Chapter 1

The Complex Genetics of *Citrus tristeza* virus

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1. Introduction

The 2000 x 11 nm long bipolar flexuous filamentous particles of *Citrus tristeza virus* (CTV) (genus *Closterovirus*, family *Closteroviridae*) (Figure 1) contain a single-stranded positive-sense RNA genome of 19.3 kb, which is encapsidated in two different capsid proteins that coat the opposite ends of the virions [1, 2]. CTV is the largest identified RNA virus infecting plants and the second largest worldwide after the animal *Coronaviruses*. The virus is phloem limited and it is transmitted by aphids (*Hemiptera: Aphididae*) (Figure 1), and mechanically by graft propagation of virus-infected plant tissues. CTV isolates from different hosts and areas display great variability either biologically or genetically. There are wild CTV isolates that consist basically of a main genotype and its quasispecies, but others could contain a mixture of strains (groups of viral variants with similar sequence) that differ in symptomology and in viral transmission efficiency by aphids. These CTV strains could bear divergent CTV genotypes. Additionally, wild isolates are also composed by a population of defective RNAs (D-RNAs) that could change by aphid or graft transmission or by host passage [3].

The *Tristeza* syndrome, induced by CTV, has devastated entire commercial citrus industries around the world, since it has caused the death of hundred million trees worldwide. In point of fact, this virus is present in most of the citrus producing areas infecting nearly all species, cultivars and hybrids of *Citrus* spp. and related genera. Phenotypically, CTV induces different grade and wide range of symptoms in *Citrus* species. In effect, depending on the virus isolate and the variety/rootstock combination, CTV strains can cause different syndromes in the field like 'decline' (QD) or 'stem pitting' (SP). Some CTV isolates induce a third syndrome, in glasshouse conditions, that is referred as 'seedling yellows' (SY). Furthermore, CTV causes a myriad of different symptom combinations in indicator plants depending on the CTV strain, or the mixture of strains, present in the plant host indexed. Remarkably, there are mild CTV strains that cause a complete lack of symptoms in almost all species and varieties of citrus, including those present in the citrus orchards, even though these mild viruses multiply to high titers [4, 5].

The study of the CTV genetics and the virus-host interactions have been hampered during long time as a consequence of the difficulties of experimenting with a virus with a large RNA genome, assembled in fragile particles and present in reduced amounts in a tree, where CTV could take long time to colonize the entire plant and to induce symptomatology. For that reason, CTV was for decades a virus complicated to isolate and characterize. Moreover, the elevated diversity of CTV populations impeded the separation of the sequence variants, composing a specific isolate, to analyze each of the genotypes independently in order to understand every aspect of viral infection. Likewise, the myriad of diseases induced by CTV, depending on the *Citrus* host, viral strain and environmental conditions, challenged the study of the host-plant interactions. In the last century, the study of CTV genetics was focused in generating molecular techniques to improve CTV detection and genotype differentiation [5]. However, in a decade, a remarkable progress has been achieved in developing the genetic engineering tools to overcome the challenges of examining CTV genetics. A cDNA clone (*T36-CTV9*) of the Florida isolate T36 was generated and an *in vitro* genetic system was developed to analyze CTV genotypes, D-RNAs, mutants and self-replicating constructs in *Nicotiana benthamiana* protoplasts or indexing plants [6, 7, 8, 9, 10]. The last advances in CTV genetics and the different biotechnological approaches used to study CTV are discussed in this chapter.

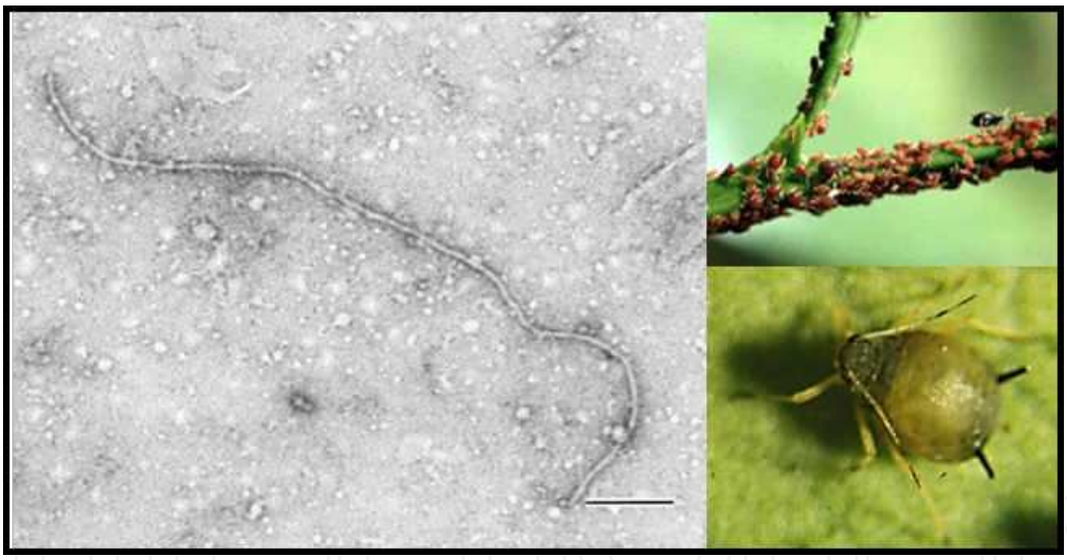

Figure 1. Left: Viral particles from *Swinglea glutinosa (Blanco) Merr.* protoplasts, transfected with CTV isolate T36, collected at 4 dpi and examined by SSEM electron microscopy. The bar indicates 200 nm. From Albiach-Marti et al. [72]. Top right: colony of *Toxoptera citricida* (Photo: Dr. A. Urbaneja). Bottom right: *Aphis gossiipi* (Photo: Dr. A. Hermoso de Mendoza)

2. *Citrus tristeza virus* genome structure, organization and gene function

The CTV RNA genome structure resembles that of *Coronaviruses,* and it is organized in twelve open reading frames (ORFs) and two non-translated regions (NTR) at the 5′ and 3′ terminus (Figure 2) [2]. The 5′ termini of the CTV genome is protected with a cap structure [2]. The 5′

NTR of around 107 nt contains the sequences necessary for both replication and particle assembly [6, 23, 24]. Remarkably, the CTV 5′NTR predicted secondary structure is similar even for divergent genotypes and folded in two stem-loops separated by a short spacer region [23, 25]. The 3′NTR (273 nt) lacks a poly-A tract and does not appear to fold in a tRNA-like structure [2] but instead consists a predicted secondary structure of minimum energy of 10 stem-loop (SL) structures [26].

The CTV genome maintains the two characteristic clusters of genes of the family *Closteroviridae* (Figure 2) [11]. The *replication gene block*, which is also conserved in the supergroup of sindbis-like viruses, comprises ORF 1a and 1b and makes up the 5′ half of the viral genome [2] (Figure 2). The ORF1a encodes a 349 kDa polyprotein with two papain-like protease domains, a type I methyltransferase-like domain, and a helicase-like domain bearing the motifs of the superfamily I helicases. The ORF1b encodes a 54 kDa protein with RNA-dependent RNA polymerase (RdRp) domains. When ORF 1 is are directly translated from the positive-strand gRNA yield a 400 kDa polyprotein [2]. The conserved *quintuple gene block* (Figure 2) is related with virion assembly and trafficking in the plant [11]. This consists of the major coat protein (CP) of 25kDa, the minor coat protein (CPm) of 27kDa and other three proteins, p61, HSP70h and p6. HSP70h is a 65 kDa protein homologue of the HSP70 plant heat-shock proteins [2], a family of plant chaperones involved in protein-protein interactions, translocation into organelles, and intracellular trafficking [12]. The p6 gene encodes a small hydrophobic protein that belongs to the single-span transmembrane proteins [2]. While CP, CPm, p61 and HSP70h are necessary for proper particle assembly, p6 is required for systemic invasion of host plant [13, 14]. The additional five ORFs located at the 3′ half of the genome (Figure 2) are the p20, an homologue of p21 of *Beet yellows virus* (BYV) (genus *Closterovirus*), and four genes encoding proteins with no homologue in other closteroviruses (p33, p18, p13 and p23) (Figure 2) [11]. The p20 protein is the main component of the CTV-induced amorphous inclusion bodies [15] and it is essential for systemic infection [14]. The multifunctional protein p23 contains a Zn finger domain that binds cooperatively both single-stranded (ss) and double-stranded (ds) RNA molecules in a non-sequence specific manner [16]. In addition, p23 controls asymmetrical accumulation of positive and negative RNA strands during viral replication, ensuring the presence of enough quantity of positive genomic RNA (gRNA) ready for virion assembly [17].

