes vaporariorum.

coat protein and a region of the replication-associated polyprotein genes (Liu, unpublished data).

Abutilon yellows virus was the first crinivirus known to be transmitted exclusively by *T. abutilonea* Haldeman (banded-wing whitefly) but there is limited information on its host range and its geographic distribution. No crop plants have been identified as hosts; however, the virus can infect members of the Malvaceae, and the experimental solanaceous species, *Nicotiana clevelandii* A. Grey (Liu et al., 1997). AYV symptoms of foliar vein yellowing appear 2–3 weeks after inoculation on the malvaceous weed *Anoda abutiloides* A. Gray (Wisler and Duffus, 2001), symptoms that are highly unusual for criniviruses.

Like other members of the genus, AYV is not mechanically transmissible. To date, the only known vector of AYV remains *T. abutilonea*, and the virus can be retained by the whitefly for up to 3 days (Wisler and Duffus, 2001). Transmission efficiency varied from 4% for individual whiteflies to 81% for 50 whiteflies with acquisition access periods (AAP) of 24 h and inoculation access periods (IAP) of 48 h; whereas efficiency of virus acquisition varied from 19% for single whiteflies to 77% when 50 of the insects were used (Wisler and Duffus, 2001).

BEET PSEUDO-YELLOWS VIRUS

Beet pseudo-yellows virus was first described in 1965 (Duffus, 1965) from sugar beet grown in greenhouses for the sugar beet indexing programs in California and subsequently found to be worldwide in distribution wherever the vector, *T. vaporariorum* Westwood (greenhouse whitefly) is found (Wisler et al., 1998a). The range of *T. vaporariorum* has increased dramatically in recent years with the movement of plant material as has BPYV. Both virus and vector have become serious problems for greenhouse production of vegetables, fruits, and ornamentals worldwide. BPYV is transmitted very efficiently by its vector (Wisler et al., 1998a; Tzanetakis et al., 2006b), a property uncommon among criniviruses (Wintermantel, 2004). Additionally, once introduced into areas where *T. vaporariorum* does well outside the protected environment of greenhouses, the vector has often become naturalized and BPYV often becomes problematic in field-grown crops, as was the case in the western United States (Wintermantel, 2004). Another unique feature of BPYV is its broad host range infecting plants in at least 12 plant families including many vegetable, ornamental, and berry crops. Typical symptoms include interveinal chlorosis as leaves mature (**Figure 1**), reduced growth and fruit size, and early senescence in cucurbits (Wisler et al., 1998a). BPYV was first reported in a rosaceous host, strawberry in 2002 and is one of the criniviruses that can induce strawberry pallidosis disease in *Fragaria virginiana* Duchesne clones UC-10 and UC-11 (Tzanetakis et al., 2003). In California, where the vector has become naturalized, BPYV is now quite common in strawberry (Martin and Tzanetakis, 2013). It was also reported from blackberry in the southeastern United States in plants that exhibited symptoms of blackberry yellow vein disease (BYVD; Tzanetakis and Martin, 2004b). At present, BPYV is rare

in blackberry (Tzanetakis, unpublished). If the vector becomes naturalized in the southeastern United States, BPYV will likely become a greater problem in blackberry given that many weed hosts are present in and around blackberry fields in that region (Martin et al., 2013).

Two isolates of BPYV have been fully sequenced, the first from Japan (Hartono et al., 2003), originally named Cucumber yellows virus, that will be referred to as the cucumber isolate here, and a strawberry isolate from the United States (Tzanetakis and Martin, 2004a). The genome size ranges from 15.5 to 15.9 kb with features found in other members of genus; with two or three open reading frames (ORFs) in RNAs 1 and 7 or eight in RNA2. The differences between isolates is noteworthy; the first being a 147 nucleotide insertion after the methyltransferase domain in the replication-associated polyprotein of the strawberry isolate (Tzanetakis and Martin, 2004a). The nucleotide sequence identity of the two isolates before the insertion is 86%, whereas after the insertion the identity is elevated to 94% indicating a possible recombination event. Additionally, the cucumber isolate lacks an ORF at the 3′ end of RNA1 that is present in the strawberry isolate. There are also significant differences between the two BPYV isolates on RNA2. RNA2 of the cucumber isolate contains seven ORFs whereas the strawberry isolate has eight. The extra ORF in the strawberry isolate codes for a putative 6 kDa protein with counterparts in several other criniviruses. Based on criteria used to differentiate species in the genus *Crinivirus*, these two isolates of BPYV are clearly distinct strains of the same virus based on amino acid sequence identities of key gene products differing by less than 25% [RNA-dependent RNA polymerase, 98% identical; coat proteins, 99% identical; heat shock protein 70 homolog (HSP70h), 99% identical at the amino acid level; Martelli et al., 2011]. Still, the strawberry strain appears to be the dominant variant in the Americas and as noted affects a wide range of crop and weed hosts (Ramírez-Fonseca et al., 2008; Tzanetakis et al., unpublished).

Because BPYV symptoms are often confused with physiological and nutritional disorders it is likely that the impact and significance of the virus in vegetables and other crops has been underestimated. Additionally, since in most cases symptoms caused by BPYV are those of general plant stress it is important to do virus testing before taking corrective action. Given the great variability among strains, it may be more appropriate to use degenerate primers for virus detection (Wintermantel and Hladky, 2010) that will minimize the possibility of false negatives in testing. To date no sources of resistance have been identified against BPYV.

BLACKBERRY YELLOW VEIN ASSOCIATED VIRUS

Blackberry yellow vein disease was first observed in the North and South Carolina in 2000 and has since become the most important disease affecting blackberry production in the southeastern United States (Martin et al., 2013). Symptoms of BYVD only occur when blackberry plants are infected with more than one virus. Symptoms include vein yellowing, oak-leaf or irregular patterns of chlorosis, ringspots, and line patterns (**Figure 3**; Susaimuthu et al., 2007, 2008). Floricanes can also be severely affected leading to misshapen fruit and cane dieback. In the past, this disease was thought to be caused by *Tobacco ringspot virus* (TRSV) as this was the only virus that was mechanically transmissible from plants with such symptoms. This was questioned when blackberry plants were infected with TRSV using nematodes and infected plants did not develop symptoms over a 3-year period. The first virus characterized from blackberry plants that exhibited BYVD symptoms from South Carolina was a crinivirus, and named *Blackberry yellow vein associated virus* (BYVaV; Martin et al., 2004).

Blackberry yellow vein associated virus is a typical crinivirus with a bipartite genome. RNA1 is 7.8 kb in length and encodes only the replication-associated polyprotein whereas RNA2 is about 7.9 kb and contains the eight ORFs typical of other criniviruses. BYVaV RNA2 contains an additional ORF at the 5′ end that encodes for a second transmembrane protein, that is absent from RNA2 of other criniviruses (Tzanetakis et al., 2006a).

