CITRUS TRISTEZA VIRUS

The Continuous Challenge of *Citrus tristeza virus* Molecular Research*

M. Bar-Joseph, X. Che, M. Mawassi, S. Gowda, T. Satyanarayana, Maria A. Ayllón, Maria R. Albiach-Martí, S. M. Garnsey, and W. O. Dawson

ABSTRACT. *Citrus tristeza virus* (CTV) infections of *Citrus* spp. are mainly manifested in two diseases: (i) Quick decline (QD) of many scions on sour orange, and (ii) Stem pitting (SP) of grapefruit, pummelo, and certain orange varieties. A third disease, seedling yellows of young sour orange, lemon, and grapefruit plants, is basically a controlled disease under experimental condition. Damages cause by CTV have been costly in areas infested with severe QD or SP isolates and sensitive citrus varieties, while in areas with mild isolates, trees on sour orange have remained productive for long periods. Basic research on CTV started in Brazil with the association of thread-like-particles with the tristeza disease, followed by the finding that these particles were similar in biophysical characteristics to other known elongated viruses such as *Beet yellows virus*. The complete sequence of the CTV genome was published for the Florida T36 isolate in 1995, although sequences of the viral coat protein gene and major parts from the 3' terminus were reported earlier. Two interesting findings followed: First, the observation that large portions from the 5' parts of the T36 and VT strains differ considerably, and second, the association of multiple defective RNAs in many CTV isolates. This paper describes past and present research from our laboratories on three main topics: 1) Development of infectious clones of dRNA and genomic RNA, 2) Subgenomic RNAs and strategies of CTV replication and encapsidation, and 3) The development of tools for mapping pathogenicity determinants on the genomic RNA.

Citrus tristeza virus (CTV) is the causal agent of two important diseases: 1) Quick decline (QD) of some *Citrus* sp. scions on sour orange rootstock, and 2) Stem pitting (SP) of grapefruit, pummelo and certain sweet orange varieties. A third disease, seedling yellows (SY) of young sour orange, lemon and grapefruit plants, is mainly observed under experimental conditions. Damages caused by CTV have been extremely costly in places with severe QD and SP isolates, while in locations with mild isolates, trees remain productive for long periods.

The virus nature of diseases now recognized to be caused by CTV was suspected from 1925 onwards (15, 26, 34, 37), however the basic virological research on CTV commenced only after electron microscope studies conducted in Brazil by Kitajima et al. (20) had associated thread-like-particles 2000 nm in length and 10-12 nm in diameter with infected plants. The development of methods for isolating concentrated preparations of these particles from infected bark led to their partial biophysical and molecular characterization, including their encapsidation by a coat protein with an estimated MW of 25 kDa (8). Further studies in California showed that plants infected with CTV contain relatively large amounts of different size dsRNAs, the larger one, was expected to represent an RF of 13.3×10^6 (13). Further improvements on CTV particle purification allowed the isolation of sufficient quantities of intact viral RNA molecules with an estimated molecular size of 6.5×10^6 Da (6), the largest RNA genome of known plant viruses. It is interesting to note that both estimates suggested a genomic size of about 20 kb, well within the range of the size

*Invited presentation

defined later by direct sequencing. Clones of cDNAs from the VT strain of CTV were obtained in the early 1980's (30) and certain cDNA fragments were shown to differentiate between CTV isolates, although no trait specific distinction could be established for any of these probes (29). The complete sequence of the T36 isolate CTV genome from Florida was published in 1995 (19), although major parts of this isolate including the coat protein sequence (33) and large parts from the 3' of the virus had been reported earlier (28). In a time that molecular information was doubling every decade, genomic characterization of CTV reached the molecular era relatively late, especially when compared with *Tobacco mosaic virus* whose nucleotide sequence was published in 1982 (16). Two main causes were involved in this delay, the lack of molecular biology expertise among the citrus virologists including our own group, and the lack of interest among basic molecular virologists in a task involving the sequencing of the immensely large CTV genome. In retrospect, it is however worth noting that completing such a task at the pre-PCR-era (27), was both difficult and costly beyond the resources of most CTV laboratories.