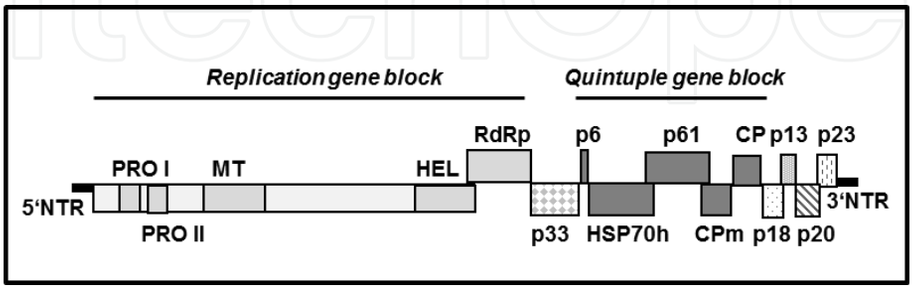

Figure 2. Scheme of CTV genome structure and organization. CTV ORFs are delimited by boxes. The acronyms PRO, MT, HEL and RdRp indicate protein domains of papain-like protease, methyltranferase, helicase and RNA-dependent RNA polymerase, respectively. HSP70h, CPm and CP indicate ORFs encoding a homologue of heat shock protein 70, the minor and the major coat proteins, respectively. From Karasev et al. [2].

In relation to host-plant interactions, CTV is a virus with a large genome and complex genetics, while the citrus host includes many species, varieties, and intergenic hybrids with which the virus could interact causing a range of physiological and biochemical responses. In fact, CTV evolved ending up with three proteins, CP, p20 and p23, which are suppressors of the plant RNA silencing mechanism in *N. benthamiana* and *N. tabacum* plants [18]. Unexpectedly, the ORFs that encode proteins p33, p18 and p13 are not required either for replication or assembly [6, 13] or for systemic infection of Mexican lime [*C. aurantifolia* (Christm.) Swing.] and *C. macrophylla* Wester plants [14]. Nevertheless, they are involved in CTV infection and movement in other citrus hosts [19].

Furthermore, several CTV genomic regions have been found to be related with viral symptom development in citrus hosts. The symptomatology determinant of SY syndrome was located at the 3′ region composed by p23 ORF and the 3′NTR [20]. Nevertheless, the p33, p18 and p13 are involved in the SP syndrome development [21], although the participation in this process of other CTV regions, undetected until the moment, has not been discarded. Mild strain cross protection has been widely applied for millions of citrus trees in Australia, Brazil and South Africa [4, 5] to protect against SP economic losses. The mechanism of this type of viral superinfection exclusion is mainly a mystery. Recently, it has been found that the lack of the functional CTV p33 protein completely eliminated the ability of the virus to exclude superinfection by the same or closely related virus [22].

3. *Citrus tristeza virus* sequence diversity

Sequencing the complete genome of CTV was the first breakthrough towards the study of CTV genetics [2]. Actually, there are twenty CTV genomic sequences available. These are T36 and T30 from Florida [2, 27]; VT from Israel [28]; SY568R from California [29, 30]; T385 and T318A from Spain [31, 32]; NuagA from Japan [33]; Qaha (AY340974) from Egypt; Mexican isolate (DQ272579); B165 form India [34]; NZ-M16, NZ-B18, NZRB-TH28, NZRB-TH30, NZRB-M12, NZRB-M17 and NZRB-G9 from New Zealand [35, 36]; HA16-5 and HA18-9 from Hawaii [37] and Kpg3 from China [38]. Genetic comparison of these CTV genomes revealed an extreme genomic divergence for genotypes of the same viral species (Figure 3). Nevertheless, these divergent CTV genotypes retained the same genomic organization [3].

Phylogenetic analysis classified the twenty CTV genomic sequences in seven main genotypes [35, 37, 38]. Six of them induce severe syndromes: (1) T36-like (T36, Qaha and Mexican); (2) the RB group plus HA18-9; (3) the VT-like (VT, NUagA, T318A, SY568 and Kpg3); (4) HA16-5; (5) B165 and NZ-B18; and the (6) NZ-M16 genomic sequences [35, 37, 38]. The group 7 consisted in the T30-like asymptomatic or mild genotypes (T30, T385). Sequence comparison of complete CTV genomes yielded nucleotide identities from 79.9%, between Qaha (a T36-like strain) and VT, to 99.3% (between T30 and T385) (Figure 3) [37]. The most conserved sequences were located in the 3′NTR region, which is almost identical in most of the cases (Figure 3). The nucleotide divergence was mostly concentrated at the 5′ half of the CTV genome and increased towards the 5′NTR region to raise, in some cases, nucleotide identities as low as 42% [27, 37,

39] (Figure 3). This pattern of genomic divergence was more evident between the T36-like genotypes and close relatives (groups 1 and 2) and the other five CTV groups [36]. However, two paths of sequence divergence were observed [39]. The sequence divergence between CTV genotype groups 3 to 7, although slightly increased in the 5′NTR region, was relatively constant in proportion and distribution along the genome [37, 39]. On the other hand, the T36-like genotypes and close relatives showed considerable genetic distance to the other five main CTV genotypes [36]. Actually, the comparison of the genomic sequences of T30 and T36 diverged from 5% in the 3′ NTR to as high as 58% in the 5′NTR (Figure 3) [27]. Based in these two paths of sequence divergence detected between CTV genomic sequences [39], it was speculate that the T36 genotype and relatives evolved from a recombinant of a CTV genome and an unknown virus millions of years ago in Asia [28].

