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## Molecular Virology and Pathogenicity of *Citrus tristeza virus*

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### 1. Introduction

*Citrus tristeza virus* (CTV) (genus *Closterovirus*, family *Closteroviridae*) is the largest identified RNA virus infecting plants and the second largest worldwide after the animal *Coronaviruses*. Unlike from most elongated viruses, CTV particles are bipolar flexuous helicoidal filaments of 2000 × 11 nm, having two different capsid proteins that coat the opposite ends of the virions (Febres et al., 1996; Kitajima et al., 1964; Satyanarayana; et al., 2004). The viral genome consists of a long single-stranded positive-sense RNA molecule (gRNA) of around 19.3 kb (Karasev et al., 1995), a size that defies the theoretical predictions on the upper limit of RNA size found in nature, based on the high error frequency of RNA polymerases (Domingo & Holland, 1997). The viral genome encodes twelve open reading frames (ORFs), which potentially express at least nineteen protein products, and contains two non-translated regions (NTR) at the 5' and 3' terminus (Figure 1) (Karasev et al., 1995). CTV infects most species, cultivars and hybrids of *Citrus* and related genera, and it is transmitted vegetatively (via infected budwood) and by aphids (*Hemiptera: Aphididae*) in a semipersistent manner. While aphid transmission is responsible of local spread, CTV dispersal to new areas or countries occurs by graft propagation of virus-infected plant tissues (Bar-Joseph et al., 1989).

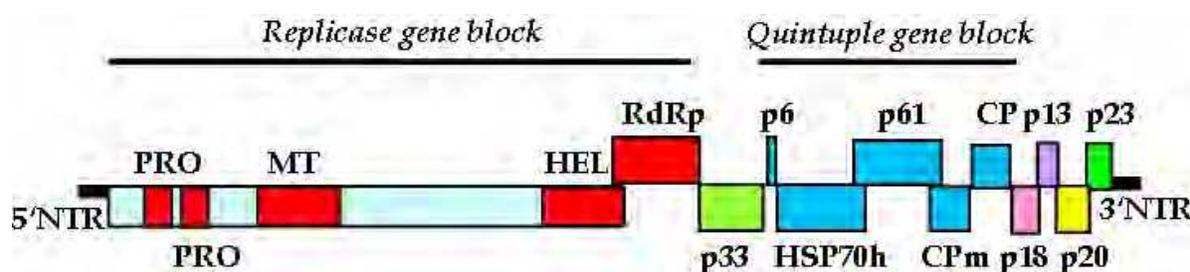


Fig. 1. Organization of CTV genome. PRO, MT, HEL and RdRp indicate protein domains of papain-like protease, methyltransferase, helicase and RNA-dependent RNA polymerase, respectively. HSP70h, CPm and CP indicate ORFs encoding a homologue of heat shock protein 70, the minor and the major capsid proteins, respectively. NTR indicates non-translated region.

*Citrus* and related genera were originated 20 million years ago in South Eastern Asia and the Malayan archipelago (Scora, 1988). CTV probably co-evolved with its host plant during centuries. There are at least four *Citrus* progenitors reported to be the origin of all of the

current citrus varieties in agriculture: citron (*C. medica* L.), pummelo [*C. grandis* (L.) Osb.], mandarin (*C. reticulata* Blanco) and *C. micrantha* L. (Nicolosi et al., 2000). The fact that many CTV isolates are symptomless in most of these citrus species supports this hypothesis (Moreno et al., 2008). The CTV-citrus pathosystem was created by mankind with the advent of the commercial citrus industries during the last two hundred years (Bar-Joseph et al., 1989). Since transport of citrus plants was difficult for centuries, they were transported and grown from seeds, which were free of CTV. Improvements in maritime transport during the XIX and XX centuries enabled the movement of intact citrus plants, which often contained different CTV genotypes, from Asia to other regions, where the virus interacted and evolved in different environmental conditions. The establishment of the modern commercial citrus industries also brought the use of new varieties and cultivars, along with new combinations citrus variety/rootstocks (which is the way as the commercial citrus trees are generally grown in the field), to increase the fruit productivity. After two hundred years, both mankind and aphid transmission have made this virus endemic in most of the citrus-growing areas, with only a few places in the Mediterranean basin and Western USA free of CTV infections. The spreading of the virus to new citrus areas resulted in the death of one hundred million trees. The actual economic damages to the citrus industries worldwide depend on the environmental conditions, the resident CTV pathotypes and the sensitivity to CTV infection of the local citrus varieties or scion-rootstock combinations. The endemic CTV population of some citrus areas is composed by mild or severe, but controllable, strains. These commercial citrus industries are continuously threatened by the possible introduction of exotic and almost uncontrollable CTV isolates of higher virulence (Bar-Joseph & Dawson, 2008). In order to avoid risks derived from introduction and dispersal of highly virulent isolates, methods are needed to discriminate the indigenous from any newly introduced severe isolates. Consequently, there is a considerable interest in mapping CTV disease determinants as well as studying their pathogenicity mechanisms.

In order to infect a plant, the virus first needs to enter in the cell and to overcome the constitutive and/or inducible plant defences to program the plant cellular machinery for its viral multiplication, followed by the systemic invasion of the plant, thus interacting with the host and inducing disease (Culver & Padmanabhan, 2007). *Citrus* genus contains many species, varieties, and intergenic hybrids with which CTV could interact causing a range of physiological and biochemical responses. In some cases, CTV invades the plant and is asymptomatic, in others the virus induces disease, and in others the plant is resistant to all or some CTV genotypes (Garnsey et al., 1987, 1996, 2005). Furthermore, within the CTV-*Citrus* pathosystem, the plant interacts not only with the virus, but also with the insect vector. In other plant virus pathosystems, also involving aphid transmission, the biochemical interactions between the virus and its host plant affect the fitness of its arthropod vectors, therefore modulating their own spread and connecting pathogenicity with effective viral transmission (Ferreles & Moreno, 2009). In order to develop methods to discriminate between pathotypes (severe, disease-causing isolates and mild or symptomless isolates) and to engineer reliable and enduring biotechnological strategies to control the diseases induced by CTV, it is essential to understand the processes that occur during CTV-citrus interactions and that connect CTV-infected plants and insect vector interactions, which lead to symptoms development and viral spread.

The genetic research on CTV was hindered for a long time due to the difficulties for experimenting with a virus with a large RNA genome, encapsidated in fragile particles and

present in reduced amounts in a tree host, thus difficult to isolate and characterize. Afterwards, the elevated complexity of CTV genetics, aphid transmission and viral populations of highly divergent genotypes, and the myriad of phenotypes induced depending on the *Citrus* host and the CTV strain, challenged the study of the viral genetics and the virus-host interactions (Bar-Joseph & Dawson, 2008; Moreno et al., 2008). In the last decades, efforts were made to develop molecular techniques to improve CTV detection and genotype differentiation. However, a remarkable achievement was the development of reverse genetics to overcome the challenges of mapping the CTV genes involved in CTV-citrus interactions, particularly the pathogenic determinants (Albiach-Marti et al., 2010; Satyanarayana et al., 1999, 2001). Therefore, the generation of a cDNA clone (*T36-CTV9*, Figure 2, left panel) of the Florida (USA) isolate T36 and the development of a *in vitro* genetic system to analyze CTV genotypes, mutants and self replicating constructs in *Nicotiana benthamiana* protoplasts or indexing citrus plants (Satyanarayana et al., 1999; 2001), allowed examining CTV viral replication, gene expression and assembly. Furthermore, the T36 genetic system was modified by Gowda et al. (2005) to allow CTV agroinoculation and replication in *N. benthamiana* plants. Afterwards, the *T36-CTV9* construct was adapted to be employed as a virus-based vector (Folimonov et al., 2007) (Figure 2, center and right panels) for the study of movement and virus-host interactions (Folimonova et al., 2008; Tatineni et al., 2008). The complexity of the CTV genetics and the pathosystem established by the interaction of *Citrus*, CTV and the insect vector, plus the molecular virology advances in identification of the genetic determinants of the diseases induced by CTV, and the different technology approaches used in these studies, are discussed.