Once detection primers were developed it was observed that BYVaV could be detected in both symptomless and symptomatic plants of several blackberry cultivars, suggesting a complex etiology for BYVD. Since that time multiple viruses have been characterized from blackberry with BYVD symptoms and in all cases symptomatic plants had mixed virus infections (Martin et al., 2013). BYVaV is the most common virus found in plants that exhibit BYVD symptoms. BYVaV does not cause symptoms on the standard woody indicators used for graft indexing in *Rubus* certification programs, which explains its presence in nursery stocks prior to the development and application of PCR-based detection assays (Susaimuthu et al., 2007). Studies with several isolates of BYVaV from cultivated and wild blackberry from diverse geographic areas showed diversity at the nucleotide level as high as 12% and suggested that recombination between isolates is likely a factor in the evolution of the virus (Poudel et al., 2012). In addition, the study on virus diversity has resulted in the development of a set of detection primers based on conserved sequences from all isolates studied, whereas previous detection primers did not detect all of these isolates (Poudel et al., 2013).

Blackberry yellow vein associated virus can be transmitted efficiently from blackberry to blackberry with efficiencies of approximately 50% for *T. abutilonea* and 25% for *T. vaporariorum* when 50 whiteflies were used for inoculation following 18–24 h AAP and 48 h IAP (Poudel et al., 2013). BYVaV was not detected in any of 25 plant species that were common in or near blackberry fields with a high incidence of BYVaV infection. Even though BYVaV could be graft transmitted to rose, it was not detected in 40 samples of rose tested in native settings with high BYVaV pressure (Poudel et al., 2013) suggesting that wild rose likely is not an important component of the epidemiology of BYVaV. The virus has been detected throughout the southeastern United States, in California and Oklahoma and as far north as Illinois and West Virginia, but with surprisingly low incidence in Georgia and Florida. Overall, 145 of 234 samples of cultivated and native blackberries that exhibited BYVD symptoms tested were positive for BYVaV (Poudel et al., 2013). Given the complexity of BYVD there have not been efforts to introduce resistance for to BYVaV.

DIODIA VEIN CHLOROSIS VIRUS

Virginia buttonweed (*Diodia virginiana* L.) is a member of the Rubiaceae (coffee family). Its natural habitat is in wetlands of

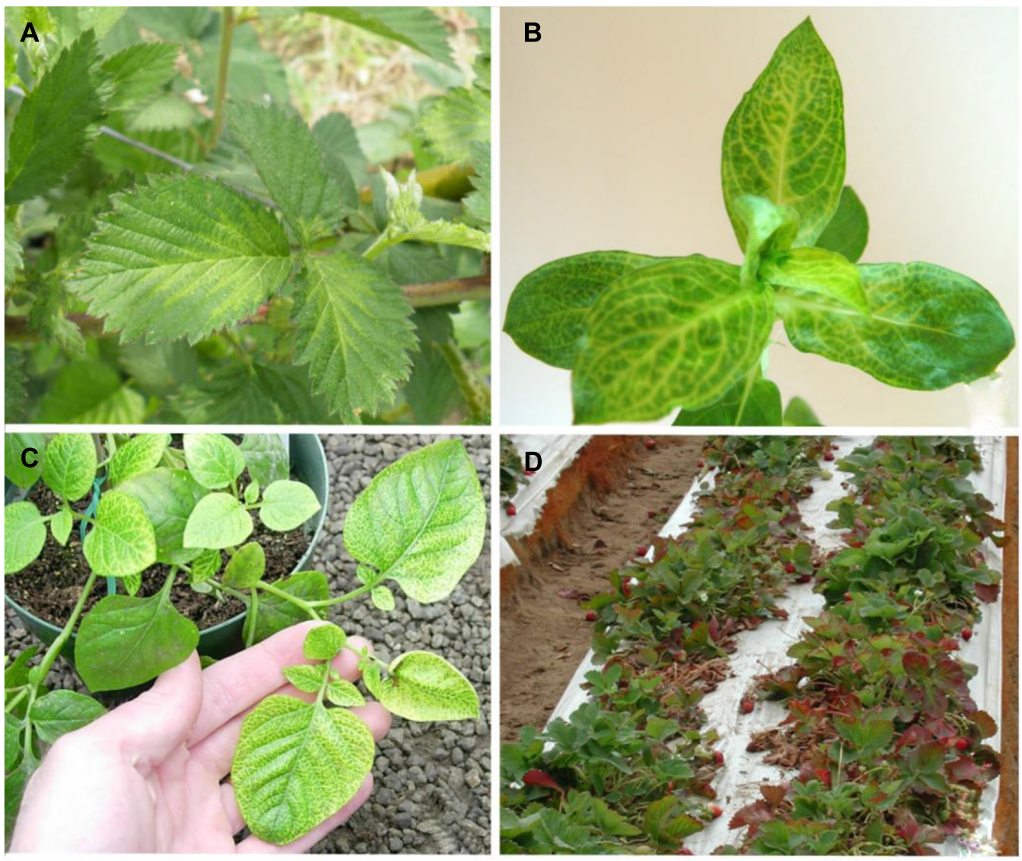

FIGURE 3 | (A) Blackberry infected with *Blackberry yellow vein associated virus* and *Blackberry chlorotic ringspot virus* showing symptoms of yellow vein disease; **(B)** *Diodia virginiana* infected with *Diodia vein chlorosis virus* showing vein netting symptoms; **(C)** potato infected with *Potato yellow vein virus* showing yellow vein disease symptoms; **(D)** strawberry decline symptoms of leaf reddening and dieback associated with *Beet pseudo-yellows virus* and *Strawberry pallidosis associated virus* co-infection with other viruses.

the Americas, extending between the 45th parallels of both continents. It propagates in a prolific manner through stolons and seed, making it one of the most noxious weeds of turfgrass. Several Virginia buttonweed populations in the southern United States show distinct vein chlorosis or vein netting symptoms, typical of virus infection (**Figure 3**). Larsen et al. (1991) studied the disease and discovered virus aggregates in infected material similar to those found in closterovirus-infected plants. The putative virus produced double-stranded RNA was similar in size to that of LIYV and *T. abutilonea* was experimentally verified as a vector. All these properties indicated that *Diodia vein chlorosis virus* (DVCV) is a member of the genus *Crinivirus* but no molecular information was available until recently when an isolate from a clone of a plant used in the Larsen et al.'s (1991) study was sequenced (Tzanetakis et al., 2011). DVCV genome is composed of 16.2 kb with RNA1 coding for the replication-associated polyprotein and RNA2 having the normal array of eight genes found in most members of the genus. Phylogenetic analysis clearly placed DVCV in group-1 of the genus. Given that all members of the group have been proven transmissible with *T. vaporariorum*, this was evaluated for DVCV. Indeed, both *T. abutilonea* and *T. vaporariorum* transmit the virus with efficiencies of over 36 and 12%, respectively when plants were inoculated with 50 whiteflies after 48-h AAP and IAP (Tzanetakis et al., 2011). The phylogenetic placement of DVCV, its vectors and the co-habitat of *D. virginiana* and berry crops resulted in a decision to conduct a series of experiments to determine the ability of the virus to infect strawberry and blackberry. Those experiments failed to identify additional hosts for DVCV other than *D. virginiana*. Given that the only known host for DVCV is a weed, control measures are not employed for this virus other than the elimination of Virginia buttonweed through the use of herbicides.