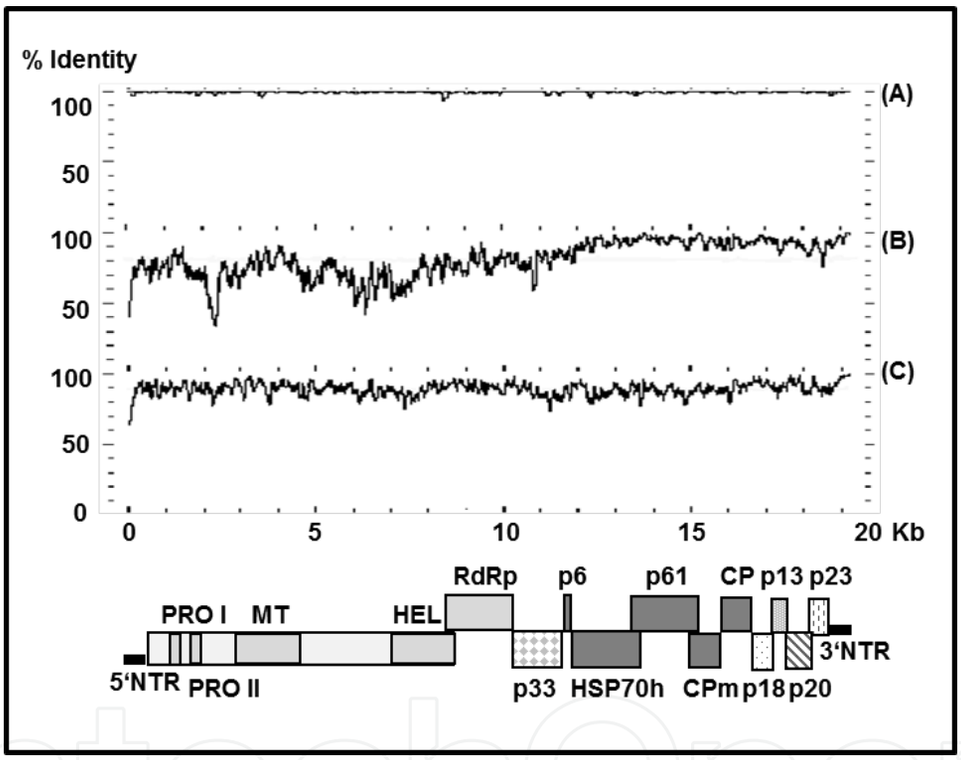

Figure 3. Graphic of the nucleotide identity along the CTV genome when comparing T30 genomic sequence with the sequences of (A) T385 (B) T36 and (C) VT genomes. From Albiach-Marti et al. [27].

Comparison of each of the CTV regions pointed to an unevenly distributed sequence variation along the CTV genome, likely reflecting different selective pressures along the genomic RNA [26, 37, 39]. Analysis of nucleotide diversity in some coding regions between CTV strains yield values higher than 0.13. However, most of the nucleotide exchanges were reported at the third codon position, indicating the preservation of the protein sequence among divergent genotypes. Actually, the ratio between non-synonymous and synonymous substitutions assessed for CTV coding regions was below the value 1, thus suggesting selective pressure for amino acid conservation [40]. In addition, analysis of the CTV genomic and D-RNAs sequences indicate homologous and non-homologous recombination events among different genotypes

[30, 32, 41, 42, 43], possibly as a result of mixed infections on trees that are recurrently inoculated by aphid transmission.

Conversely, in spite of this genetic variability, sequence comparisons of some CTV genomes revealed a remarkable viral genetic stasis as the genomes of some CTV strains, separated geographically and in time, were found essentially identical [27]. This genetic stability has been explained as a consequence of strong selection and competition between the mutants that arise in each replication cycle, which creates equilibrium in the viral quasispecies distribution [27]. In this context, there is a hypothesis to explain the high sequence variability found in the wild CTV isolates [3, 27]. In a fist stage, each of the main genotypes evolved separately in different *Citrus* species at their point of origin in Asia. This was followed by the dispersal of the main CTV genotypes to different environments around the world with the advent of the modern citrus industry in the XIX century. After that, RNA virus mutation, due the error-prone nature of RNA-dependent RNA polymerases, in addition to recombination events between diverged sequence variants, plus selection, genetic drift and gene flow could have been promoted rapid evolution [3, 5, 27].

4. *Citrus tristeza virus* replication and gene expression

CTV replication is an extraordinary process that generates at least 35 different species of viral RNA in CTV-infected cells (Figure 4) [44] plus a myriad of D-RNAs [45, 46, 47] (Figure 5). The viral genomic sequences necessary for CTV replication are the *replication gene block* plus the 3′ and 5′NTRs, which contain the cis-acting elements indispensable for this process (Figure 4). In fact, a T36 CTV replicon consisting in only these genomic regions is able to self-replicate in protoplasts of *N. benthamiana* [6]. As indicated previously, the CTV 5′NTR predicted secondary structure folded into two SL separated by a short spacer region [25]. Directed mutations disrupting this predicted secondary structure were shown to abolish replication, whereas compensatory mutations resumed replication, suggesting that the secondary structure of the 5′ NTR is more important than the primary structure for CTV replication [23]. Conversely, the basic function of the 3′ NTR (273 nt) is minus-strand initiation for the CTV gRNA and the subgenomic (sg) RNAs [26]. The 3′NTR consists in a predicted secondary structure of 10 SL structures. While the core of the 3′ replication signal was located in the primary structure of three of the central stem-loops (SL4, SL6 and SL8), the secondary structure of the other stem-loops (SL3, SL5, SL7 and SL9) proved dispensable but required for efficient replication [26]. In addition, all CTV genomes retain a CCA triplet at the 3′ termini necessary to initiate replication [26].

Wild CTV populations could be composed by divergent genotypes [3]. In the case of mixed infections in the same plant cell, it is essential to determine whether a specific replicase complex is able to recognize the cis–acting elements of the 3′or 5′ NTR of other genomic variants. The exchange of 5′NTR and 3′NTR sequences, from different main genotypes, into the *T36-CTV9* infectious clone decreases replication as the degree of sequence divergence increases. Therefore, indicating partial compatibility of the T36 replicase complex with diverged 5′ and 3′ cis-acting elements, thus suggesting limited heterologous replication in mixed viral infections [6].