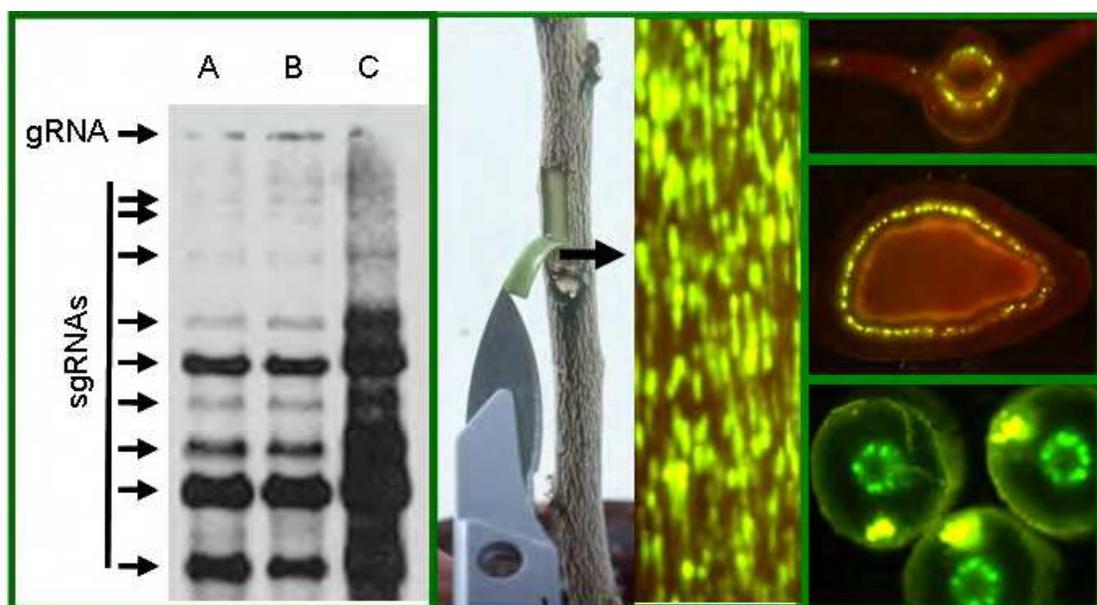


Fig. 2. *T36-CTV9* infectious clone and the *in vitro* genetic system to manage CTV. (Left panel): Northern-Blot hybridization using minus-stranded riboprobes specific to the 3' proximal genomic region. Accumulation of total RNAs from (line A) construct *T36-CTV9* in *N. benthamiana* protoplast; (line B) T36 isolate in *C. macrophylla* plants; (line C) dsRNA accumulation of construct *T36-CTV9* in *C. macrophylla* plants. (Center and right panels): Sections of *C. macrophylla* infected with construct *BCN5-GFP* visualized in a confocal microscope under UV light, indicating location of CTV in plants. (Center panel): bark flap; (Right panel): (Top) leaf, (Center) shoot, (Bottom) roots. Photos from Folimonov et al. (2007).

## 2. The complexity of the *Citrus tristeza virus* molecular genetics

The virus needs to program the plant cellular machinery for the viral multiplication and for the viral movement through the plasmodesmata and the plant vascular system to colonize the plant. Although each viral gene product seems to have primary functions that are required for the survival of the virus, there are secondary interactions that cause disease or trigger resistance response in the plant host (Culver & Padmanabhan, 2007). Additionally, the genetic variability of CTV viral populations is extremely important in order to design strategies to study pathogenic determinants and to develop reliable and perdurable biotechnological strategies of viral control (Albiach-Marti et al., 2010; Folimonova et al., 2010). In this section, the primary functions of CTV genes and the genetic variability of CTV genotypes, composing a wild isolate, will be reviewed.

### 2.1 Genome organization and functions of viral proteins

The expression of the twelve CTV ORFs is a remarkable process that 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'-coterminally sub-genomic RNAs (sgRNA) (Karasev et al., 1997). The organization and expression of the 19.3 Kbs of the CTV genome resembles that of *Coronaviruses*, but phylogenetically the CTV polymerase, like in other *Closteroviruses*, belongs to the Sindbis virus-like lineage (Karasev et al., 1997). The *replication gene block*, which is conserved in the family *Closteroviridae* and in the supergroup of sindbis-like viruses, comprises ORF 1a and 1b and makes up the 5' half of the genome and encodes, as indicated the replication machinery (Figure 1) (Dolja et al., 2006). The ORFs 1a and 1b are directly translated from the positive stranded gRNA to yield a 400 kDa polyprotein that is later proteolytically processed into at least nine protein products (Karasev et al., 1995). 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 that is occasionally translated after ORF 1a by a +1 ribosomal frameshifting (Karasev et al., 1995).

The other 10 ORFs, located at the 3' half of the CTV genome, are expressed by the synthesis of a set of 3' co-terminal subgenomic RNAs (sgRNAs). Each 3' sgRNA serves as a messenger for the translation of its 5' proximal ORF (Hilf et al., 1995; Karasev et al., 1997) and the expression of each of the ten 3' proximal ORFs is regulated independently both in amount and timing (Hilf et al., 1995; Navas-Castillo et al., 1997). Part of the CTV 3' ORFs are enclosed in the conserved *quintuple gene block* (Figure 1), another hallmark of the *Closteroviridae* family that is related with virion assembly and trafficking (Dolja et al., 2006). This consists of the major coat protein (CP of 25kDa), the minor capsid protein (CPm of 27kDa) (Febres et al., 1996), p61, HSP70h (a p65 kDa protein homologue of the HSP70 plant heat-shock proteins) and p6 (a small hydrophobic protein that belongs to the single-span transmembrane proteins) (Karasev et al., 1995). About the 97% of the CTV genome is coated by CP, while the remainder 3% is encapsidated by CPm resulting in viral particles with the emblematic tail of the members of the *Closteroviridae* family (Dolja et al., 2006; Febres et al., 1996; Satyanarayana et al., 2004). The coordinate action of HSP70h and p61, in addition to

the CP and CPm coat proteins, are required for proper assembly of CTV virions (Satyanarayana et al., 2000). During CTV assembly, HSP70h or p61 bind to the transition zone between CP and CPm (around 630 nt) and restrict CPm to the virion tail (Satyanarayana et al., 2004; Tatineni et al., 2010). The protein homologous to HSP70h and p61 in *Beet yellows virus* (BYV) (genus *Closterovirus*) are coordinately assembled with CPm in the virion structure and remain attached to the viral particles (Dolja et al., 2006). However, the assembly of HSP70h and p61 has not been directly confirmed for CTV (Satyanarayana et al., 2004). Although unnecessary for virus assembly or replication, p6 is required for systemic invasion of host plant (Tatineni et al., 2008) and probably functions as a movement protein, similarly to its homologue in BYV (Dolja et al., 2006).

The additional five CTV ORFs located at the 3' half of the genome (Figure 1) are the p20 ORF, an homologue of p21 of BYV, and four genes encoding proteins with no homologue in other closteroviruses (p33, p18, p13 and p23) (Dolja et al., 2006). The p20 protein is the main component of the CTV-induced amorphous inclusion bodies (Gowda et al., 2000) and, as well as p6, is needed for CTV systemic infection, thus suggesting a possible role in CTV translocation in the citrus plant (Satyanarayana et al., 2001; Tatineni et al., 2008). Unexpectedly, ORFs that encode proteins p33, p18 and p13 are not required either for replication or assembly (Satyanarayana et al., 1999, 2000) or for systemic infection of Mexican lime [*C. aurantifolia* (Christm.) Swing.] and *C. macrophylla* Wester plants (Tatineni et al., 2008). These three genes are CTV host range determinants (Tatineni et al., 2011). The p33 gene is essential for complete infection of sour orange (*C. aurantium* L.) and lemon [*C. limon* (L.) Burm. f.] trees. The p33 plus p18 and the p33 plus p13 are required for systemic infection of grapefruit (*C. paradisi* Macf.) and of calamondin (*C. madurensis* Lour.) trees, respectively (Tatineni et al., 2011). Part of the plant antiviral defense consists of the post-transcriptional gene silencing (PTGS) mechanism. Viruses have evolved developing genes to suppress this plant mechanism (Qu & Morris, 2005; Voinnet, 2005). Unusually, CTV evolved ending up with three proteins that act as RNA silencing suppressors in *N. benthamiana* and *N. tabacum* plants. The p23 inhibits intercellular RNA silencing, while CP impedes intracellular RNA silencing and p20 obstructs both inter and intracellular RNA silencing (Lu et al., 2000). Additionally, the multifunction protein p23 contains a Zn finger domain that binds cooperatively both ssRNA and dsRNA molecules in a non-sequence specific manner (López et al., 2000) and it controls asymmetrical accumulation of positive and negative RNA strands during viral replication, ensuring the presence of enough quantity of positive gRNA ready for virion assembly (Satyanarayana et al., 2002b). The CTV genes or sequences related with aphid transmission are unknown. However, for viral transmission, unknown *helper component* or CTV virions have to interact with the mouthparts and the foregut of the aphids (Ng & Falk, 2006). Therefore, the structural proteins, CP, HSP70h, p61 (Satyanarayana et al., 2004) and especially the CPm, which composes the CTV particle tail structure, are suspected to affect aphid transmission (Barzegar et al., 2009; Febres et al., 1996). In fact, the CPm of *Lettuce infectious yellows virus* (LIYV), a close relative to CTV (genus *Crinivirus*, family *Closteroviridae*), is involved in viral transmission (Steward et al., 2010).