POTATO YELLOW VEIN VIRUS

Potato, a plant native to South America, is a host of several viruses. Many are asymptomatic in single infections, and as many cause devastating diseases that lead to major losses (Salazar, 2006). Plants affected by potato yellow vein disease can suffer losses reaching as much as 50%. Typical symptoms include vein yellowing that gives leaves the appearance of a yellow net (**Figure 3**). The disease was first identified in 1943 and has since been reported in Venezuela, Columbia, Ecuador, and Peru (Diazmore, 1963; Salazar, 2006). It was not until the turn of the century that the putative causal agent was identified and characterized (Salazar

et al., 2000). The agent was transmitted by *T. vaporariorum* and named PYVV. Virus purifications and cloning of the HSP70h gene of the virus indicated that is a member of the genus *Crinivirus* (Salazar et al., 2000). PYVV is a unique crinivirus as the only member of the genus with a tripartite genome. RNA1 is organized similarly to other criniviruses, encoding the replication-associated proteins and small peptide with a transmembrane domain. RNA2 encodes five proteins that are found in the 5′ terminus of the crinivirus orthologous molecule whereas RNA3 has three ORFs commonly found at the 3′ terminus of RNA2 in other crinivirus species, indicating that PYVV is probably a product of an ancestral virus segmentation in which the ancestral RNA2 segregated into PYVV RNAs 2 and 3 (Livieratos et al., 2004) although, in phylogenetic terms, it appears ancestral to the bipartite members of group-1 (**Figure 2**). The host range of the virus is rather restricted, and includes species in the genera *Solanum*, *Polygonum*, *Rumex*, *Tagetes*, *Catharanthus*, and *Malva* able to sustain virus replication whereas many common crinivirus indicators including *Nicotiana*, *Datura*, and *Physalis* species are resistant to infection (Salazar et al., 2000; Guzman and Rodriguez, 2010). The limited host range of the virus is reinforced by the fact that studied isolates present rather limited diversity (Offei et al., 2004; Guzmán et al., 2006; Rodriguez-Burgos et al., 2010). PYVV is closely associated with yellow vein disease symptoms but Koch's postulates have not been fulfilled as the virus can remain asymptomatic in potato. The importance of the disease, the ease of transmission, as recorded with the transmission of the virus in greenhouses in the UK, in combination with the asexual propagation and the cosmopolitan growth of the potato industry has made the development of advanced detection methods obligatory for the industry, and there are reports of sensitive detection protocols available (López et al., 2006). Virus control is based on insecticide use and strict quarantine directives that would not allow virus spread outside the countries where it is already present.

STRAWBERRY PALLIDOSIS ASSOCIATED VIRUS

Strawberries (family Rosaceae) are known to be natural hosts for about 30 viruses (Martin and Tzanetakis, 2006; Tzanetakis, 2010), several of which occur wherever the crop is grown and can cause significant losses (Spiegel and Martin, 1998). Pallidosis disease initially was identified in the United States during the 1950s (Frazier and Stubbs, 1969). Symptoms on indicator plants of *F. virginiana* clones "UC-10" or "UC-11," can include leaf distortion, chlorosis, and some dwarfing, though under less than optimal conditions for symptom development it is easy to overlook symptoms. Two viruses have been consistently associated with the disease, BPYV and *Strawberry pallidosis associated virus* (SPaV). Sequencing of the genome of SPaV confirmed it as a crinivirus (Tzanetakis et al., 2005). It contains two RNAs, both approximately 8 kb, with typical crinivirus genome organization. SPaV is most closely related to BPYV and AYV based on phylogenetic analysis (Tzanetakis et al., 2005).

There have been reports of severe strains of the pallidosis agents that are lethal on indicators. Graft transmission of multiple isolates from the eastern and western United States caused only mild symptoms and it is most likely that these "severe strains" actually represented mixed virus infections involving not only a crinivirus, but likely another partner virus (Hokanson et al., 2000; Tzanetakis et al., 2004).

Strawberry pallidosis associated virus is transmitted by *T. vaporariorum*, although somewhat inefficiently compared to BPYV (Tzanetakis et al., 2006b). Surprisingly, SPaV was more common in strawberry than BPYV in field settings. Both viruses were found in the majority of plants that exhibited decline symptoms due to mixed virus infections in California in the 2002–2003 periods (**Figure 3**). The decline epidemic was estimated to cause losses of about 50 million dollars for the two seasons (Martin and Tzanetakis, 2013). Plants were infected with at least one of the two criniviruses (BPYV or SPaV) and one of the common aphid-transmitted strawberry viruses (*Strawberry crinkle virus*, *Strawberry vein banding virus*, *Strawberry mottle virus*, or *Strawberry mild yellow edge virus*); incidence of SPaV was as high as 90% compared to 40% for BPYV. In plants from the Mid-Atlantic states that indexed positive for pallidosis disease based on symptoms, 37 of 38 plants were positive for SPaV and only about 25% were positive for BPYV (Tzanetakis et al., 2006b). Either virus can cause pallidosis symptoms in indicator plants. In other comparisons, SPaV was always more common in strawberry plants in side-by-side field comparisons than BPYV. This suggests that in nature there are other factors that contribute to virus transmission efficiency than what is typically measured in greenhouse or growth chamber studies. It is possible that the colony of whiteflies used in the greenhouse studies is better adapted to transmission of BPYV than SPaV or there are other, yet to be identified, vectors that are more efficient for transmission of SPaV. SPaV had a very limited host range in greenhouse studies, where it did not infect *Urtica urens* L., but was found in an *Urtica* species in the field in an area with high *T. vaporariorum* populations, though this could have been a different *Urtica* species (Tzanetakis et al., 2006b). The virus has been reported in strawberry production areas throughout the Americas, Australia, and Egypt (Wintermantel et al., 2006; Ragab et al., 2009; Constable et al., 2010; Martin and Tzanetakis, 2013). Both BPYV and SPaV are asymptomatic in single or mixed infections in "Hood" and "Noreaster" strawberry (Tzanetakis, 2004). Given the annual plasticulture that has been adapted in most production areas in the world it is imperative that plants do not become infected within the nursery system. Infections with the strawberry criniviruses may be asymptomatic but when plants accumulate additional viruses in the field, they can decline rapidly. The titer of SPaV declines in summertime and for this reason testing for this virus is recommended in spring or late fall using younger but fully expanded leaves (Tzanetakis et al., 2004). As in the case of BYVaV, the symptomless single infections and the complexity of disease-causing virus complexes have discouraged work toward identification of accessions which preclude virus replication.