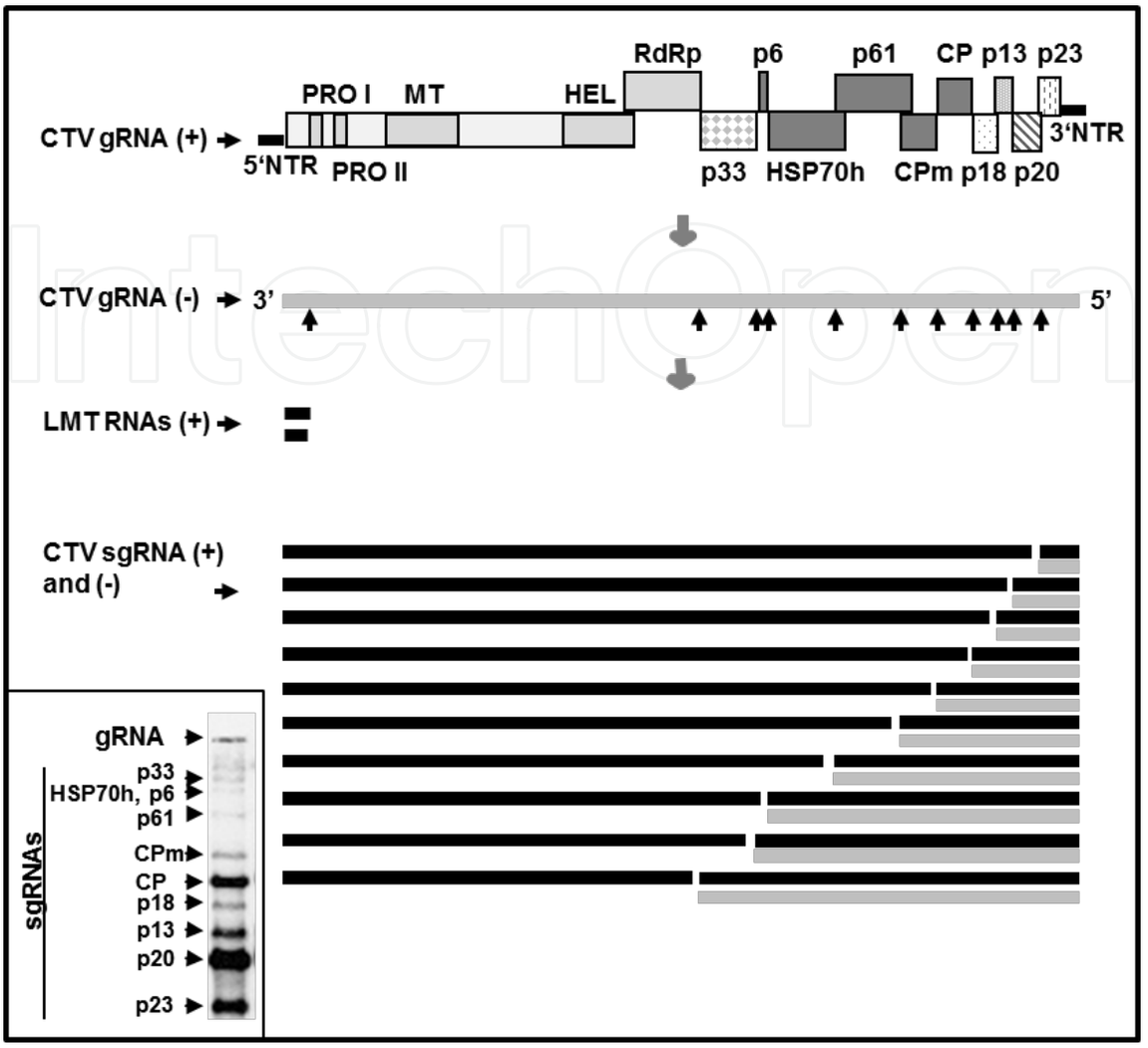

Figure 4. RNA species produced *in cis* during CTV replication. Main panel: Scheme of the different CTV RNA species. Black lines: single-stranded positive-sense RNAs. Grey lines: single-stranded negative-sense RNAs. The acronyms gRNA, sgRNA and LMT RNA indicate genomic, subgenomic and *low molecular-weight tristeza* RNAs, respectively. The signs (+) and (-) specify plus and minus-strand RNAs, respectively. Black arrows on the CTV gRNA(-) line designate the approximate position of the CTV controller elements in the CTV genome. Small left panel: Accumulation of T36 double strain (ds) RNAs in citrus plants showing the 3´coterminal sgRNAs produced during replication and expression of the ten 3´half ORFs of the CTV genome. Northern-blot hybridization performed using a single-stranded negative-sense riboprobes specific to the 3' end of T36 genomic sequence. From Karasev et al. [2, 50], Gowda et al. [44, 51, 52] and Ayllon et al. [49, 48].

In the first step of the CTV genome replication, the viral replicase uses the single stranded positive-sense gRNA (CTV gRNA (+)) from the uncoated viral particles, as template to generate a homologous single-stranded negative –sense CTV gRNA (CTV gRNA (-)) (Figure 4). The CTV gRNA(-) molecules will function as basis for the synthesis of the CTV progeny of positive-strand gRNAs. The CTVgRNA(+) molecules would act as RNA messenger for expression of viral proteins or as a pool of CTV gRNAs ready to be incorporated into virions to produce newly infectious viral particles. The new CTV gRNA(+) could also serve as template for the synthesis of fresh CTVgRNA(-) molecules to start all over the process [44].

Another function of the CTV gRNA (-) is to serve as template to produce high quantities of single and double strain sgRNAs during the expression mechanism of the ten ORFs situated at the 3′half of the genome (Figure 4 and 5) [44, 48, 49, 50]. Unlike the large animal viruses of the *Nidovirales*, the 3′ sgRNAs of CTV do not share a common 5′ terminus and the sgRNA transcription mechanism resembles the transcriptional mechanism of other Sindbis-like viruses [50]. The synthesis of each 3′ coterminal sgRNA is controlled by its corresponding cis-acting element (controller element (CE)) (Figure 4). Probably each CTV CE could act as promoter or terminator of the CTV RNAs during the replication process [44, 48, 49, 50]. However, if the CEs function as internal promoters for the generation of positive-strand sgRNAs, using as template the CTVgRNA(-) molecules (Figure 4), or act as terminators for the synthesis of negative-strand gRNAs (by premature termination at the CE site), or both, is still unclear [44]. In addition to the plus and minus- sense 3′coterminal sgRNAs, the CEs corresponding to each of the ten 3′ORFs produce a reduced amount of a set of 5′coterminal positive-strand sgRNAs (Figure 4), probably due to premature termination during the synthesis of the CTV gRNA(+) [44]. Moreover, CTV generates significant amounts of *low molecular-weight tristeza* (LMT1 and LMT2), two positive-strand 5′co-terminal sgRNAs population with heterogeneous 3′termini at nt 842-854 and 744-746, respectively (Figure 4 and 5) [46, 47]. LMT 1 and LMT 2 are generated and accumulated differently [51, 52]. LMT1 is likely created by premature termination during CTV gRNA(+) synthesis at a 5′ CE situated in the PRO I domain of the replicase (Figure 4). This 5′ CE acts as a strong promoter when placed immediately upstream of the ORFs near the 3′ terminus [51]. In contrast of the 3′ CEs, which are able to generate plus and minus-strand sgRNAs, the 5′ CE of the LMT 1 only promoted the synthesis of positive-strand sgRNAs (Figure 4) [51]. In fact, the RNA termination and initiation sites of the 5′ CE, compared to those of 3′ CEs, occur at opposite ends of the corresponding minimal active CE site [49]. Therefore, as a result of the replication process, CTV produces high amounts of viral RNA species in the infected cell (Figure 4). The total (gRNAs plus sgRNAs) positive to negative-strand RNA ratio (approximately 40 to 50:1) falls within the range of the genomic RNAs of most positive-strand RNA viruses, particularly the more similar alphavirus supergroup and large complex viruses of the *Nidovirales* [26]. However, during CTV replication, only the positive-strand gRNA accumulates approximately 10 to 20 times more than their negative-strand gRNA homologues, a rather lower ratio compared to those generated during other RNA viruses replication [17].

The expression of the CTV genome, which potentially yields at least nineteen protein products, resembles that of *Coronaviruses* [50]. This remarkable process includes at least three different RNA expression mechanisms widely used by positive-strand RNA viruses: proteolytic processing of the polyprotein precursor, translational frameshifting and the generation of a nested set of ten 3′-coterminal sgRNAs [50]. Therefore, the ORFs 1a and 1b are directly translated, from the positive-strand gRNA, to yield a 400 kDa polyprotein that is later proteolytically processed in, at least, nine protein products. The ORF1b encodes a 54 kDa protein with RdRp domains that is occasionally translated after ORF 1a by a +1 ribosomal frameshifting [2]. Additionally, as indicated above, the 10 ORFs located at the 3′ half of the CTV genome are expressed by the synthesis of ten 3′ co-terminal sgRNAs (Figure 4). Each 3′ sgRNA serve as RNA messenger for the translation of its 5′ proximal ORF [13, 46] and the

expression of each of the ten 3′ proximal ORFs is regulated independently both in amount and timing [46, 47].

5. *Citrus tristeza virus* defective RNAs

In addition to the 35 different species of RNA created during replication, CTV could accumulate considerable amounts of D-RNAs in infected cells (Figure 5) [46]. CTV D-RNAs vary in size, abundance and sequence [41, 45, 46] and could be encapsidated into particles and could be transmitted by aphids [45].

Generally, D-RNAs bear a genome from 2.0 to 5.0 kb and are composed by variable portions of the 3′ and 5′ termini of CTV genomic RNA with large internal deletions (Figure 5). Nevertheless, some D-RNAs comprising the two termini and a non-contiguous internal sequence or a non-viral sequence, plus large D-RNAs of 10-12 kb including in their 5′ proximal region the ORFs 1a and 1b, or with a 3′ region homologous to the ten CTV 3′ terminal ORFs, have been described [41, 43, 46, 55]. These large D-RNAs resembled the RNAs 1 and 2 distinctive of the bipartite *Criniviruses*, also included in the *Closteroviridae* family. Moreover, the D-RNA containing the complete CTV replicase constitutes a novel class of large self-replicating D-RNAs [47].