The 3' and 5' non-translated regions (Figure 1) contain the cis-acting elements indispensable for CTV replication (Satyanarayana et al., 1999). The 5' termini of the CTV genome is protected with a cap structure (Karasev et al., 1995). CTV 5' NTR is the most variable genomic region with nucleotide identities as low as 42% among some CTV isolates (Figure

3) (Albiach-Marti et al., 2000b; López et al., 1998). Remarkably, the CTV 5'NTR secondary structure is similar even for divergent genotypes and folded in two stem-loops separated by a short spacer region (Gowda et al., 2003b, 2009; López et al., 1998). This secondary structure contains the sequences necessary for both replication and particle assembly (Gowda et al., 2003b; Satyanarayana et al., 2004; Tatineni et al., 2010). Opposite to the sequence divergence of the CTV 5'NTRs, the 3' NTR sequences are almost identical (Figure 3) (Harper et al., 2010; López et al., 1998). The 3'NTR lacks a poly-A tract and does not appear to fold in a tRNA-like structure (Karasev et al., 1995) but instead consist in a secondary structure of 10 stem-loop structures, which contain the sequences necessary for minus-strand initiation for the CTV gRNA and the sgRNAs (Satyanarayana et al., 2002a)

## 2.2 Viral RNA species generated during *Citrus tristeza virus* replication

CTV replication is an extraordinary process that generates at least 35 different species of viral RNA in CTV-infected cells (Gowda et al., 2001) plus a myriad of defective RNAs (D-RNAs) (Albiach-Marti et al., 2000a; Ayllon et al., 1999a; Mawassi et al., 1995). The positive to negative-stranded total RNA (gRNAs plus sgRNAs) ratio, approximately 40 to 50:1, falls within the range of the genomic RNAs of most positive-stranded RNA viruses, particularly the more similar *Alphavirus* supergroup and large complex viruses of the *Nidovirales* (Satyanarayana et al., 2002a). Viral replication starts when the CTV replicase generates a negative-stranded gRNA using as template the positive stranded CTV genome. Apart from the gRNA molecules, CTV accumulates high quantities of single- and double- stranded sgRNAs generated during the expression of the ten ORFs situated at the 3' half of the CTV genome (Hilf et al., 1995). Unlike the large animal viruses of the *Nidovirales*, the 3' sgRNAs of CTV do not share a common 5' terminus. The synthesis of each sgRNA is controlled by its corresponding cis-acting element [controller elements (CEs)] (Ayllon et al., 2005; Gowda et al., 2001; Karasev et al., 1997). In addition of the plus and minus stranded 3' coterminal sgRNAs, the CEs corresponding to each of the ten 3' ORFs produce a reduced amount of a set of 5' coterminal positive-stranded sgRNAs, probably due to premature termination during the synthesis of the gRNA (Gowda et al., 2001). In addition, CTV generates significant amounts of *low molecular-weight tristeza*, LMT1 and LMT2, a two positive-stranded 5' coterminal sgRNAs population with heterogeneous 3' termini at nt 842-854 and 744-746, respectively (Che et al., 2001), which are produced and accumulated differently (Gowda et al., 2003a; 2009). LMT1 is likely created by premature termination during genomic RNA synthesis at a 5' CE sited in the PRO I domain of replicase (Gowda et al., 2003a). Nevertheless, the LMT2 production is correlated to virion assembly although its exact viral function is unknown (Gowda et al., 2009).

In addition to the 35 different species of RNA created during replication, CTV could accumulate considerable amounts of D-RNAs in infected cells, probably originated during the generation of the positive-stranded sgRNA or gRNA by a template-switching mechanism (Ayllón et al., 1999a; G. Yang et al., 1997). Generally, D-RNAs bear a genome from about 2.0 to 12.0 kb and are composed by variable portions of the 3' and 5' termini of CTV genomic RNA with large internal deletions (Mawassi et al., 1995a; Ayllón et al., 1999a; Che et al., 2003). Moreover, some of them resembled the RNAs 1 and 2 distinctive of the bipartite *Criniviruses*, also included in the *Closteroviridae* family (Che et al., 2003). Apart from of their size, they vary also in sequence, and could be encapsidated into particles and be

transmitted by aphids (Albiach-Martí et al., 2000a; Mawassi et al., 1995a, 1995b). D-RNAs need to use the viral machinery for their survival (Pathak & Nagy, 2009). CTV D-RNAs replication *in trans* required a minimal 5' proximal region of 1kb and a 3' terminus limited to the 3' NTR. In addition, efficient replication 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 (Mawassi et al., 2000). In other viral pathosystems, D-RNAs have the capacity of interfering in the viral replication of their helper virus (named defective interfering (DI) RNAs) (Pathak & Nagy, 2009), but this function was not confirmed for CTV (Mawassi et al., 2000) and thus, their biological role is presently unknown.

### **2.3 *Citrus tristeza virus* viral diversity: From the extreme genomic divergence to the genetic stasis**

CTV isolates from different host and areas exhibit great variability either biologically or genetically (Moreno et al., 2008). There are CTV isolates that consist basically of a main genotype and its quasispecies (Albiach-Martí et al., 2000b; Satyanarayana et al., 2001), but others are composed by two or more different CTV genotype-related groups which are recognized as strains. These could vary in the pathogenicity induced depending on the citrus host and in transmission efficiency by aphids (Bar-Joseph & Dawson, 2008; Moreno et al., 2008). The strains composing a field population are unequally distributed within a CTV infected tree (d'Urso et al., 2000) and could be somehow separated from the former mixture by aphid or graft transmission or by host passage creating a new mixture of strains, thus a new isolate, which could generate a completely distinct symptomatology in citrus plants (Albiach et al., 2000a; Ayllon et al., 1999b; Moreno et al., 1993; Roy & Brlansky, 2009; Velazquez-Monreal et al., 2009; Weng et al., 2007). Additionally, aphid or graft transmission and host passage could modify the composition of the D-RNAs population in the CTV isolates (Albiach-Martí et al., 2000a; Mawassi et al., 1995b)

The sequencing of the genomes of nineteen CTV isolates from distant places in the planet, which represented a subset of its local CTV population, helped the understanding of the evolution of CTV and the complex structure of the actual CTV isolates. These genomic sequences are T36 and T30 from Florida, USA (Albiach-Martí et al., 2000b; Karasev et al., 1995); VT from Israel (Mawassi et al., 1996); SY568R from California (Z.N. Yang et al., 1999; Vives et al., 2005); T385 and T318A from Spain (Ruiz-Ruiz et al., 2006; Vives et al., 1999); NuagA from Japan (Suastika et al., 2001); Qaha (AY340974) from Egypt; Mexican isolate (DQ272579); B165 from India (Roy & Brlansky, 2010); NZ-M16, NZ-B18, NZRB-TH28, NZRB-TH30, NZRB-M12, NZRB-M17 and NZRB-G9 from New Zealand (Harper et al., 2009, 2010); and HA16-5 and HA18-9 from Hawaii (Melzer et al., 2010). Comparison of CTV genomes yielded nucleotide identities from 79.9% (between Qaha and VT) to 99.3% (between T30 and T385) (Melzer et al. 2010). Phylogenetic analysis grouped the CTV genome diversity in seven clades reflecting six main severe sequence-related groups [(1) T36-like (T36, Qaha, Mexican); (2) the NZRB strains plus HA18-9; (3) the VT-like (VT, NUagA, T318A, SY568); (4) HA16-5; (5) B165 and NZ-B18; (6) NZ-M16] and one asymptomatic genotype [the T30-like (T30, T385)] (Harper et al., 2009; Melzer et al. 2010). Moreover, the T36-like genotypes and close relatives [clades (1) and (2)] show an unusually high genetic distance to the other CTV genotypes in spite of belonging to the same taxonomic entity. The divergence between these two groups of genotypes is mostly concentrated at the 5' half of

the genome and increases towards the 5'NTR (Figure 3) (Bar-Joseph & Dawson, 2008; Hilf et al., 1999; Mawassi et al., 1996). In this way, 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) (Albiach-Martí et al., 2000b). Conversely, genomic sequence divergence between the CTV genotypes included in clades (3) to (7) increase slightly in the 5'NTR region but is relatively constant in proportion and distribution along the genome (Figure 3) (Hilf et al., 1999; Melzer et al. 2010). Based in this two paths of sequence divergence (Hilf et al., 1999) it was speculated that the T36 genotype and relatives evolved from a recombinant of a CTV genome and an unknown virus millions of years ago in Asia (Mawassi et al., 1996). This high genomic divergence between distinct genotypes confirmed the genetic variability found between strains composing the CTV isolates.