GROUP-2

BEAN YELLOW DISORDER VIRUS

Legumes (family Fabaceae) are infected by numerous viruses, several of which cause significant losses with many regularly identified in new locations around the world (de Oliveira et al., 2011; Zhou et al., 2011). This was also the case of a disease observed in common bean (*Phaseolus vulgaris* L.) in Spain in

2003. Symptoms were similar to nutritional disorders with yellowing of the leaf blade, whereas pods appeared malformed. Leaves were brittle and whitefly transmission with *B. tabaci* Gennadius yielded reproducible symptoms. These observations pointed to a crinivirus infection. Confirmation came with the cloning of the HSP70h gene of the virus, which was named *Bean yellow disorder virus* (BnYDV; Segundo et al., 2004). An extended study in greenhouses in Spain, the only country the virus is known to exist, showed BnYDV incidence of about 6%, indicating that the virus was an emerging problem for bean growers (Segundo et al., 2008). BnYDV genome is 17.5 kb; encoding four proteins in RNA1 and nine in RNA2 (Martín et al., 2008). Phylogenetic analysis indicated the close relationship of BnYDV with vegetable-infecting criniviruses that are efficiently transmitted by *B. tabaci* (Martín et al., 2008). Transmission experiments revealed efficiencies that exceeded 35% using single whiteflies with 24 h AAP and IAP, respectively. A much more surprising result was the retention ability of *B. tabaci* which reached 2 weeks when most other criniviruses are retained for less than a week (Martín et al., 2011). More than 30 species belonging to the families Asteraceae, Boraginaceae, Cucurbitaceae, Fabaceae, Geraniaceae, Lamiaceae, Malvaceae, Scrophulariaceae, Solanaceae, Thymelaeaceae, and Verbenaceae were evaluated as hosts but only four legume species (*P. vulgaris* L., *Pisum sativum* L., *Lens culinaris* Medik., and *Vicia faba* L.) were able to sustain virus replication. Given the high incidence of the virus in greenhouses, control measures have primarily focused in these production systems. Beans grown in screenhouses had 14 times fewer whiteflies per plant. The incidence of the virus under screenhouse protection never exceeded 12.5% unlike that in conventional greenhouses which reached over 80% (Janssen et al., 2011). Given the incidence of the virus in the confined environment of a greenhouse, the physical barrier of fine mesh screenhouses appears to be the most efficient approach to minimize vector presence and associated virus transmission.

CUCURBIT CHLOROTIC YELLOWS VIRUS

Cucurbits are grown throughout the world and are exposed to a wide array of production environments and pests. These crops are known to be infected by more than 60 viruses (Lecoq and Desbiez, 2012), and several are discovered each year (Brown et al., 2011; Lecoq et al., 2011; Dong et al., 2012). Melon plants with severe yellowing symptoms in Kumamoto, Japan tested negative for known cucurbit viruses. Further research revealed that the disease agent was transmissible with *B. tabaci* biotypes B and Q whereas limited sequence data revealed that the agent shared similarities with criniviruses (Gyoutoku et al., 2009). The virus, now known as *Cucurbit chlorotic yellows virus* (CCYV), has a typical bipartite crinivirus genome, encoding four proteins in RNA1 and eight in RNA2 (Okuda et al., 2010). Phylogenetic analysis revealed the placement of CCYV into group-2. Okuda et al. (2010) studied the ability of the virus to replicate and move systemically in 19 additional hosts belonging to the families Asteraceae, Chenopodiaceae, Convolvulaceae, Cucurbitaceae, Fabaceae, and Solanaceae. The majority were shown to accommodate systemic movement, expanding the known CCYV host range. Since its first report in 2004, CCYV has spread to Taiwan, China, North Africa, and the Middle East, always found in association with severe disease outbreaks in cucurbits (Huang et al., 2010; Gu et al., 2011; Hamed et al., 2011; Abrahamian et al., 2012). Virus infection can significantly reduce crop characteristics in melon and watermelon, with significant brix reduction and yield losses that can reach a third of the crop when virus incidence is higher than 75% (Peng and Huang, 2011). Gyoutoku et al. (2009) have developed an efficient RT-PCR test for the virus but the widespread presence of the virus led to the need for high-throughput detection protocols. For this reason, Kubota et al. (2011) developed antibodies against the recombinant coat protein able to detect the virus using immunoelectron microscopy, tissue blot and ELISA. The importance of the virus and the significant yield losses have led to efforts toward identification of resistance in melon with five accessions from the Indian subcontinent exhibiting promising results (Okuda et al., 2013). Until resistance is incorporated into commercial cultivars, control will require insecticide treatment of whitefly-infested areas.

CUCURBIT YELLOW STUNTING DISORDER VIRUS

Cucurbit yellow stunting disorder virus (CYSDV) was initially discovered in the United Arab Emirates in 1982 (Hassan and Duffus, 1991). Virus particles range from 825 to 900 nm in length (Celix et al., 1996), and the two RNAs are 9.1 and 8 kb, with genome organization similar to other criniviruses.

Cucurbit yellow stunting disorder virus has been very successful in spreading from the Middle East to many cucurbit production regions throughout the world. Affected production regions include, in addition to the Middle East, the Mediterranean Basin including Lebanon, Israel, North Africa, and Southern Europe as well as the Canary Islands (Celix et al., 1996; Wisler et al., 1998a; Abou-Jawdah et al., 2000; Desbiez et al., 2000; Kao et al., 2000; Louro et al., 2000). The virus has recently become a significant production threat throughout cucurbit production regions in the southern United States, Mexico, and Central America. CYSDV is latent for up to 3 weeks but when symptoms develop they appear similar to those of other whitefly-transmitted viruses on cucurbits, with mottle symptoms early followed by extensive interveinal chlorosis (**Figure 4**). As with other criniviruses, symptoms are more prominent on older leaves with younger leaves remaining symptomless. CYSDV infections result in reduced plant vigor, and can significantly reduce fruit sugar production, resulting in poor tasting, unmarketable fruit.