CTV D-RNAs characteristic genomic structure suggests an origin in the recombination events during viral replication. In this way, some large D-RNA bear a 5′ termini identical o slightly larger than the 5′ sgRNA generated by the CE of the p33 ORF [47], and the small ones usually contain a 3′ termini identical to 3′ sgRNA of p23 ORF [43]. Additionally, a repeated 4-5 nt, (corresponding to two CTV genomic regions) was reported flanking the D-RNA 3′ and 5′ termini junction sites indicating that D-RNAs are probably created during the generation of the positive-strand sgRNA or gRNA by a template-switching mechanism [41, 43].

D-RNAs require the viral machinery for their survival. The D-RNA replication *in trans* was examined using infectious D-RNAs and the *in vitro* genetic system of *T36/CTV9* [10, 6]. The minimal D-RNA sequence required for replication are a 5′ proximal region of 1kb and a 3′ termini limited to the CTV 3′NTR. In addition, efficient replication of D-RNAs involves some spacing between these terminal cis-acting signals and a continuous ORF through most of the 5′ proximal regions of the D-RNA sequence [10, 56]. CTV field isolates are composed by viral populations of divergent genotypes. In this case, an important point is to understand the dynamics of generation and accumulation of D-RNAs in a specific plant cell infected with distinct CTV genotypes. Mawassi et al., [56] demonstrate that some wild-type populations of CTV are capable of supporting the replication of synthetic divergent D-RNAs. However, replacement of 5′ region (which is the most variable among CTV strains) of a particular synthetic D-RNA, with the corresponding sequence from different main CTV genotypes, resulted in chimeric D-RNAs that were replicated to detectable levels by some CTV genotypes but not with the others. Consequently, differential specificities of distinct CTV replicase complexes with divergent D-RNA replication signals are possibly affecting the maintenance of D-RNA population structures in the infected plant cell.

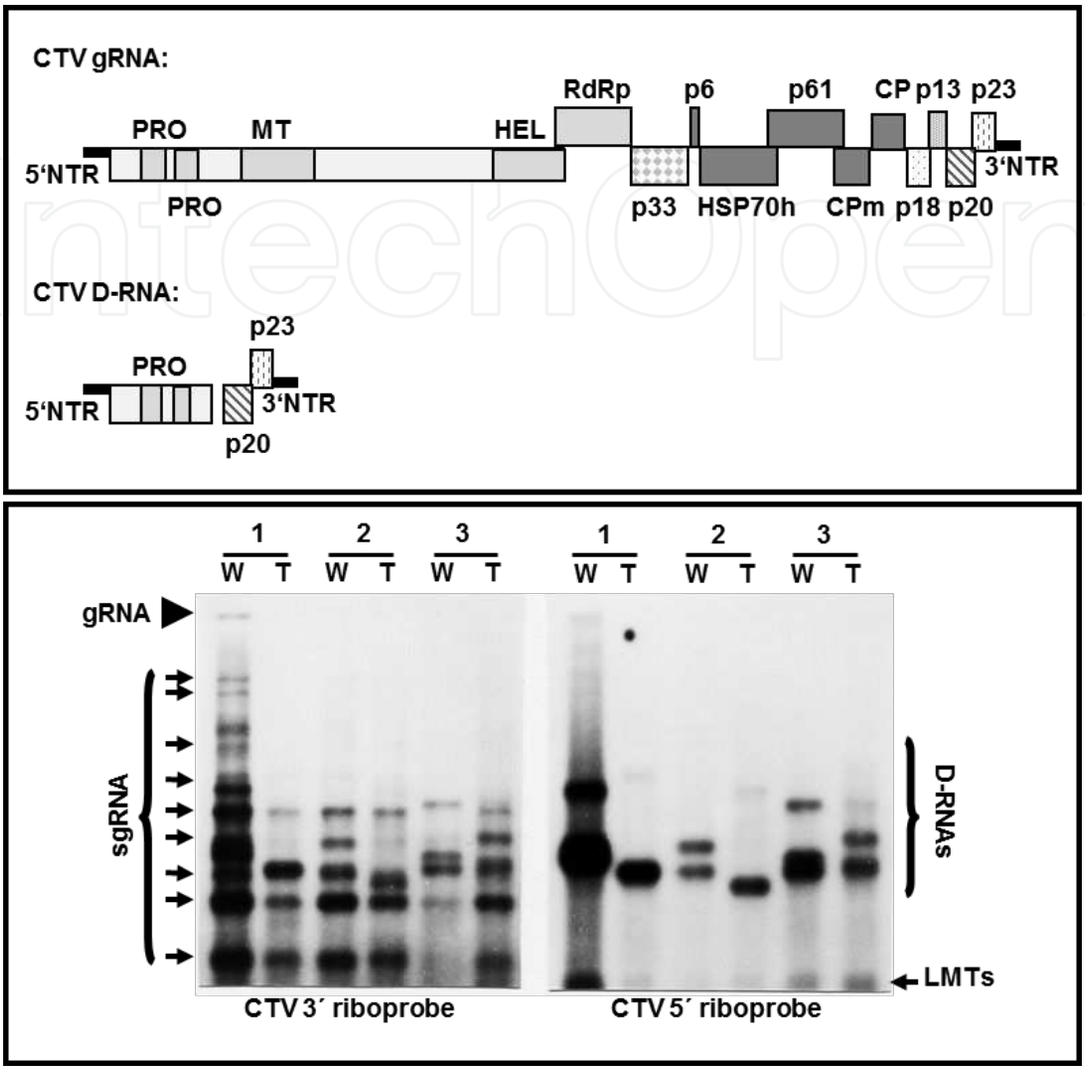

Figure 5. Different species of D-RNAs in CTV populations. Top panel: graphic representation of a usual small CTV D-RNA compared with the CTV genome. Bottom panel: accumulation of CTV RNAs in bark extracts from sweet orange plants infected with three field isolates (1 to 3 lines), before (lines w) and after (lines T) aphid transmission. Northern-blot hybridization performed using a single-stranded minus-sense riboprobes specific to the 3' end (left panel) and the 5´end (right panel) of the T36 genome. From Mawassi et al. [46] and Albiach-Marti et al. [45]

In other viral pathosystems, D-RNAs have the capacity of interfering with the viral replication process of their helper virus (named defective interfering (DI) RNAs), but those function was not reported for CTV [10]. Although their biological role is presently unknown, at least in one case, the presence of D-RNAs was suggested to modulate symptom development either increasing or decreasing CTV symptom expression [57]. Most of the CTV D-RNAs contain a complete region p23 and the 3´NTR [43] that is associated with SY development [20], thus they could have a role in symptom modulation. Therefore, it will be necessary to promote further research to elucidate the role of the D-RNAs (or DI-RNAs) in CTV replication or in modulation of pathogenic responses in the infected plant host.