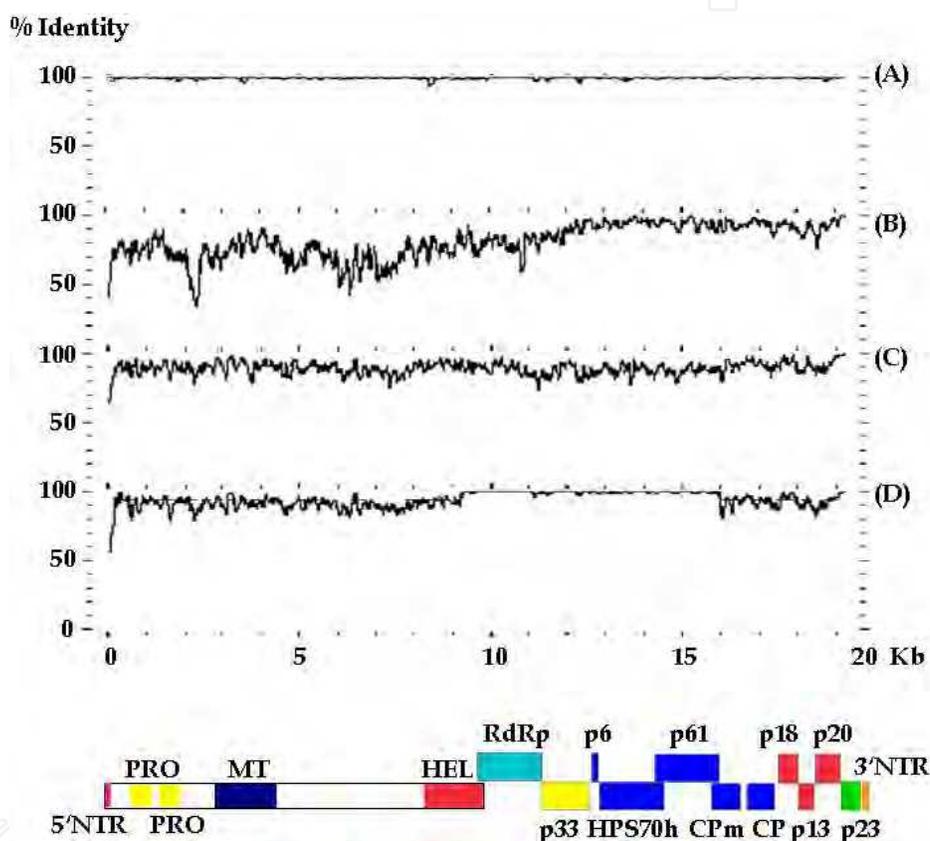


Fig. 3. Graphic of the sequence identities along the CTV genome between T30 genotype and (A) T385 (B) T36, (C) VT and (D) SY568 genotypes (Albiach-Martí et al., 2000b). PRO, MT, HEL and RdRp indicate protein domains of papain-like protease, methyltransferase, helicase and RNA-dependent RNA polymerase, respectively. HSP70h, CPm and CP indicate ORFs encoding a homologue of heat shock protein 70, the minor and the major capsid proteins, respectively. NTR indicates non-translated region.

In spite of this genetic variability, Albiach-Martí et al. (2000b) reported that the genomes of the symptomless isolates T30 from Florida and T385 from Spain, which were separated geographically and in time, were essentially identical (Figure 3). Moreover, these authors demonstrated that the T30/T385 genotype was distributed around the world and it could have been stable at least 500 years, which suggests that the T30/T385 genotype is well adapted to the citrus environment. Afterwards, this genetic similarity was found for other

CTV genotypes separated geographically and in time like T36 from Florida and Qaha from Egypt. Although, a great capacity for rapid evolution is a common feature of RNA viruses (Domingo & Holland, 1997), there are examples of long genetic stability in animal (Nakajima et al., 1978) and plant (Blok et al., 1987; Fraile et al., 1997) viral RNA populations, which were reported to be nearly identical after 22 years and from 100 to 13,000-14,000 years, respectively. This genetic stasis 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 (Blok et al., 1987; Fraile et al., 1997). Therefore, if some CTV sequences tend to remain relatively stable over periods of years, sequence-based control strategies like transgenic plants based in PTGS or cross-protection using recombinant mild CTV strains, have a higher probability of success (Albiach-Martí et al., 2000b, 2010; Folimonova et al., 2010).

A possible hypothesis to explain the actual high sequence variability found in the CTV isolates could be based in the fact that the main CTV genotypes evolved in different *Citrus* progenitors at its point of origin in Asia. Afterwards, the high viral diversity found *intra* and *inter* CTV isolates could have been generated by two processes acting in parallel. In one hand, the dispersal of the main CTV genotypes to different environments around the world by vegetative propagation of citrus, followed by the exposure during decades of the citrus-infected trees to repetitive inoculation by the natural aphid population, and by cultural practices like graft transmission, would create founder effects or bottlenecks. These could change the frequency of sequence variants within field isolates. On the other hand, RNA virus mutation due to the error-prone nature of RNA-dependent RNA polymerases (Domingo & Holland, 1997), in addition to recombination events between diverged sequence variants, plus selection, genetic drift and gene flow, possibly might allow newly arising mutants to shift the sequence variants distribution of the isolates, becoming prevalent in CTV populations, and promoting rapid evolution (Moreno et al., 2008). In this context, identifying a specific genetic determinant that is responsible for a specific disease symptom under field or glasshouse conditions is, in the case of CTV, a real challenge.

### 3. Virus-host interactions in the *Citrus tristeza virus* pathosystem

*Citrus tristeza virus* natural plant hosts belong to the order *Geraniales*, family *Rutaceae*, subfamily *Aurantoidea*. Most of them are included in the genus *Citrus* L. except for kumquats (*Fortunella* spp) and other *Citrus* relatives (*Aegle*, *Aeglopsis*, *Afraegle*, *Atalantia*, *Citropsis*, *Clausena*, *Eremocitrus*, *Hesperthusia*, *Merrillia*, *Microcitrus*, *Pamburus*, *Pleiospermium*, and *Swinglea*). However, there are also non-rutaceous hosts that have been experimentally infected with CTV strains like *Passiflora gracilis* and *Passiflora coerulea* (Bar-Joseph et al, 1989; Moreno et al., 2008), and *N. benthamiana* protoplasts or agroinfiltrated leaves (Gowda et al., 2005; Navas-Castillo et al., 1997). When CTV interacts with the plant host there could be several plant responses. Depending on the CTV strain and the specific citrus host or variety/rootstock combination, CTV interactions with a particular citrus host might be pathogenesis or asymptomatic or from limited to complete plant resistance. In this way, Citron, Mexican lime, *C. macrophylla*, sour orange and lemon seedlings are usually susceptible to CTV infection. In addition, mandarins, clementine (*C. clementina* Hort. ex Tan.), satsuma [*C. unshiu* (Mak.) Marc.] and the citrus hybrids tangelos (mandarin × grapefruit or pummelo) and tangors (mandarin × sweet orange), as well as some pummelos and citrumelos (grapefruit × *P. trifoliata*), are among the commonly tolerant hosts to CTV.

Sweet orange [*C. sinensis* (L.) Osb.] and grapefruit could be susceptible or tolerant depending on the CTV pathotype (Bar-Joseph et al., 1989; Garnsey et al., 2005). Finally, pummelos, grapefruit, sour orange and the rootstock Swingle citrumelo exhibit a differential degree of resistance depending on the CTV strain (Bernet et al., 2008; Garnsey et al., 1996; Fang & Roose, 1999; Folimonova et al., 2008). Whereas, some *Citrus* relatives, within subfamily *Aurantioideae* like *Poncirus trifoliata* (L.) Raf., *Swinglea glutinosa* (Blanco) Merr., and *Severinia buxifolia* (Poir) Ten, as well as *P. trifoliata* intergeneric hybrids like citranges (sweet orange × *P. trifoliata*), remain resistant or immune to most of the CTV strains (Garnsey et al., 1987; Yoshida, 1985, 1993). Therefore, there is an elevated complexity in the *Citrus*-CTV interactions that generate processes like pathogenesis or plant host resistance. Although there have been considerable advances in the study of the CTV genetics, the interaction between CTV and *Citrus* and in particular the mechanisms involved in the development of disease or plant resistance, are still poorly understood. Interactions between CTV and citrus could also affect the performance of the CTV arthropod vector, therefore affecting virus spread (Ferreles & Moreno, 2009). Consequently, the different CTV-*Citrus* interactions as well the possible connection between CTV infected plant interactions and modulation of aphid transmission are reviewed in this section.