The host range of CYSDV was originally believed to be restricted to members of the Cucurbitaceae (Celix et al., 1996); however, more recent studies have demonstrated CYSDV can infect plant species from at least nine families (Wintermantel et al., 2009a). Although cucurbits are the predominant and most significant agricultural hosts of the virus, common bean can be severely affected, resulting in severe stunting and virtual elimination of yield when infected at an early age. Lettuce is another host of the virus (Wisler et al., 1998a), and can be a reservoir for transmission to other crops, but symptoms are mild and agronomically insignificant (Wintermantel et al., 2009a). Numerous common weeds are also hosts of the virus, but in most cases these plants are symptomless and vary in their ability to serve as effective virus reservoirs for transmission to crop hosts (Wintermantel et al., 2009a).

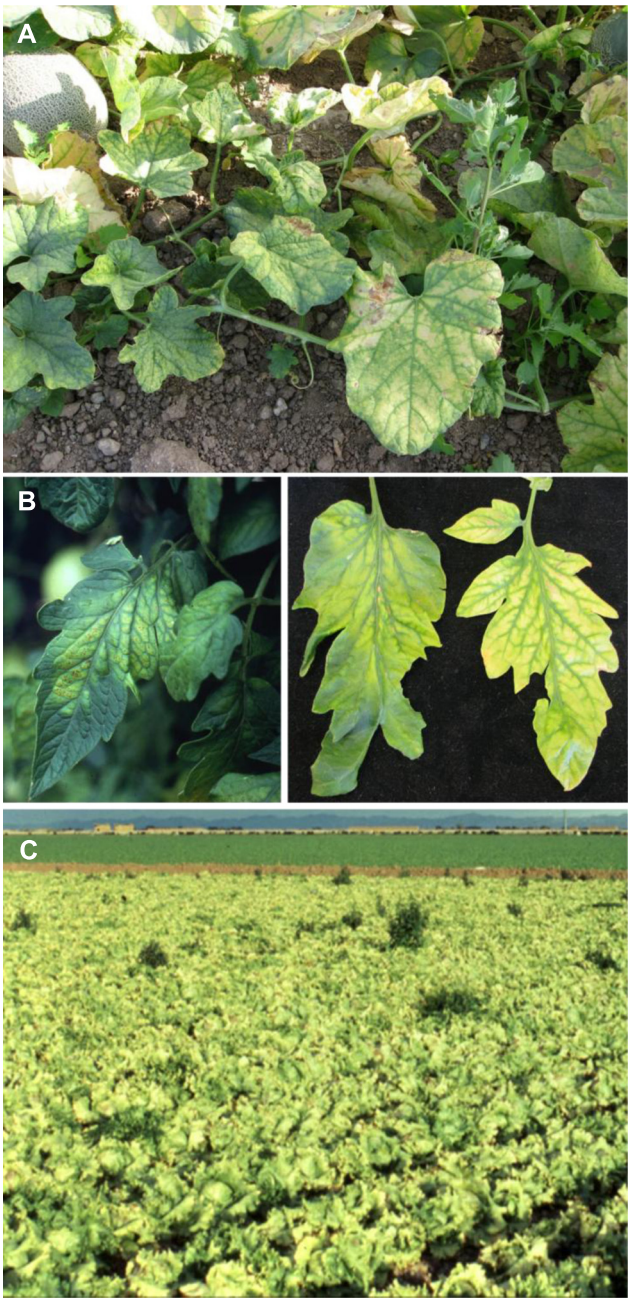

FIGURE 4 | (A) Severe interveinal chlorosis in melon caused by *Cucurbit yellow stunting disorder virus*; **(B)** typical symptoms of *Tomato chlorosis virus* (left) and *Tomato infectious chlorosis virus* (right) on tomato leaflets, illustrating the range of similar symptoms produced by both viruses; **(C)** lettuce field exhibiting classic yellowing symptom due to *Lettuce infectious yellows virus*.

CYSDV is transmitted very efficiently by at least three biotypes of *B. tabaci* A, B, and Q (Wisler et al., 1998a; Berdiales et al., 1999). The A biotype has become rare after its displacement from its native range in the American Southwest by the B biotype. Both B and Q biotypes are prevalent in many significant cucurbit production regions of the world, and are highly efficient in transmission. When vector populations are high it is virtually impossible to prevent infection of cucurbits. When CYSDV emerged in the American Southwest nearly all cucurbit production was affected during the first year due to the presence of excessively high vector populations.

Studies conducted on isolates collected over geographically distinct regions (Rubio et al., 2001) as well as local populations (Marco and Aranda, 2005), demonstrated most isolates are highly conserved genetically. Proteins show significant variation and among them the coat protein region seems to exhibit the most substantial variability, illustrating the divergence of a cluster of isolates from Saudi Arabia from other isolates identified from throughout the world (Rubio et al., 2001). Examination over time of a CYSDV collection from a localized region in Spain demonstrated an exceptionally high level of conservation within the virus population compared with other plant viruses (Marco and Aranda, 2005). It is speculated that genetic bottlenecks may influence the low genetic diversity within local populations. Similarly, genetic bottlenecks may also influence emergence of unique variants as observed for Arabian isolates (Marco and Aranda, 2005).

Management of CYSDV is predominantly through insecticide based vector control, which reduces vector numbers and results in slower rates of symptom development, but does not prevent virus transmission. Increasing efforts are focusing on development of virus resistance, particularly in cucumber and melon (Lopez-Sesé and Gomez-Guillamon, 2000; Marco et al., 2003; Aguilar et al., 2006; Eid et al., 2006; McCreight and Wintermantel, 2011), in which new sources of resistance to the virus have been identified in recent years. Efforts are progressing toward characterization of resistance in both hosts and toward combining resistance sources in melon.

LETTUCE CHLOROSIS VIRUS

Yellowing symptoms, normally associated with the crinivirus, LIYV, were observed in vegetable fields in the southwestern United States in the 1990s. At that point in time LIYV had virtually been eliminated following displacement of its primary vector, *B. tabaci* biotype A. This fact lead Duffus et al. (1996b) to investigate the possibility that other viruses might be present in the region, and ultimately to the discovery of *Lettuce chlorosis virus* (LChV). The virus is transmitted by *B. tabaci* biotypes A and B with similar efficiencies. Whiteflies can acquire and transmit the virus with AAP/IAP of 1 h each. Transmission was more efficient after 24 h of feeding whereas retention did not exceed 4 days. The host range includes at least 31 species belonging to 13 families, with several noteworthy hosts including spinach, sugar beet, and several weed species commonly found in the southwestern United States (Duffus et al., 1996b; McLain et al., 1998). The two genomic RNAs of the virus are contain in individual particles of 800–850 × 12 nm. The 17-kb genome is arranged similarly to that of other members of group-2, encoding four proteins in RNA1 and 10 in RNA2 (Salem et al., 2009). Insecticide applications can minimize virus incidence, something that is particularly important in early season where LChV can have a significant impact in lettuce yield (McLain et al., 1998). Infected lettuce can exhibit foliar yellowing, but also head deformation if infection occurs early. LChV has not spread

to areas outside the southwest United States and is not usually a significant production threat, probably as a result of lettuce-free periods and the inability of the virus to infect other significant crop hosts during the fall season when whitefly populations are elevated.