6. *Citrus tristeza virus* encapsidation

Differently of most elongated viruses, CTV particles are encapsidated by two different capsid proteins that coat the opposite ends of the virion [24]. About the 97% of the CTV genome is coated by CP, while the remainder 3% is coated by CPm resulting in viral particles with the emblematic tail of the members of the *Closteroviridae* family [11, 24]. The coordinate action of HSP70h and p61, in addition to the CP and CPm coat proteins, are required for proper assembly of CTV particles [13]. The previously described CTV 5′NTR conserved SL structures contains the origin of assembly of CPm, overlapping the sequences that function as a cis-acting element required for gRNA synthesis [24]. During CTV assembly, CPm begins coating the gRNA at the 5′ NTR to about nt 630. However, in the absence of HSP70h or p61, CPm may coat larger segments or even the complete gRNA. Probably, HSP70h or p61 bind to the transition zone between CP and CPm (around 630 nt) and restrict CPm to the virion tail [24]. The protein homologous to HSP70h and p61 in BYV are coordinately assembled with CPm in the virion structure and remain attached to the viral particles [58]. Although the assembly of HSP70h and p61 is not directly confirmed for CTV, RNA transcripts lacking one or both of these ORFs were unable to produce infective CTV virions [24]. Several strains with divergent genotypes could coexist in the same citrus cell. Analysis of the encapsidation of heterologous CPms in absence of HSP70h and p61 indicated reduction or lack of CPm assembly. Nevertheless, the presence of HSP70h and p61 restored CPm assembly to wild-type levels. This indicated that the HSP70h and p61 could play an important role facilitating heterologous CPm assembly in mixed infections [59].

The region coated by CPm also overlaps the LMT2 5′ sgRNA (650 nts). Actually, LMT2 production is correlated to virion assembly since mutations in the 5′ NTR that abolish encapsidation also eliminate accumulation of LMT2. Although this represents the first evidence of a viral RNA processed by the assembly mechanism, the exact function of LMT2 in CTV assembly is unknown at the moment [52].

7. *Citrus tristeza virus* genetics and plant-host interactions

In order to infect a plant, CTV needs to enter in the cell, and to overcome the constitutive and/or inducible plant defences, to re-program the plant cellular machinery for its viral multiplication. The infection process will continue with the assembly of new viral particles that will move cell to cell through the plasmodesmata. This process will be completed with the viral long distance movement through the plant vascular structure to colonize systemically the plant. Each CTV gene product seems to have a primary genetic function required for the survival of the virus. However, there are secondary genetic interactions, which cause or trigger resistance or pathogenic responses in the citrus host [60, 61]. *Citrus* genus contains multitude of species, cultivars and intergenic hybrids, with which CTV could interact causing a range of physiological and biochemical responses. These could be from either pathogenic or asymptomatic phenotypes to limited or complete plant resistance [5]. Although most of these mechanisms are

still a mystery, new discoveries towards the understanding of the genetics of CTV movement in the plant, host-range, host resistance and pathogenicity have been reported recently [19, 20, 21, 62, 63, 64].

7.1. *Citrus tristeza virus* host range and plant systemic infection

Citrus tristeza virus natural plant hosts belong to the order *Geraniales*, family *Rutaceae*, subfamily *Aurantoidea*. There are also non-rutaceous hosts that have been experimentally infected with CTV strains like *Passiflora gracilis* or *Passiflora coerulea*. Some citrus hosts are usually susceptible to CTV infection like Mexican lime or *C. macrophylla*. Other citrus host are tolerant to some CTV strains like sweet orange [*C. sinensis* (L.) Osb.] and grapefruit (*C. paradisi* Macf.), or tolerant to almost all known CTV strains as mandarins (*C. reticulata* Blanco). Finally, pummelos [*C. grandis* (L.) Osb.], sour orange (*C. aurantium* L.) and the hybrid rootstock Swingle citrumelo exhibit a differential degree of resistance depending on the CTV strain. In addition, some *Citrus* relatives within subfamily *Aurantioideae,* like *Poncirus trifoliata* (L.) Raf., as well as *P. trifoliata* intergenic hybrids remain resistant or immune to most of the CTV strains [4, 5]. Consequently, these data highlight an elevated complexity in the CTV systemic infection and host range genetics.

Several CTV genes are related with systemic infection of citrus plants [14, 62]. Viral mutants with a deletion in the p6 and p20 ORFs failed to infect citrus plants systemically, suggesting their possible roles in virus translocation or infection of the whole plant. Likewise, the p6 homologue in BYV is a movement protein [65], and similarly to homologous proteins function in BYV [11], CP, CPm, HSP70h and p61 probably participate in the viral movement. CTV genome has several ORFs that are non-conserved in the family *Closteroviridae,* thus unique for CTV. Unexpectedly, three of these ORFs (p33, p18 and p13) neither are required for replication and assembly [6, 13] nor for systemic infection of Mexican lime and *C. macrophylla* [14]. However, p33, p18 and p13 were demonstrated to be CTV host range determinants. The p33 ORF is necessary for the systemic infection of sour orange and lemon trees. Likewise, either p33 or p18 ORF is enough for systemic infection of grapefruit trees. Similarly, p33 or p13 ORF is sufficient to invade whole calamondin (*C. mitis*) plants. As a result of the acquisition of multiple non-conserved genes (p33, p18, and p13), probably CTV increased the possibilities to interact with multiple hosts, thus extending its host range during the course of its evolution [19].

7.2. *Citrus tristeza virus* suppressing genes of plant silencing mechanism

The plant constitutive defence consists of the RNA mediated post-transcriptional silencing mechanism (PTGS) that implies the specific degradation of the viral dsRNA in small interfering RNAs (siRNAs), which guides a specific plant ribonuclease to disintegrate the viral genomes in the cytoplasm. Besides the antiviral role, the plant silencing mechanism has important functions in regulating plant gene expression (miRNA metabolism) [60]. In order to infect plants, viruses developed a strategy to block this silencing mechanism: the suppressing genes. This strategy allows viral replication but interfere with host gene expression, thus inducing disease [60, 66]. As indicated previously, CTV evolved ending up with three proteins that are

suppressors of the plant RNA silencing mechanism in *N. benthamiana* and *N. tabacum* plants. The p23 inhibits intercellular RNA silencing, while CP impedes intracellular RNA silencing and p20 limits both inter and intracellular RNA silencing [18]. Although, CP, p20 and p23 have not been yet reported as suppressors of the citrus silencing mechanism, their presence in the CTV genome is in concordance with wide host range among citrus species and hybrids, previously described, and consequently, with the complexity of CTV-citrus interactions. In fact, in spite of the existence of these three silencing suppressors, accumulation of siRNAs in CTV-infected susceptible hosts is 50% of the total RNAs in the plant [64]. The CTV siRNAs accumulation in infected plants is directly proportional to the virus accumulation and varies depending on the citrus host. Deep sequencing analysis of these siRNAs, from CTV-infected plants, indicated that they mainly consisted in small RNAs of 21-22 nt derived essentially from the CTV genome [64].

7.3. Genetic determinants of the *Citrus tristeza virus* pathogenic syndromes

Viruses possess the potential to disrupt host physiology either by usurpation of substantial amount of plant metabolic resources or by the interaction of a specific viral product with the host components [60]. CTV induces three hallmark syndromes, plus a myriad different symptom patterns in indexing plants. *Tristeza* disease or QD syndrome consists in overgrowth of the scion at the bud union, loss of root mass, and therefore death of citrus commercial varieties grafted on sour orange rootstock [5]. The SP syndrome consists in deep pits in the wood under depressed areas of bark in commercial varieties of sweet orange and grapefruit trees grafted on any rootstock. Usually SP do not cause tree death, but severe stunting and unmarketable fruit, thus causing elevated economic damages [5]. The SY syndrome is characterized by stunting, leaf chlorosis and sometimes a complete cessation of growth on sour orange, grapefruit or lemon [*C. limon* (L.) Burm. f.] seedlings (Figure 6). Although, SY syndrome might be found at the field in top–grafted plants and it is not economically valuable, it could be examined in the greenhouse in a timely manner and has a substantial diagnostic value for CTV pathotype differentiation [5]. On the contrary, the development of QD and SP extends over 10 to 40 years at the field [1], a period too long to screen the CTV isolates. Although SP pathotype could be likely examined in glasshouse conditions, there are no reliable methods to reproduce the QD in those conditions [5]. Therefore, the degree of severity of a specific CTV isolate, strain or genotype usually is assessed by using indexing plants (Mexican lime, *C. macrophylla*, sour orange, sweet orange and Duncan grapefruit) [60]. In this case, the degree of CTV symptomology ranges from the mild phenotypes, which are almost asymptomatic, to the highly virulent CTV isolates that could generate vein clearing, leaf cupping, dwarfing, stem pitting and the plant death [5]. This diversity and grade of symptom responses to CTV infection suggests the possible presence of more than one mechanism of pathogenicity taking place during the CTV-*Citrus* interactions.