### 3.1 *Citrus tristeza virus* pathotypes

Phenotypically, CTV is a very complex virus, with three hallmark diseases, plus a myriad different symptom patterns in indexing plants. After the *Phytophthora* epidemics in 1836, commercial citrus varieties were mainly propagated on sour orange, a *Phytophthora* resistant rootstock exceptionally adaptable to all soil types that generates excellent fruit quality and elevated productivity. *Tristeza* disease or *Quick decline* (QD), the first known syndrome of CTV, appeared in 1930 as sour orange rootstock resulted to be highly sensitive to CTV (Figure 4). The QD syndrome consists in the rapid death of the commercial varieties sweet orange, mandarin, grapefruit, Kumquats or limes on sour orange rootstock in field conditions (Bar-Joseph & Dawson, 2008; Moreno et al., 2008). During the development of QD the sieve tubes and companion cells close to the bud union between the scion and the rootstock collapse and necroses, producing an excessive amount of non-functional phloem (Schneider, 1959). This generates overgrowth of the scion at the bud union, loss of root mass, and therefore, drought sensitivity, stunting, leave chlorosis, reduced fruit size, poor growth, dieback, wilting and finally death of the tree. The QD symptomatology explains the disease name of *Tristeza*, which means "sadness" in Spanish and Portuguese. The CTV QD pathotype has been devastating for the commercial citrus industries around the world, since it has caused the death of hundred million trees worldwide. Moreover, the QD syndrome forced the transformation of a citrus industry based in sour orange rootstock to other established on *Tristeza*-tolerant rootstocks, which generate damages from soil salinity or alkalinity, water logging in heavy soils, or sensitivity to soil fungi and lower fruit yield than sour orange rootstock. The QD pathotypes are distributed in most of the citrus-growing areas, except a few places in the Mediterranean basin and Western USA. The second pathogenic interaction between citrus and CTV, *Stem pitting* (SP) (Figure 4) was first observed in orchards replanted with *Tristeza* resistant or tolerant rootstocks. The disease is produced by highly virulent strains that affect commercial lime, sweet orange, and grapefruit trees grafted on any rootstock. SP consists of deep pits in the wood under depressed areas of

bark. Contrasting with QD, the SP pathotypes usually do not cause tree death, but chronically limit profitable growth of different varieties, significant reduction of plant vigor, severe stunting and low yield of unmarketable fruit, thus causing high economic losses (Bar-Joseph et al., 1989). The SP pathotypes are restricted to regions of Asia, Australia, South Africa and South America. However, these pathotypes have also been found, although at lower frequency, in California, Florida, and the Mediterranean area ((Bar-Joseph & Dawson, 2008; Moreno et al., 2008).



Fig. 4. CTV pathotypes. (Left panel): *Quick decline syndrome*. (Right panel): *Stem pitting syndrome*.

The third CTV-induced syndrome, *Seedling yellows* (SY) is observed in the greenhouse (Figure 5) but might also be found in the field in top-grafted plants. SY is characterized by stunting, leaf chlorosis and sometimes a complete cessation of growth of sour orange, grapefruit or lemon seedlings (Fraser, 1952). Although SY is not economically valuable, it can be examined in the greenhouse in a timely manner and has a substantial diagnostic value for CTV pathotype differentiation (Garnsey et al., 2005). In contrast, there are mild CTV strains (as the T30-like genotypes) that cause a complete lack of symptoms in almost all varieties of citrus, including those propagated on sour orange rootstocks, even though the virus multiplies to reach high titers (Albiach-Marti et al., 2000b). These mild isolates are common in almost all the citrus growing areas (Albiach-Marti et al., 2000b; Hilf et al., 2005; Roy et al., 2010), although their presence is frequently masked when they are present in mixed infections with severe isolates (Moreno et al., 2008).

The development of QD and SP extends over 10 to 40 years in the field, a period too long to screen the CTV pathotypes. Although the SP pathotype can be examined somehow in glasshouse conditions, there are no reliable methods to reproduce QD in those conditions (Moreno et al., 2008). Therefore, the degree of severity of a specific CTV isolate, strain or genotype usually is assessed by using indexing plants (seedlings from Mexican lime, sour orange, Madame vinous sweet orange and Duncan grapefruit), where the development of severe pathotypes could be determined in months (Garnsey et al., 2005). The majority of CTV isolates induce symptoms (vein clearing, leaf cupping, dwarfing and stem pitting), in Mexican Lime (Figure 5), the most sensitive host for CTV. In this case, the degree of CTV symptomatology ranges from the mild phenotypes, which are almost asymptomatic, to the

highly virulent CTV isolates that could kill the plant (Garnsey et al., 2005). However, there are exceptions, such as severe isolates that induce symptoms in sweet orange but not in Mexican lime (Harper et al., 2009).

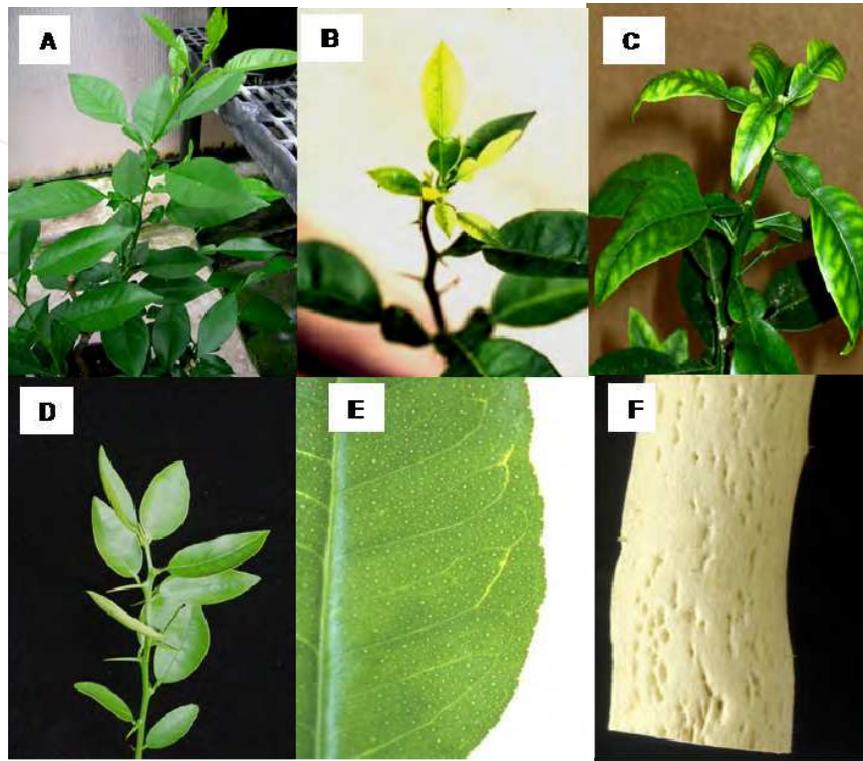


Fig. 5. CTV symptomatology in greenhouse conditions. (A) healthy sour orange plant. *Seedling yellows* syndrome in (B) sour orange and (C) grapefruit. Symptoms induced in Mexican lime: (D) leaf cupping, (E) vein clearing and (F) stem pitting.

### 3.2 Genetic determinants of the *Citrus tristeza virus* syndromes

Viruses possess the potential to disrupt host physiology by the interaction of specific viral components with the host components. During viral infection, the virus has to overcome the constitutive and/or inducible plant defences. The plant inducible defence could confine the virus and prevent systemic infection (Culver & Padmanabhan, 2007). The plant constitutive defense consist in the PTGS or the RNA interference (RNAi) pathway that implies the specific degradation of the viral dsRNA in small interfering RNAs (siRNAs), which guides a specific plant ribonuclease to degrade the viral genomes in the cytoplasm of the plant cell. Besides of the antiviral role, the gene silencing mechanism has important functions in regulating plant gene expression (miRNA metabolism) (Voinnet, 2005). Viruses contain RNAi suppressing genes or RNA silencing suppressors, allowing viral multiplication and interfering in host gene expression, thus inducing disease (Qu & Morris, 2005; Voinnet, 2005). The CTV genome contains three suppressors (CP, p20 and p23, see section 2) that block intracellular and/or intercellular RNA silencing mechanism in *N. benthamiana* and *N. tabacum* plants (Lu et al., 2000). In spite of the presence of these three silencing suppressors, accumulation of siRNAs in CTV-infected susceptible hosts could be 50% of the total RNAs in the plant (Ruiz-Ruiz et al., 2011). Moreover, the siRNAs accumulation is directly proportional

to the virus accumulation and varies depending on the CTV strain and the citrus host (Comellas, 2009). Deep sequencing analysis of siRNAs from CTV-infected plants indicated that they mainly consisted in small RNAs of 21-22 nt derived from essentially all the CTV genome (Ruiz-Ruiz et al., 2011). Although, CP, p20 and p23 have not been yet reported as RNAi suppressing genes in citrus, these three CTV genes could be candidates for symptom determinants.