SWEET POTATO CHLOROTIC STUNT VIRUS

Sweet potato is one of the most nutritious vegetables, rich in vitamins and microelements and one of the most important staple foods available today in sub-Saharan Africa (Loebenstein and Thottappilly, 2009). Virus-like diseases of sweet potato have been reported for more than 50 years in Africa with several aphid-borne and whitefly-borne agents known to cause significant losses (Schaefers and Terry, 1976). Schaefers and Terry (1976) provided the first evidence that one of the components of the sweet potato virus disease (SPVD), the most important sweet potato disease in sub-Saharan Africa was whitefly-transmitted (Chavi et al., 1997; Gibson et al., 1998; Ateka et al., 2004). About 25 years later the virus, named *Sweet potato chlorotic stunt virus* (SPCSV) was partially characterized at the biochemical level (Winter et al., 1992) and a decade later was fully characterized at the molecular level (Kreuze et al., 2002). SPCSV is the crinivirus with largest genome identified to date with particles of 900–1000 nm in length and two genomic RNAs exceeding 17.6 kb (Winter et al., 1992; Kreuze et al., 2002). RNA1 encodes the replication-associated polyprotein and two or three additional genes depending on the isolate, similar to what is observed for BPYV (Cuellar et al., 2011a). RNA2 has similar architecture to most criniviruses with seven ORFs speculated to be involved in assembly and movement. SPCSV is transmitted by *B. tabaci*, *B. afer* sensu lato, and *T. vaporariorum* (Sim et al., 2000; Gamarra et al., 2010) and has spread to most areas where sweet potato is grown (Yun et al., 2002; Lozano et al., 2004; Abad et al., 2007; Qiao et al., 2011).

While SPCSV appears to exhibit minimal yield effects in single infections as is also the case for some of the other criniviruses presented here, it has a major effect when occurring together with *Sweet potato feathery mottle virus* or other potyviruses, resulting in SPVD. In a seminal paper by Karyeija et al. (2000) it was shown that co-infection of the two viruses leads to a 600-fold titer increase of the *Potyvirus* and subsequent development of SPVD symptoms. It was later shown that similar effects can be observed when the virus exists in mixed infections with viruses of other genera and families, further signifying the importance of the SPCSV in SPVDs (Untiveros et al., 2007; Cuellar et al., 2011b).

There have been several studies on the population structure of SPCSV (Alicai et al., 1999; Fenby et al., 2002; Tairo et al., 2005). There are distinct populations of the virus that show diversity in excess of 25% at the nucleotide level although there is less diversity at the amino acid level. Those studies have identified distinct virus populations, also reinforced by the variability in gene numbers between isolates (Cuellar et al., 2011a), indicating that SPCSV presents a polyphyletic evolutionary pattern.

Given the asymptomatic infection of SPCSV in single infections and its importance in SPVD sensitive cultivars, efficient detection protocols are important for testing propagation stock and minimizing virus movement to areas where the virus is absent. For this reason there are several reports of detection protocols for the virus, both immunological and molecular (Kokkinos and Clark, 2006; Opiyo et al., 2010). There has also been extensive work on identification of resistance for the viruses involved in SPVD using traditional and modern approaches with promising results (Karyeija et al., 1998; Mwanga et al., 2002; Kreuze et al., 2008; Miano et al., 2008). Still, the complexity of the disease and the apparent diversity of the virus make incorporation of viable resistance into commercial cultivars a challenging undertaking.

TOMATO CHLOROSIS VIRUS

Tomato chlorosis virus (ToCV) was originally identified in 1996 from greenhouse-grown tomatoes (*Lycopersicon esculentum* Mill.) from Florida (Wisler et al., 1998b), and exhibits a moderate host range of at least 24 plant species from seven different families (Wintermantel and Wisler, 2006). Symptoms on tomato include interveinal chlorosis, leaf brittleness, and limited necrotic flecking or leaf bronzing, and are nearly identical to those associated with infection by TICV (**Figure 4**), although genetically the two viruses vary significantly. Several methods are now available to differentiate ToCV from TICV, including RT-PCR (Wintermantel and Hladky, 2010; Papayiannis et al., 2011), molecular probes (Garcia-Cano et al., 2010), or virus-specific antiserum (Duffus et al., 1996; Jacquemond et al., 2009; Wintermantel, unpublished).

The 16.8 kb genome of ToCV is typical of criniviruses and is encapsidated as long flexuous virions approximately 800–850 nm in length (Liu et al., 2000). RNA1 encodes four ORFs including proteins associated with virus replication, and suppression of gene silencing (Wintermantel et al., 2005; Cañizares et al., 2008), and RNA2 encodes up to nine ORFs encoding proteins involved in a multitude of functions including virus encapsidation, cell-to-cell movement, membrane association, and whitefly transmission (Stewart et al., 2010; Chen et al., 2011).

The host range of ToCV extends, in addition to tomato, to other solanaceous hosts including pepper (Lozano et al., 2003), potato (Fortes et al., 2012), and tomatillo (Trenado et al., 2007). Several weed species can also harbor ToCV (Font et al., 2004; Wintermantel and Wisler, 2006), and the presence of weed hosts near production areas can provide an alternate host for the virus between cropping seasons, as well as providing an acquisition source for whitefly vectors that can carry the virus back to cultivated hosts.

Tomato chlorosis virus is unique among members of the genus as transmission by at least five different whiteflies has been documented (Navas-Castillo et al., 2000; Wintermantel and Wisler, 2006). The virus AAP is short, but transmission occurs more readily when vector whiteflies have IAP of several hours. Transmission efficiency varies among whitefly species, with *T. abutilonea* and *B. tabaci* biotype B, highly efficient vectors, yielding high rates of transmission, whereas *B. tabaci* biotype A and *T. vaporariorum* transmit ToCV with much lower efficiency (Wintermantel and Wisler, 2006). *B. tabaci* biotype Q is also an efficient vector, and has emerged as the predominant vector in southern Europe (Navas-Castillo et al., 2000). Each vector also differs in its ability to retain the virus, with *T. abutilonea* able to transmit for up to 5 days following virus acquisition, whereas *B. tabaci* biotype B loses its ability to transmit ToCV after 3 days.