CTV multiplication generates great quantities of viral products like, at least, 19 viral proteins, 35 RNA species (gRNAs, sgRNAs and LMTs) and D-RNAs along with a complicated process of replication, gene expression, assembly and movement, where the interaction with host factors is essential. Consequently, during the CTV-*Citrus* interaction there are multiple opportunities to generate disease. In fact, analysis of Mexican lime transcriptome using microarrays, after infection with a severe CTV isolate, showed altered expression of 334 genes

and about half of them without significant similarity with other known sequences [63]. In this context, identifying a specific genetic determinant that is responsible for a specific disease symptom under field or glasshouse conditions could be a real challenge [20].

Although serological or molecular markers were correlated with some CTV pathotypes [5], direct linkage of these markers to symptom development has not been established. Nevertheless, the CP, p20 and p23, reported as suppressors of the plant silencing mechanism [18], could be candidates for symptom determinants since they could potentially disrupt the miRNA metabolism, thus possibly inducing disease. Indeed, several viral suppressors of RNA-mediated gene silencing have been identified as pathogenicity determinants [66]. Actually, when p23 is ectopically expressed in transgenic limes or transgenic sour orange plants induces virus-like symptoms. However, the symptomatology pattern developed in these transgenic plants is different than those induced by natural virus infection. Additionally, the grade of symptom severity observed in these p23 transgenic plants is directly proportional to the p23 production level, and independent of the viral source or sequence of the p23 gene [67, 68]. Nevertheless, the symptom intensity in wild virus-infected limes or sour oranges is radically different between severe and mild isolates of virus. Yet, the different response in transgenic plants could be related to the fact that, in this case, the p23 protein is produced constitutively in most cells, while the expression of p23 is limited to phloem-associated cells in nature [20].

As previously described, a distinctive phenotype of some isolates of CTV is the ability to induce *Seedling yellows* in sour orange, lemon and grapefruit seedlings (Figure 6). To delimit the viral sequences associated with the SY syndrome, T36/T30 hybrids were generated by substituting severe sequences, located in the 3′ moiety of the T36-CTV9 infectious clone, for homologous asymptomatic sequences from the T30 genome. The T36/T30 hybrids were analyzed in *N. benthamiana* and citrus plants [20]. The SY determinant was mapped to the region encompassing the p23 gene and the 3' NTR (nt 18394-19296) (Figure 6) [20]. The 3'NTR has been used to generate transgenic plants resistant to CTV [69] and it has also been related to symptom development for other virus [70]. Likewise, the p23 is an obvious candidate for SY symptom determinant since it is one of the most highly expressed CTV proteins [54], a RNA-binding protein responsible for asymmetric replication [16, 17], and it is a viral suppressor of RNA-mediated gene silencing mechanism [18]. Additionally, p23 ORF has been used to produce transgenic plants searching for resistance to CTV [71].

The study of the devastating QD syndrome is especially important under de economical point of view. In this case, the extremely difficult task of reproducing this syndrome in glasshouse conditions hinders the study of the QD genetic determinants. However, since a strong correlation between SY and QD has been observed in the biological evaluation of a wide range of CTV isolates [5], it could be possible, but not yet confirmed, that determinant(s) for the decline disease map similarly to that of SY. Therefore, the CTV hybrids, used for evaluation of the SY genetics determinant [20], have been directly assessed in decline-susceptible grafted combinations of scion and rootstock in field conditions. In addition, since the hybrids are made by recombinant DNA technologies, these assays require special permits from the plant protection and environmental safety authorities [20].

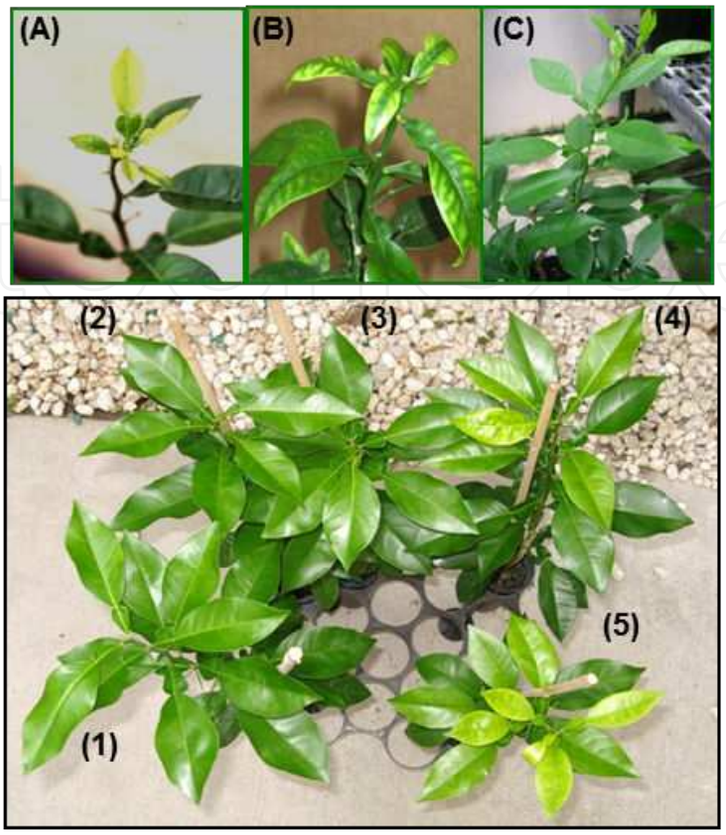

Figure 6. Development of seedling yellows syndrome (SY) in CTV infected plants. Top panel: SY symptoms in (A) sour orange and (B) Duncan grapefruit seedlings compared to (C) a healthy sour orange plant. Bottom panel: SY symptoms in (1) T36/T30 hybrid [P23-3´NTR], (2) isolate T30 (3) healthy (4) T36 infectious clone CTV9 and (5) T36/T30 hybrid [HSP70h-P61] sour orange seedlings. From Albiach-Marti et al. [20].

In order to map the stem pitting determinants, the effect on symptom development in *C. macrophylla* of deletions in p33, p18, and p13 ORFs were evaluated [21]. Although the T36 full-length construct (*T36-CTV9*) causes only very mild SP symptoms in this host, certain deletion combinations (p33 and p18 and/or p13) greatly increased SP symptoms, while other combinations (p13 or p13 plus p18) resulted in reduced SP [21]. Remarkably, the stem-pitting phenotype seems to be induced as result of a balance between the expressions of different viral genes.

7.4. Host resistance to *Citrus tristeza virus* infection

There are different *Citrus* species and relatives that exhibit total or limited resistance to CTV infection. Pummelos, sour orange and the rootstock Swingle citrumelo display a differential degree of resistance depending on the CTV strain. However, some *Citrus* relatives, within subfamily *Aurantioideae,* like *P. trifoliata*, *Swinglea glutinosa* (Blanco) Merr., and *Severinia buxifolia* (Poir) Ten, as well as *P. trifoliata* intergenic hybrids like citranges (sweet orange × *P. trifoliata*), remain resistant or immune to most of the CTV strains [4, 5].