Apart of CP, p20 and p23, CTV multiplication generates great quantities of other viral products (35 RNA species, 16 protein products and D-RNAs, see section 2.1 and 2.2) along with a complicated process of replication, gene expression, assembly and movement, where the interaction with host factors is essential. Consequently, during CTV-Citrus interactions there are multiple opportunities to generate disease. However, determination of which of the viral products induce a specific symptomology is a complicated task. Analysis of Mexican lime transcriptome 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, thus indicating elevated complexity in the citrus-CTV interaction during symptoms development (Gandia et al., 2007). Many attempts have been made to develop rapid diagnostics for specific CTV syndromes. Their application has led to the establishment of some correlations between various serological and molecular markers with CTV pathotypes (Hilf et al., 2005; López et al., 1998; Permar et al., 1990; Roy et al., 2010; Sambade et al., 2003). Although these molecular markers are a valuable tool for genotyping a particular CTV population, recombinants between genotypes affecting the disease determinants could be present, thus invalidating these methodologies for severe/mild strain differentiation. Additionally, direct linkage of these markers to symptom development has yet not been established. An important step through the identification of disease determinants was the sequencing of the nineteen CTV complete sequences. Their sequence comparison yielded an intriguing correspondence of the CTV phylogenetic clades (section 2.3 of this chapter) with CTV pathotypes that could point to a distribution of the symptom determinants along the CTV genome.

### 3.2.1 Genetic determinant of *Seedling yellows syndrome*

A distinctive phenotype of some isolates of CTV is the ability to induce *Seedling yellows* in sour orange, lemon and grapefruit seedlings (Fraser, 1952). The recombinant virus *T36-CTV9* and the original wild type T36 isolate produce identical SY symptoms in sour orange and grapefruit seedlings (Satyanarayana et al., 2001). T30, the type isolate of the widely distributed asymptomatic genotype T30 (T385), does not induce SY and consists of one genotype and its quasispecies (Albiach-Martí et al., 2000b). To delimit the viral sequences associated with the SY syndrome, eleven T36/T30 hybrids were generated by substituting T36 sequences for homologous T30 sequences into the 3' moiety of *T36-CTV9*, where both genome sequences (T30 and T36) are about 90% similar (Figure 3) (Albiach-Martí et al., 2010). However, hybrid constructs, which carried exchanges of T30 CP and CPm into the T36 genome, failed to passage through successive sets of *N. benthamiana* protoplasts (Albiach-Martí et al., 2010), probably due to deficient heteroencapsidation since incomplete virions do not withstand this procedure (Satyanarayana et al., 2000; 2004). Nevertheless, hybrid T30/T36 constructs [P23-3'NTR], [P13], [P61], [P18-3'NTR] and [HSP70h-P61] were sufficiently amplified to allow successful infection of the highly susceptible host *C.*

*macrophylla* by stem-slash or bark-flap inoculation (Robertson et al., 2005; Satyanarayana et al., 2001). Sour orange and Duncan grapefruit seedlings were graft-inoculated with tissues from the *C. macrophylla* plants infected with the five hybrid constructs as well as plants infected with T36 and T30 (as controls). Finally, analysis of the SY development demonstrated that the parental T36 and three of the T36/T30 hybrids induced SY symptoms while hybrid constructs [P23-3'NTR] and [P18-3'NTR] and the wild type T30 remained symptomless like the healthy controls (Figure 6, Left panel). Therefore, Albiach-Martí et al. (2010) demonstrated that SY is mapped to the region encompassing the p23 gene and the 3' NTR.

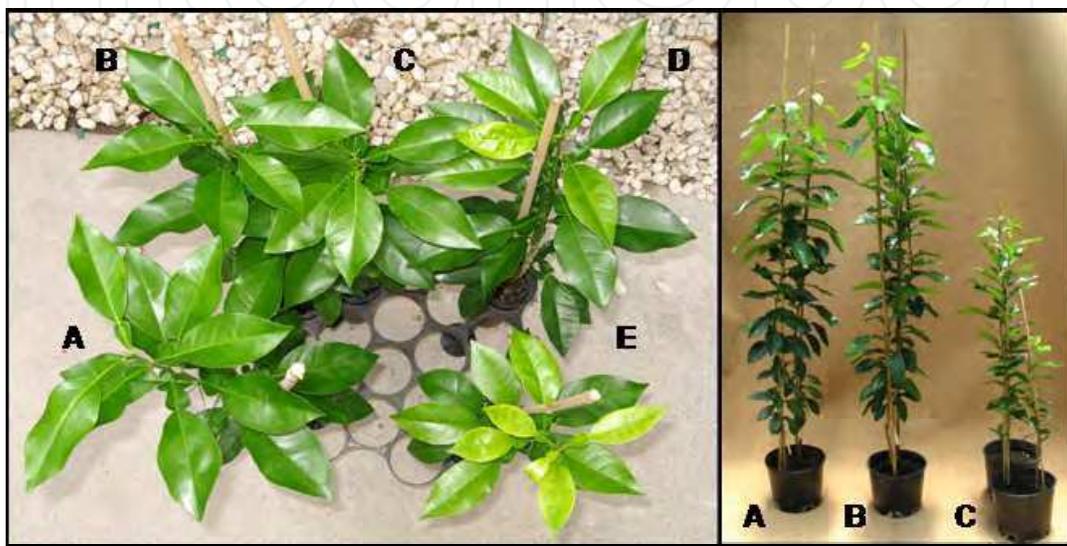


Fig. 6. (Left panel) Seedling yellows syndrome assay in sour orange seedlings inoculated with (A) T36/T30 hybrid [P23-3'NTR], (B) isolate T30 (C) healthy (D) construct T36 CTV9 and (E) T36/T30 hybrid [HSP70h-P61]. (Right panel) T36/T30 hybrid [P23-3'NTR] protects against development of SY symptoms in sour orange seedlings. Sour orange plants inoculated with (A) T36/T30 hybrid [P23-3'UTR] (B) hybrid [P23-3'UTR] and then challenged with construct T36-CTV9, and (C) T36 CTV9. Pictures from Albiach-Martí et al. (2010).

Other methodology used to map disease determinants was the expression of CTV proteins in transgenic plants (Fagoaga et al., 2005; Ghorbel et al., 2001). When p23 is ectopically expressed in transgenic citrus induces virus-like symptoms. However, transgenic Mexican lime plants develop more intense vein clearing in the plant leaves and symptomatology like chlorotic pinpoint in leaves, stem necrosis and collapse (Ghorbel et al., 2001) that differs from those induced by natural virus infection (Figure 5). Additionally, transgenic sour orange plants expressing p23 develop vein clearing, leaf deformation, defoliation, and shoot necrosis (Fagoaga et al., 2005). However, these transgene-induced symptoms differ substantially from the virus-induced SY of uniform chlorosis and stunting of new shoot growth in sour orange (Figure 5). Transgenic limes differ from virus-infected limes in that symptom severity is proportional to the levels of p23 production, not to the source or sequence of the gene (Fagoaga et al., 2005; Ghorbel et al., 2001), whereas the symptom intensity in virus-infected limes is dramatically different between severe and mild isolates of virus. Yet, the different response in transgenic plants could be related to the fact that the p23 protein is produced constitutively in most cells, while in nature the expression of p23 ORF from the viral genome is limited to phloem-associated cells (Albiach-Martí et al., 2010).

If the symptoms induced by CTV in sour orange are determined by p23, they should be related to p23 sequence and not to protein expression levels, since there was no correlation between the amount of p23 and the intensity of the SY symptoms induced by T36 or by the T36/T30 hybrids, which did not induce SY in sour orange plants (Albiach-Marti et al., 2010). Since p23 is a suppressor of RNA-mediated gene silencing, it could potentially disrupt the miRNA metabolism thus inducing the SY syndrome. Several viral silencing suppressors have been identified as pathogenicity determinants (Qu & Morris, 2005) and p23 could be the obvious candidate for being the CTV determinant of SY syndrome development in sour orange and Duncan grapefruit seedlings. However, since a viral 3' NTR has also been related to symptom development (Rodriguez-Cerezo et al., 1991), it cannot yet be concluded that the p23 protein directly induces SY. Additionally, the SY reaction is specific to only certain citrus hosts of CTV, such as lemons, sour orange and grapefruit, indicating that there are specific host factors involved in its expression in addition to the isolate-specific factors identified. Although Albiach-Marti et al. (2010) were able to map a determinant of the SY syndrome in T36, since this genotype is highly divergent from the other CTV genotypes (Harper et al., 2010; Mawassi et al., 1996), it is essential to assess whether this determinant is common to other CTV genotypes that also induce SY or if there are other possible SY determinants.