B. tabaci biotype A and *T. vaporariorum* lose their transmissibility after only a day (Wintermantel and Wisler, 2006). ToCV has a relatively long latent period in infected host plants, often not inducing symptoms until 3 weeks after infection. If nursery plants are exposed to viruliferous vector populations at an early age, it is possible for ToCV-infected plants to be carried to new areas through movement of transplants prior to symptom development.

Management of ToCV is primarily through the management of vector populations using both chemical and cultural practices. Since criniviruses cannot spread without whitefly vectors, suppression of vector populations can keep crinivirus spread to a minimum. Although insecticides can reduce whitefly populations, such control methods are inefficient for virus control, since whiteflies can transmit viruses before being killed by an insecticide. In addition to vector control, it is important to limit availability of alternate host plants that can serve as virus reservoirs. Testing of nursery stock and ornamental host plants for the presence of these viruses can also reduce movement of ToCV to new areas. Importantly, resistance to ToCV was recently identified in crosses between *Solanum lycopersicum* (tomato) and *S. peruvianum* L., as well as *S. chilense* (Dunal) Reiche (Garcia-Cano et al., 2010). Introgression of this resistance into cultivated tomato should greatly strengthen future management of ToCV.

GROUP-3

LETTUCE INFECTIOUS YELLOWS VIRUS

Lettuce infectious yellows virus is the most thoroughly studied virus in the genus *Crinivirus*. It was discovered in the southwestern desert agricultural regions of the United States in 1981 (Duffus and Flock, 1982), and was the first crinivirus sequenced (Klaassen et al., 1995). Its 15.3 kb genome partially defined the characteristics of the genus.

Lettuce infectious yellows virus has a relatively large host range, infecting at least 45 species of plants in 15 families, and caused significant yield losses for lettuce, melon, and sugar beet. LIYV causes interveinal yellowing symptoms in melon and sugar beet, and a severe yellowing symptom on lettuce that gave the virus its name and resulted in widespread field yellowing (**Figure 4**). Unlike most other criniviruses affecting commercial agriculture, which have effectively been distributed around the world, LIYV remained predominantly confined to southwestern United States and northern Mexico. This is due to its close relationship with the *B. tabaci* biotype A, which shared a common geographical range with the virus (Brown and Nelson, 1986; Duffus et al., 1986). The virus persisted in the region throughout the 1980s, but quickly faded from prevalence with the emergence of the *B. tabaci* biotype B in the early 1990s (Cohen et al., 1992; Brown et al., 1995). As the B biotype became established, the A biotype gradually disappeared from fields, and along with it LIYV. Studies have shown a biological basis for this, with LIYV exhibiting over 100 times greater transmission using the *B. tabaci* biotype A than biotype B (Wisler and Duffus, 2001). LIYV has not been identified in the American Southwest for well over a decade, and although it is possible the virus may still exist in long-term reservoir hosts, the likelihood that it would reemerge is slim, since it is transmitted poorly by current *B. tabaci* biotypes, and the A biotype is no longer present in the field.

TOMATO INFECTIOUS CHLOROSIS VIRUS

Tomato infectious chlorosis virus was discovered in tomato from southern California in 1993 (Duffus et al., 1996a) and has since been identified as a problem for tomato production in many parts of the world including Mexico, Europe, the Middle East, as well as East and Southeast Asia (Wintermantel et al., 2009b). Symptoms on tomato include, similarly to ToCV, interveinal yellowing (**Figure 4**) with leaves becoming thickened and crispy, breaking easily when bent. Yield is affected through decreased fruit size and number (Wisler et al., 1996), as well as decreased plant longevity (Wintermantel, 2004).

Tomato infectious chlorosis virus virions consist of long flexuous rods varying from 850 to 900 nm in length (Liu et al., 2000) containing the two RNAs of about 8.3 and 7.9 kb. Similarity between TICV and other criniviruses varies throughout the genome but TICV is related much more closely to LIYV than to any other crinivirus, and together the two form a distinct clade within the genus (Wintermantel et al., 2009b).

The virus is transmitted exclusively by *T. vaporariorum* (Duffus et al., 1996a). TICV can be acquired and transmitted after a 1-h AAP; however, transmission efficiency increases steadily with longer AAPs. A 48-h AAP using 30 whiteflies per plant was most efficient and resulted in 94% transmission. Individual whiteflies given a 24-h AAP on infected source plants transmit TICV at an 8% rate; whereas an 83% transmission rate is found when plants are exposed to 40 viruliferous whiteflies each. Transmission by viruliferous whiteflies also varies over time with transmission using 30 viruliferous whiteflies per plant increasing from 16% transmission with 1 h transmission access periods to 80% when whiteflies are exposed to test plants for 48 h. TICV can persist in whiteflies for up to 4 days, but transmission efficiency drops off dramatically after 24 h (Duffus et al., 1996a).

Although tomato is considered the principal host of TICV, the virus also infects a number of important vegetable and ornamental host plants (Duffus et al., 1996a; Wisler et al., 1996). Lettuce, potato, petunia, artichoke, ranunculus, and China aster can also be infected by TICV. Like other criniviruses, TICV symptoms take up to 3 weeks to develop, and during this period movement of infected plant material by the nursery industry or by commercial vendors can be responsible for distribution of TICV to new regions (Wisler et al., 1998a). The virus can survive during non-crop seasons in a wide range of weed hosts near production areas and move into crops as whitefly populations develop and become active. Similarly, some ornamentals or alternate crops such as lettuce can serve as reservoirs for virus transmission to tomato (Duffus et al., 1996a; Wisler et al., 1998a; Font et al., 2004).

Management of TICV, like other criniviruses, involves both chemical and cultural practices. Since criniviruses cannot spread without whitefly vectors, suppression of vector populations can keep crinivirus spread to a minimum. In addition to vector control, it is important to limit availability of alternate host plants that can serve as virus reservoirs. Although insecticides can reduce whitefly populations, such control methods are inefficient for virus control, since whiteflies can transmit viruses before being killed

by an insecticide. Resistance to TICV is not available in cultivated tomato; however, preliminary studies have indicated resistance to whitefly feeding can slow TICV disease progress in cultivated tomato (Mutschler and Wintermantel, 2006).

DISCUSSION

Closteroviruses cause diseases of great economic importance. *Citrus tristeza virus* has changed the map of citrus production around the world and the Grapevine leafroll associated viruses have had a major impact on vine health and wine quality, both affecting multi-billion dollar industries worldwide. Criniviruses have recently emerged as major pathogens in world agriculture, primarily because of the movement and establishment of their whitefly vectors in temperate regions around the world.