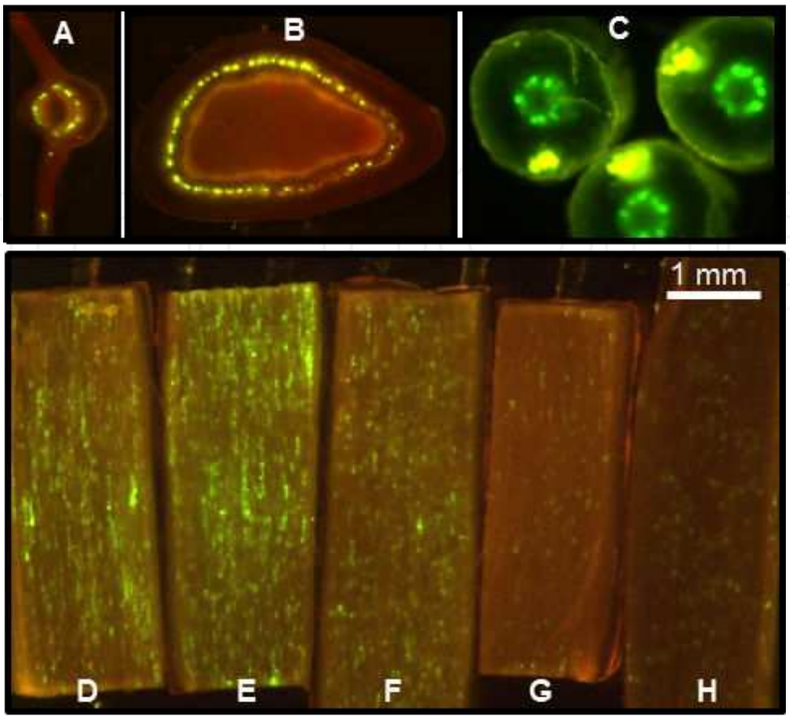

Figure 7. Systemic infection of CTV in different citrus host. Top panel: localization of construct *CTV-BCN5-GFP*, derived from the recombinant virus *T36-CTV9, in* (A) leaf, (B) shoot and (C) roots of tolerant host *C. macrophylla*. Bottom panel: localization of *BCN5-GFP* in a bark flap of (D) Mexican lime, (E) *C. macrophylla*, (F) sweet orange, (G) sour orange and (H) Duncan grapefruit. Pictures were taken in a confocal microscope under UV light. Pictures from Folimonov et al. [9] and Folimonova et al. [62].

Resistance of plants to viruses results from blockage of a basic step in the virus life cycle. This blockage can result from the lack of a factor(s) in the plant that is necessary for virus multiplication and movement (passive resistance) or activation of a defense mechanism (active resistance) [60]. One of the most effective methods of characterizing resistance mechanisms is to determine whether the resistance is expressed at the single-cell level. Albiach-Martí et al., [72] studied the nature of this CTV resistance mechanism and reported efficient multiplication of CTV in resistant *P. trifoliata* and its hybrids (Carrizo citrange, US119 and Swingle citrumelo) and *S. buxifolia* and *S. glutinosa* protoplasts (Figure 1). Thus, the resistance mechanism in these plant species affects a viral step subsequent to replication and assembly of viral particles, probably preventing CTV movement. Similar results were obtained in CTV inoculation experiments of resistant pummelo and sour orange protoplasts (Albiach-Martí, unpublished data). Likewise, the CTV systemic infection of Duncan grapefruit (a descent of pummelo) and sour orange plants was examined using a stable virus-based vector *CTV-BC5/GFP*, which was generated from the *T36-CTV9* recombinant virus (Figure 7) [9]. The susceptible host *C. macrophylla* and Mexican lime and the tolerant host sweet orange were used as controls [62]. CTV infection sites, after cell to cell movement, consisted of clusters of 3 to 12 cells in the susceptible species, while in Duncan grapefruit and sour orange there were fewer CTV

infection sites and they were usually single cells, indicating absence of cell to cell movement in both cases (Figure 7) [62]. However, the long-distance movement mechanism of CTV appears to be inefficient in some extend, since the majority of phloem-associated cells in the bark flaps have not been infected, even for *C. macrophylla* and Mexican lime susceptible hosts (Figure 7) [62]. In these experiments, accumulation of T36 seemed related to host susceptibility. Actually, the hypothesis points to plant silencing as a probable cause of this resistance mechanism [62]. However, inadequate interactions of the CTV host range determinants (p33, p18 and p13) [19] with the host factors, which allow viral movement, have not been discarded.

8. *Citrus tristeza virus* genetic determinants related with aphid transmission

While CTV dispersal between new areas or countries occurs by graft propagation of virus-infected plant tissues, aphid transmission is responsible of local spread [1]. Viruliferous aphids of *Toxoptera citricida* (Kirkaldy) and *Aphis gossypii* (Glover) species are able to transmit CTV in a semipersistent manner [1] (Figure 1). However, *A. spiraecola* (Patch) and *T. aurantii* (Boyer de Fonscolombe) have also been reported as CTV vectors, although with less efficiency than *A. gossypii*. The aphid *T. citricida* is the most effective transmitting CTV and the most efficient and fast in the spatial and temporal viral spreading in citrus orchards. Moreover, when *T. citricida* appears in a new citrus area, the interaction between CTV and *T. citricida* seems to shift a specific mild or QD viral population to severe SP one [5]. This incidence suggests that *T. citricida* is more effective transmitting the minor virulent SP populations than the endemic mild or QD CTV genotypes. Citrus is the primary host of *T. citricida*, while *A. gossypii* populations build up in other crops. Probably *T. citricida* evolved with citrus and CTV and this could explain its high efficiency transmitting this virus [3].

The CTV genes or sequences related with aphid transmission are mostly unknown. However, usually for viral transmission, a *helper component* or the CTV virion has to interact with the mouthparts and the foregut of the aphids. Therefore, the protein components of the CTV particles (CP, CPm, HSP70h, p61) are candidates for aphid transmission determinants. In fact, CPm, which composes the particle tail structure of *Lettuce infectious yellows virus* (LIYV) (genus *Crinivirus*, family *Closteroviridae*), a close relative to CTV, is involved in viral transmission by *Bemisia tabaci* [75]. Similarly, the CTV CPm is suspected to affect aphid transmission [73, 74]. Comparison of CPm protein sequences from transmissible and non- transmissible CTV strains yield five mutations that appear to be conserved in transmissible CTV strains. These ones could affect aphid transmission efficiency by altering the conformation of the protein or masking motifs, which could be involved in the interaction between CPm and aphid stylet [76]. Although the special abilities of *T. citricida* are partially explained by its high efficiency in viral transmission [3], it seems that could be distinct interaction of this aphid with the coat proteins corresponding to different CTV genotypes. Additionally, the transmission mechanism of CTV by *A. gossypii* may possibly be, to some extent, different to the one by *T. citricida*.

9. Conclusions

Citrus tristeza virus research continues pushing the molecular virology technology to further limits. Molecular tools have been developed to study CTV gene expression, replication, assembly, systemic infection, viral movement, and plant-host interactions. The scientific results reveal a virus with a complex genetics that has become a model for molecular virology studies and viral biotechnology development. However, in spite of the CTV complicated genetics, further efforts need to be applied to engineer viral-based vectors, or additional biotechnological approaches, with the aim of understanding the mechanisms of viral movement, pathogenesis, resistance and aphid transmission, and the role of the D-RNAs in the CTV infection or the pathogenesis process. This valuable information will be applied to implement biotechnological strategies in order to control the devastating CTV epidemics.

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