The main developments on CTV genomic analyses had followed earlier studies by Atabekov's group in Moscow on the molecular characterization of *Beet yellows virus* (BYV) (2, 3, 9). Thus while the isolation of CTV paved the way for BYV purification and characterization (7) and eventually to the Closterovirus grouping (5), it was the information gathered from the sequencing of the BYV genome that demonstrated the uniqueness and coherence of viruses included in Closterovirus group (1, 14, 18). The second development was the entrance of Dawson's group into CTV cloning, bringing into the field for the first time a team of able scientists that completed sequencing the 19,296 nts genome of the T36 isolate (19).

Two interesting findings followed, first the finding of defective RNAs in plants infected with the VT and other isolates from different geographic locations (21, 22). These molecules turned out to form a major part of the dsRNA populations of VT infected plants and their chimeric nature explained many of the initial difficulties to sequence the virus from its 3' end (Mawassi et al., unpublished data). The second finding was an unusual variation in the sequences from the 5' halves of VT and T36 (23). The sequence variation over a substantial part of the two genomes was considerably larger than among isolates or strains from most other RNA viruses and for a while it was debated whether or not to separate these isolates into two different species. We will return to this subject later.

Recently, Dawson et al. (11) and Bar-Joseph et al. (4) summarized information on the molecular biology of CTV and the genetics of both SY and QD, respectively. This paper reviews developments on CTV and dRNA genomes and points to future possibilities for taming and controlling the virus. The themes are divided into three, mostly interconnected, topics including:

1. Development of infectious clones of dRNA and genomic RNA;
2. Subgenomic RNAs and strategies of CTV replication and encapsidation;
3. The development of tools for mapping pathogenicity determinants on the genomic RNA.

Other related topics including unusual genetic conservation and diversity among CTV isolates, coherence and diversity within the *Closteroviridae* family and some practical implications of these results will be also dealt within these subtitles.

Development of infectious clones of dRNA and genomic RNA. A cDNA clone of a dRNA molecule from the VT isolate was tagged and used to infect citrus plants.

Only the sensitive RT-PCR allowed the detection of low amounts of these molecules in the inoculated plant (35). The analysis of different artificial dRNA of the T36 isolate, allowed Mawassi et al. (24, 25) to demarcate several components necessary for dRNA infection.

A different type of artificial dRNA that consists of the entire 5' replication complex (ORFs1a+b), a truncated 3' and lacking the translation products of all 3' ORFs, replicates efficiently in *Nicotiana benthamiana* protoplasts. This construct, designated as Delta Cla (31), turned out to be a useful genetic platform for the study transcription of CTV RNAs (17). The main advantage of this construct was its efficient replication in protoplasts and the shortened size of the replicating entity, thus allowing the easy manipulation and characterization of *cis* acting elements involved in replication.

A further major breakthrough was the development of a full-length CTV clone capable of replicating in protoplasts (31). Achieving this goal for a *c.*20 kb was an unusually complicated task. Although the efficiency of full length RNA transcripts to replicate in protoplasts was extremely poor compared to virions from infected sap, a series of passages in protoplasts allowed the generation of sufficient virions to infect citrus plants (32). The authenticity of the cloned transcript was demonstrated by macroscopic and cytopathological observations that showed the identity of symptoms of plants infected with the native wild type and with cloned RNA transcripts. These results have opened now the way to examine hybrids of different CTV isolates.

Tools for evaluating genetic diversity among CTV isolates. In addition to T36 and VT, several other CTV isolates including some highly pathogenic and others extremely mild have been completely sequenced. Furthermore information to be presented in this meeting and unpublished information from other laboratories will allow now the comparison of a large number of full length sequences from different geographic origins. Added to this are hundreds of partial sequences of individual genes from different CTV genomes and many defective RNAs. The main conclusions from these collections of sequence data and from a range of hybridization studies that had started almost two decades ago (29), are surprising. We still do not know much about determinants involved in viral pathogenicity. What we know is that some isolates are widely spread within certain geographic areas and that certain mild and also certain severe isolates (e.g., VT and Mor-T) that were both spatially and temporally separated over many years are > 99% identical. We also know that some virus isolates consist of two or more genetically different isolates.