There are clear cases in which criniviruses are the causal agents of devastating diseases such as CYSDV and BPYV in cucurbits or TICV and ToCV in tomato. In addition, there are many cases in which criniviruses have been the underlying problem behind major epidemics even though they were not originally recognized as such. The examples of SPVD, strawberry decline, and BYVD illustrate how criniviruses can be asymptomatic in single infections and yet cause serious diseases in the presence of virus complexes with major impacts on plant health and yield. Furthermore, even criniviruses normally regarded as symptomatic can be asymptomatic in some hosts. Most members of the genus also require a minimum of 3 weeks for symptoms to become apparent. During this time infected plants can be moved to new areas or even new countries without evidence of infection. This fact has major implications at many levels; especially for viruses infecting clonally propagated crops (BPYV, BYVaV, PYVV, SPaV, and SPCSV) or crops associated with grafted transplants (CYSDV and CCYV). In today's global trading environment there is constant germplasm exchange among individuals and organizations. The previous examples of crinivirus-driven epidemics should become lessons for the future and provide the impetus to improve plant certification schemes. This will facilitate increasing international trade in plant and plant products while decreasing the unintentional movement of plant pathogens. Given that some of the aforementioned viruses remain confined in specific geographic areas (i.e., BYVaV in the United States, PYVV in northwestern South America) it is still feasible to minimize their future impact by eliminating movement of infected material into areas where these viruses are not present. It is also important to establish vector exclusion strategies at the nursery or propagation field level. It has been common practice in certification schemes that plants are only visually inspected at the certified plant (G4) level. Using strawberry or blackberry as an example, neither BPYV, BYVaV nor SPaV cause symptoms in single infections in modern berry cultivars. However, when singly infected plants are planted in the field they often become infected with additional viruses and the resulting mixed infections can lead to serious epidemics. Exclusion and testing at the G4 level or prior to distribution can enhance longevity and profitability of the crops within regions and prevent or reduce accidental introduction of viruses into new production areas.

Given the relatively recent identification of criniviruses as economically important disease agents, work has primarily focused on characterization, epidemiology, and in certain cases chemical control of vectors. Still, the ultimate control strategy for any pathogen is strong, stable genetic resistance. Resistance using modern methods such as RNA interference is probably the most straightforward and durable approach to prevent infection by viruses, but public resistance to genetically modified plants especially in crops that are labeled as "healthy food" or "superfoods" such as fruits and vegetables, the primary hosts for criniviruses, has minimized the application of this technology. For the majority of the criniviruses little or no work has been directed toward identification of resistance using traditional screening of germplasm resources and/or breeding to incorporate such sources into commercially acceptable cultivars. In the few cases where resistance has been identified it is almost always found in wild accessions, which requires many generations of backcrossing before the relevant genes are incorporated into marketable varieties. That is not to say progress is not being made. Sources of resistance to LIYV were identified in both lettuce and melon (McCreight, 1987, 2000), although the demise of LIYV as an agricultural threat due to shifting vector population dynamics largely rendered advancement of the material a moot point. Other efforts however offer real potential for effective crinivirus management. A source of resistance to ToCV was recently identified in tomato (Garcia-Cano et al., 2010), and two independent and complementary sources of resistance to CYSDV have been found in melon (Lopez-Sesé and Gomez-Guillamon, 2000; McCreight and Wintermantel, 2011). Sequencing of the genomes of many crops affected by criniviruses, identification of resistance sources, and the use of marker-assisted selection will speed up the incorporation of these and likely other resistance traits into commercially relevant cultivars.

Criniviruses are transmitted in a semi-persistent manner and chemical control of vectors has not always been effective for virus disease management. In addition, the development of resistance to insecticides in insect populations and the effect of insecticides on whitefly predators may have a negative impact on vector and virus control, particularly in systems using broad integrated pest management approaches. Consequently, it may be appropriate to consider a more generic approach, such as identification of resistance against whitefly vectors. There have been several cases in which insect resistance has been identified in plants (Mutschler and Wintermantel, 2006). In many cases this has been more effective and long-lived than virus resistance, possibly due to the ability of the viruses to drift toward resistance-breaking populations. In addition, vector resistance may be effective in controlling several viruses that are transmitted by a common vector. As an extreme example, aphid resistance to *Amphorphora agathonica* (Hottes) had been effective for over 50 years in controlling three aphid-borne viruses in raspberry in the North America, before new biotypes of the vector developed that overcame the resistance (Hall et al., 2009). Forms of resistance against insects can function in a number of ways, including acting as feeding deterrents, physical barriers, or oviposition inhibitors. Some plant secondary metabolites dissuade insects from settling on plants, preventing the steady feeding that can lead to toxicity or virus transmission. Others may prevent oviposition, reducing vector populations (Mutschler and Wintermantel, 2006). Studies are just beginning to

address the potential of resistance to insect feeding on control of whitefly-transmitted viruses (Mutschler and Wintermantel, 2006; Rodriguez-Lopez et al., 2011, 2012). Appropriate and effective utilization of such approaches will require specific research to confirm that methods effective in controlling one pest do not exacerbate problems with another. Integrating vector control with other means of pest and disease management; however, offers the potential to strengthen durability and effectiveness of control for not only criniviruses, but a number of insect-borne pathogens.

There have been numerous significant breakthroughs in understanding criniviruses, the diseases they cause, and their epidemiology. However, a great deal more work is needed on virus control, including an emphasis on certification to minimize virus movement, identification of resistance sources against both vectors and viruses, and introgression of resistance genes into commercially acceptable germplasm. These should be priority areas for long-term reliability of crinivirus management. Such efforts will complement or reduce the need for extensive pesticide-based programs, and will minimize the impact and spread of criniviruses in world agriculture.

ACKNOWLEDGMENTS

The authors thank Dr. Quito-Avila for his help with the phylogenetics. The majority of the work done by the authors of this review was made possible with funds from the California Strawberry Commission, the North America Strawberry Growers Association, the North American Raspberry and Blackberry Association, the Northwest Center for Small Fruits Research, the Washington Red Raspberry Commission, the Oregon Raspberry and Blackberry Commission, USDA-NIFA-SCRI (2009-51181-06022), USDA-APHIS-NCPN (10-8100-1572-CA and 10-8100-1567-IA), the Southern Region Small Fruit Consortium, the Arkansas Agricultural Experimental Station, and the United States Department of Agriculture.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 February 2013; accepted: 26 April 2013; published online: 16 May 2013.

*Citation: Tzanetakis IE, Martin RR and Wintermantel WM (2013) Epidemiology of criniviruses: an emerging problem in world agriculture. Front. Microbiol. **4**:119. doi: 10.3389/fmicb.2013.00119*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.