### 3.2.2 Genetic determinants of *Quick decline* and *Stem pitting* syndromes

From economic standpoint it would be highly valuable to map decline and stem pitting determinants, which could be developed into detection tools. It is possible, but not yet confirmed, that determinant(s) for the decline disease map similarly to that of SY, since a strong correlation between SY and decline has been observed in the biological evaluation of a wide range of CTV isolates (Garnsey et al., 2005). However, since some decline-inducing isolates do not produce obvious SY symptoms, the T36/T30 hybrids have to be directly evaluated in decline-susceptible grafted combinations of scion and rootstock. Unfortunately, clear decline assays need to be conducted during long periods in the field. In addition, since the hybrids are made by recombinant DNA technologies these assays require special permits from the plant protection and environmental safety authorities (Albiach-Marti et al., 2010).

In relation to the mapping of the stem pitting determinants, expression of p23 in transgenic plants of several citrus species, but not in tobacco plants, induced phenotypic aberrations resembling in some cases foliar symptoms induced by CTV, indicating that the stem pitting determinant could be also located in p23 (Fagoaga et al., 2005; Ghorbel et al., 2001). However, it seems that, in addition to p23, there are other genes related to the development of SP, at least in *C. macrophylla* plants infected with the four T36/T30 hybrids used to map the SY syndrome determinant, since the T36/T30 hybrid [p23-3'NTR] generate an attenuated phenotype for SP in this plant host (M.R. Albiach-Marti et al., unpublished data). Apart of p23, CTV genome codes for other two possible silencing suppressors in citrus plants (p20 and CP) that could be involved in the developing of QD and SP phenotypes. Consequently, there is no evidence that other CTV symptom determinants would map similarly to the SY determinant of the T36 isolate. Thus, it is necessary to promote the research of the mapping of the decline and stem pitting determinants and to discover the nature of these specific virus/host interactions.

### 3.2.3 The possible role of D-RNAs in *Citrus tristeza virus* pathogenicity modulation

Models for DI RNA-mediated reduction in helper virus levels and symptom modulation include the enhancement of the PTGS (Pathak & Nagy, 2009). At least in one case, the presence of CTV D-RNAs was suggested to modulate SY development either increasing or decreasing symptom expression (G. Yang et al., 1999). Most of the CTV D-RNAs contain a complete region p23 and the 3'NTR (G. Yang et al., 1997) that is associated with SY symptom development (Albiach-Martí et al., 2010), thus they could have a role in symptom modulation. Additionally, p23 could be a suppressor of PTGS in citrus (Lu et al., 2004), thus probably could act increasing symptom development. The isolate T30 usually generates elevated concentration of several small D-RNAs during replication in some species of citrus plants, while T36 generates sporadically small and large D-RNAs. Similarly, some of the T36/T30 hybrids infecting *C. macrophylla* also accumulated D-RNAs, which did not appear to affect the T36/T30 hybrid replication in *C. macrophylla* (Albiach-Martí et al., 2010). These D-RNAs, created during replication of the T36/T30 hybrids, were specific of the CTV *C. macrophylla* infection since the multiplication of the same T36/T30 hybrids in sour orange did not produce any D-RNA. These results suggest that the generation of the D-RNAs could depend in part on host factors. Further research would elucidate whether D-RNAs (or DI-RNAs) contribute in CTV disease modulation.

### 3.3 Citrus host resistance to *Citrus tristeza virus* infection

As mentioned above, while pummelos, grapefruit, sour orange and Swingle citrumelo exhibit a differential degree of resistance depending on the CTV strain, *P. trifoliata*, *Swinglea glutinosa*, *Severinia buxifolia*, and the citranges remain resistant or immune to most of the CTV strains (Bernet et al., 2008; Garnsey et al., 1996, 1987; Fang & Roose, 1999; Folimonova et al., 2008; Yoshida, 1985, 1993). The major component of CTV resistance in *P. trifoliata* appears to be a single-gene trait (*Ctv*) (Gmitter et al., 1996). There is little information concerning the nature of the resistance genes of *S. glutinosa* and *S. buxifolia*, but their resistance phenotypes seem to differ from that of *P. trifoliata* (Herrero et al., 1996; Mestre et al., 1997). Analysis of differential gene expression TAG libraries from CTV inoculated *P. trifoliata* tissues, yielded 289 sequences differentially expressed, mostly related with metabolism and defense responses indicating a complex resistance mechanism (Cristofani-Yaly et al., 2007). Additionally, resistance in Chandler pummelo [*C. maxima* (Burm.) Merrill] is controlled by a single dominant gene (*Ctv2*) different from the resistant gene of *P. trifoliata* (Fang & Roose, 1999). Resistance of plants to viruses results from blockage of some necessary 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 the plant defense mechanism (*active resistance*). 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., (2004) studied these CTV resistance mechanisms and reported efficient multiplication of CTV in resistant *P. trifoliata* and its hybrids Carrizo citrange, US119 and Swingle citrumelo, and in *S. buxifolia* and *S. glutinosa* protoplasts. 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 from CTV-inoculated protoplasts from resistant pummelo and sour orange plants (Albiach-Martí et al., 2004; M.R. Albiach-Martí, unpublished data).

CTV resistance in Duncan grapefruit (a descent of pummelo) and in sour orange have been investigated recently (Bernet et al., 2008; Comellas, 2009; Folimonova et al., 2008). The systemic invasion of the stable virus-based vector *CTV-BC5/GFP* (descendent of the *T36-CTV9* construct) in Duncan grapefruit and sour orange, compared to those of the susceptible hosts *C. macrophylla* and Mexican lime and the tolerant host Madam Vinous sweet orange, were examined (Folimonova et al., 2008). 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 displayed fewer infection sites limited to single cells, indicating absence of viral movement in both cases (Folimonova et al., 2008). After the analysis of the sour orange resistance to mild, SP and *T36-CTV9* CTV strains, Comellas (2009) found, similarly to Folimonova et al. (2008), a limitation of viral movement in this host. This limitation was more accentuated for T36 and mostly complete for the mild strain. However, after two years post inoculation, both, T36 and the mild strain, accumulated in sour orange similarly to in Mexican lime revealing a transitory viral resistance (Comellas, 2009), which was also noticed by Bernet et al. (2008) that using another CTV isolate and QTL-linked markers reported that CTV resistance in sour orange was distinct to that of *P. trifoliata*. Sour orange resistance to CTV infection could be due to the plant RNA silencing mechanism (Folimonova et al., 2008). However, the separate analysis of accumulation of RNA, concentration of siRNAs in plant, as well as changes in the transcriptome of sour orange during CTV-host resistance periode, indicated that the silencing mechanism was not activated as well as the known plant resistant genes (Comellas, 2009). Therefore, sour orange probably exhibits a *passive resistance* where an inefficient interaction between CTV and the host factors blocks viral movement. This plant-host interaction could be mediated by p33 gene (see Figure 1 and section 2.1), which is related with CTV systemic infection in sour orange (Tatineni et al., 2011). This resistance possibly is broken after the rising of movement competent CTV mutants. Similarly, the *resistant-breaking* (NZRB, see section 2) CTV genotype from New Zealand has been reported to overcome the resistance of the *P. trifoliata* and its intergenic hybrids and generate a SP syndrome in this host (Harper et al., 2010). The development of the NZRB genotype could be due to the extensive use of the *P. trifoliata* rootstock since the late 1920s, giving enough time to the adaptation of CTV to *P. trifoliata* host, followed by the rising of the NZRB genotypes able to overcome the resistance genes of this citrus host (Harper et al., 2010).

### 3.4 CTV-plant infected interactions and modulation of aphid transmission

One of the essential features of CTV, from the disease control standpoint, is that it is transmitted by aphids. In fact, without this feature, CTV would have been easy to eradicate by eliminating CTV-infected trees, and probably CTV strains would be less exposed to genetic variability, which could allow virulent genotypes to arise. Viruliferous aphids of *Toxoptera citricida* (Kirkaldy) and *Aphis gossypii* (Glover) are able to transmit CTV. However, *A. spiraecola* (Patch) and *T. aurantii* (Boyer de Fonscolombe) have also been reported as CTV vectors, although with less efficiency. The aphid *T. citricida* is able to transmit CTV 6 to 25 times more effectively than *A. gossypii* in greenhouse conditions, it enables experimental CTV transmission using single aphids and it is more efficient and fast in the spatial and temporal spreading of CTV in citrus orchards (Moreno et al., 2008). 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, thus this could explain its high efficiency transmitting this virus. *T. citricida* is present in almost all the citrus producing areas except the Mediterranean basin and areas of North America, where *A. gossypii* is the main vector (Cambra et al., 2000; Hermoso de Mendoza et al., 1988; Yokomi & Garnsey, 1987). However, recently *T. citricida* became established in Florida (Halbert et al., 2004) and has been detected in Northern Spain and Portugal (Ilharco et al., 2005), representing a risk to these citrus production areas on the southern Iberian Peninsula. When *T. citricida* appears in a new citrus area, where mild or QD CTV phenotypes are endemic, existing minor virulent SP populations, which were masked by the predominant mild or QD genotypes, have become prevalent. Therefore, the interaction between CTV and *T. citricida* seems to shift a specific CTV population from mild or QD phenotypes to severe SP ones (Halbert et al., 2004; Rocha-Peña et al., 1995). This special ability of *T. citricida* is partially explained by its high efficiency in viral transmission.