Both the high level of sequence conservation among isolates separated for long periods and the presence of multiple forms of CTV genomes within a single host pose theoretical questions. The quasi species theory and the erratic nature of RNA transcription should have had a considerable impact on the genomic variation, while cross protection could had been expected to lead a higher rate of genomic conformity. Better answers to these questions are however expected when research aimed to examine the sequence composition of plants inoculated with chimeras of T36 and other biologically different isolates, will become available.

Strategies of CTV replication and virion assembly. Infected plants contain relatively large amounts of double-stranded replicative form RNA molecules corresponding to the genomic (g) RNA, and to at least nine 3' co-terminal sub-genomic (sg) RNAs. Unlike the large animal viruses of the order Nidovirales, the 3' sgRNAs of CTV do not share a common 5' terminus (19, 35). Additionally, at least three

distinct size positive-stranded 5' sgRNAs, two of about 0.8 kb (LMT 1 and 2) and a large *c.*11 kb (LaMT) were found in CTV infected tissue. These sgRNAs differed considerably in abundance in infected cells; the LMTs were more abundant than the gRNA, while the LaMT accumulated to lower levels. The 3' termini of LaMT molecules, for which no function could be assigned yet, reside just a few nucleotides downstream of the ORF1b termination codon (10). Because of the absence of corresponding of minus strand molecules, it was suggested that the *cis*-acting element that were expected to function as the ORF2 sgRNA promoter might also act as the termination signal of the ORF1a+b 5' transcripts. Using insertions, deletions and inversions of 3' sequences, ORFs and their intergenic regions into the self replicating Delta Cla construct, allowed Gowda et al. (17) to demonstrate that each of the 3'-CTV controller elements allows the production of three types of RNA molecules, a 5'-terminal positive strand RNA transcript and 3'-terminal positive- and negative-stranded sgRNAs.

Recently Che et al. (unpublished data) characterized a novel class of large self replicating dRNAs (LadRNAs) from three CTV isolates which were infectious when transmitted mechanically to citrus plants and *N. benthamiana* protoplasts. These molecules showed 5' termini, identical or slightly larger than the 5' large single stranded sgRNA of ORF1a+1b (LaMT). These, and previous results showing dRNAs with 3' identical with the sequence of ORF11, suggest that populations of CTV dRNAs are shaped by recombination of 5' terminal sgRNAs and 3' sgRNAs and suggest on the involvement of closterovirus sgRNAs modular exchange and rearrangement of closterovirus genomes.

Closteroviruses differ from most other elongated viruses by having two different capsid proteins which are located on two opposite poles of the virions. The major coat protein of about 25 kDa encapsidates the major part (~95%) of the virion, while the remaining part is encapsidated by p27. Several reports suggested that divergent CP is encapsidating the 5' termini of closterovirus. Two other proteins, p61 and the HSP70-like protein, were reported to be tightly associated with virions of other closteroviruses. Although this association was not directly demonstrated for CTV, the finding that RNA transcripts lacking one of these genes were unable to produce infective virions is strongly supporting a similar dependence of CTV particles on four different protein products. Two other 3' proteins for which functions had been assigned include the p20, which was located within inclusion bodies, and p23, which was found to be an RNA binding protein. Recently, Satyanarayana et al. (unpublished data) showed that p23 is modulating the synthesis of plus RNAs in infected cells and replicons with truncated p23 genes tend to produce equal ratios of plus and minus molecules.