CTV transmission efficiency depends on the aphid species, the viral strain, the host plant and the environmental conditions, however it is not reported to be dependent on the CTV pathotype (Moreno et al., 2008). Although relationships between viral pathogenicity and aphid transmission have been barely studied (Froissart et al., 2010), it was reported that in viral pathosystems involving transmission by aphids, trips or whiteflies, viruses transform infected-plants in host of superior quality for their vectors, promoting changes in attractiveness, settlement or feeding host plant preference, together with changes on vector performance (development, fecundity, rate of population increase and survival), therefore increasing vector fitness that promotes viral spread and alters disease epidemiology (Belliure et al., 2005, 2008; Bosque-Pérez & Eigenbrode, 2011; Fereres & Moreno, 2009; Froissart et al., 2010). In a recent study, it was shown that CTV affects the fitness of its vector *A. gossypii* developing on sweet orange and Mexican lime infected with four distinct CTV-isolates (mild, QD and SP strains). CTV affected the performance of *A. gossypii* from negative to positive depending on the host plant and the virus strain. Assuming equal transmission efficiency, the frequency in field of the CTV isolates neutral or beneficial for *A. gossypii* should be higher than the frequency of detrimental ones (B. Belliure-Ferrer & M.R. Albiach-Martí, unpublished results). Similarly, the capability of *T. citricida* of shifting the CTV population could be explained by the existence of a specific interaction between the virulent strain and the citrus host that alters the aphid performance, increasing viral spread of severe strains. The links between determinants of CTV aphid transmission and the aphid vector together with the interactions between the CTV-infected host, CTV pathogenicity and the aphid fitness seems to depend on numerous factors. The elucidation of these complex and specific interactions will promote the development of better biotechnological methods to manage viral epidemiology and control CTV diseases.

### 3.5 Application of the strategies based on plant-host interaction for viral control

The control of the CTV diseases constitutes a continuous challenge (Bar-Joseph et al., 1989). General strategies include quarantine and budwood certification programs, elimination of infected trees and, as mentioned above, the use of *Tristeza*-tolerant rootstocks. Mild strain cross protection has been widely applied for millions of citrus trees in Australia, Brazil and South Africa to protect against SP economic losses (Bar-Joseph et al., 1989; Broadbent et al., 1991; Costa and Müller, 1980; Van Vuuren et al., 1993). This technique consists of deliberate

preimmunization of trees with a mild isolate of CTV that prevents or reduces the disease caused by a more virulent isolate (Fraser, 1998). However, mild strain cross-protection has not yet provided effective protection against QD isolates, and this remains an important goal since it would allow to recover the use of the sour orange, the rootstock with superior agronomic qualities (Bar-Joseph & Dawson, 2008). Additionally, incorporating resistance genes from *P. trifoliata* into commercial varieties as sour orange by conventional breeding is presently unfeasible and might need further research (Rai, 2006).

Advances in genetically engineered protection against viruses by the generation of transgenic plants have lately been remarkable. However, incorporation of pathogen-derived resistance by plant transformation of CP and p23 or the 3'NTR has yielded variable results (Cervera et al., 2010; Dominguez et al., 2002; Fagoaga et al., 2006; López et al., 2010). Another biotechnological approach to control the virus, and eventually turn it from a pathogen into a molecular tool for citrus improvement, is the custom engineering of a recombinant mild strain cross-protection (Albiach-Martí et al. 2010). Wider application of natural mild strain cross-protection has been limited by difficulty in finding mild isolates of CTV that effectively protect against SP and QD pathotypes (Bar-Joseph et al., 1989). Another problem is that natural mild CTV isolates may contain minor severe stem pitting variants that, upon aphid transmission, could become prevalent (Moreno et al., 1993; Velazquez-Monreal et al., 2009). Since only isolates within a closely related sequence group will cross-protect (Folimonova et al. 2010), naturally occurring mild T30-like isolates (Albiach-Martí et al., 2000b), would not protect against disease inducing isolates from other genotypes.

A valuable outcome was that the recombinant mild hybrid virus [P23-3'NTR] developed by Albiach-Martí et al. (2010) is able to protect efficiently citrus trees from SY caused by the parental virus (T36) (Figure 6, Right panel) and their hybrid genomic sequences are highly stable in citrus plants. The use of these recombinant hybrid constructs could offer a mechanism to custom engineer isolates that are both protective and free of disease induction potential. The stability noted in the T30/T36 constructs is also important for its application. This means that if naturally occurring mild strains cannot be found for stem-pitting or decline diseases control, it would be possible to map the disease determinant, remove it by recombinant DNA technology, and use the recombinant mild virus as a cross-protecting strain. Therefore, the potential feasibility of using engineered constructs of CTV for mitigating disease has been demonstrated (Albiach-Martí et al., 2010).

#### 4. Conclusions

Interactions between the different CTV strains and their citrus hosts assembled a complicated plant pathosystem. The large number of citrus species, cultivars, varieties and hybrids that could be infected with a virus with a large genome, complex genetics, as well as with an extreme diversity of viral populations, generates numerous possibilities of plant-host interactions. These factors complicate the study of the CTV pathogenicity and the development of reliable strategies for viral control. Although a remarkable advance in the knowledge of CTV genetics and the diversity of CTV viral populations have been achieved, the interaction between virus and host and particularly the mechanisms involved in the development of the disease are still mostly a mystery. Therefore, further attention needs the study of the interactions between viral products, the different citrus hosts and the vector transmission factors, which are the basis of pathogenicity, host resistance and viral

epidemiology. The success of the citrus management strategies depends on a deep understanding of these interactions, as well as on the elucidation of the diversity, and evolutionary relationships of the CTV isolates present in a particular citrus area to protect. In addition to make available methods to rapidly discriminate virulent from mild isolates in order to reduce risks derived from introduction and dispersal of virulent isolates and to properly monitor mild cross-protection.

Recently, pushing the molecular virology methodology to further limits, molecular tools have been developed to clone each of the CTV pathotypes and examine them individually in *N. benthamiana* protoplasts or in a particular citrus host to study the genetics and biology of the virus and virus-host interactions like pathogenicity and host resistance. However, further efforts are needed for developing additional methodologies to map the QD and SP determinants and to study their pathogenicity mechanism, as well as to elucidate the possible role of CTV D-RNAs in symptom modulation, in addition to determine the viral factors related to sour orange and *P. trifoliata* resistance and the relationships between CTV pathogenicity, aphid fitness and virus dispersal. This knowledge must be applied to elaborate appropriate quarantine and eradication programs as well as to develop biotechnological approaches of viral control, which exploit virus plant-host interactions for viral control, such as sequence-based control strategies. Resistant transgenic plants based on PTGS and self-immunization by scFv expression mechanisms, against specific viral sequences, are already developed. In addition, engineered mild strain cross-protection demonstrated its potential in excluding superinfection by severe strains. Both biotechnological strategies retain high possibilities of success in the proper management of devastating CTV diseases.

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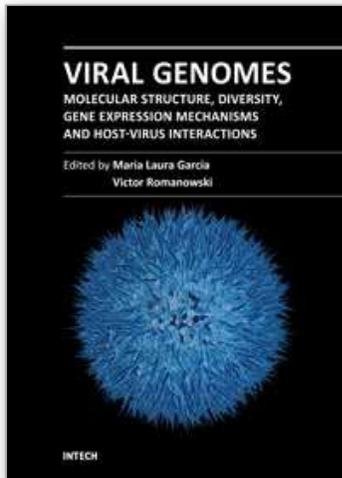
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**Viral Genomes - Molecular Structure, Diversity, Gene Expression  
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Viruses are small infectious agents that can replicate only inside the living cells of susceptible organisms. The understanding of the molecular events underlying the infectious process has been of central interest to improve strategies aimed at combating viral diseases of medical, veterinary and agricultural importance. Some of the viruses cause dreadful diseases, while others are also of interest as tools for gene transduction and expression and in non-polluting insect pest management strategies. The contributions in this book provide the reader with a perspective on the wide spectrum of virus-host systems. They are organized in sections based on the major topics covered: viral genomes organization, regulation of replication and gene expression, genome diversity and evolution, virus-host interactions, including clinically relevant features. The chapters also cover a wide range of technical approaches, including high throughput methods to assess genome variation or stability. This book should appeal to all those interested in fundamental and applied aspects of virology.

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