SOME FRAGMENTED CONCLUSIONS

Pathogen Derived Resistance (PDR). Following the finding that many virus diseases could be readily controlled after transforming hosts with constructs either expressing the viral coat protein or other viral genomic components such as a truncated viral RNA polymerase, several groups have attempted to use similar techniques for controlling CTV. Research on CTV mediated resistance and on a possible different source of resistance based on the isolation of resistance gene from *Poncirus trifoliata* is currently being conducted (12, 36). Naturally, successful results with transgenic citrus will bring up the question of applicability. Public acceptance is of considerable importance, especially

when considering fruits which are promoted by boosting the health consciences of consumers. Restricting the transformation to the rootstock should however ease these problems, although the use of resistant/tolerant rootstock will not provide protection to SP problems of the scion.

Is there an additional CTV-like virus with a different 3' and how it will affect the future utilization of PDR? The taxonomy of Closteroviruses had facilitated CTV research in many ways. It is interesting to note that unless some other fruit trees and vines which carry several different viruses of this family, only a single species of Closterovirus was so far found to infect citrus. The considerable variation between the 5' of T36 and many other strains, hints that there might be a hidden reservoir for a second virus with a different 3' half. Will such a virus be recognized by antibodies reacting with CTV, or will it blocked by virus mediating constructs based on CTV genes? Most probably not, and it might be worth searching for the presence of such a virus, before it overcomes resistance expected from this novel technologies.

The biology of CTV and dRNAs. The biology of the virus and its interaction with different citrus hosts remains mostly unknown, both situations of high conservation and variability in reactions to CTV isolates were noticed. Thus while *P. trifoliata* is resistant to almost all CTV isolates, sour orange plants are mostly resistant to CTV infection but some isolates replicate to high levels. An association of a population of large (4.5kb) dRNA consisting of >4.0kb 5' terminus was associated with VT and Mor-T effectively replicating in sour orange, but this correlation will have to be supported more detailed studies using the infectious clone. It is interesting to note that CTV isolates that were showing a mild reaction when infected on sweet orange/sour orange combinations caused severe SP reactions on sensitive Star Ruby grapefruit. The two main categories of CTV pathologies seem therefore to be determined by different genetic factors, probably residing on different genomic regions.

Toward the mapping of pathogenicity and vector transmission determinants. Several other aspects of CTV biology including, the modes of vector transmission, factors affecting transmissibility by aphids, will also benefit from the new tools for genetic dissection and chimerization of differently transmitted CTV genomes.

The future of Tristeza? The citrus industries in many countries have changed considerably, in terms of the profile of producers, of varieties and rootstocks, and the areas of planted groves. Naturally, the phytosanitary conditions have also changed with many diseases almost completely eliminated from new groves while others becoming of epidemic proportions throughout the citrus industries of the world. CTV belongs to the second class and the number of citrus areas remaining free of CTV has been recently reduced dramatically. Yet during this period, and especially during the last decade, considerable advances have been made by several research groups on the basic molecular aspects of this virus. Some of these achievements have already been utilized for practical purposes, mainly in the field of improved reagents for CTV detection and improved ability to differentiate between CTV isolates. However, there are indications from related fields that these are just the preludes of much more impressive possibilities to control the virus and eventually turn it from a pathogen into a molecular tool for citrus improvement.

How far away are we from these goals and when these dreams are going to be materialized will much depend on the continuous readiness of the citrus industries to support these programs.

LITERATURE CITED

1. Agranovsky, A. A.
   1996. Principles of molecular organization, expression and evolution of closteroviruses: Over the barriers. Adv. Virus Res. 47: 119-158.
2. Agranovsky, A. A., D. E. Lesemann, E. Maiss, R. Hull, and J. G. Atabekov
   1995. "Rattlesnake" structure of a filamentous plant virus built of two capsid proteins. Proc. Natl. Acad. Sci. USA 92: 2470-2473.
3. Agranovsky, A. A., V. P. Boyko, A. V. Karasev, E. V. Koonin, and V. V. Dolja
   1991. Nucleotide sequence of the 3' terminal half of beet yellows closterovirus RNA genome. Unique arrangement of eight virus genes. J. Gen. Virol. 72: 15-23.
4. Bar-Joseph, M., X. Che, D. Piestun, O. Botuman, R. Gofman, Y. Ben-Shalom, and G. Yang
   2000. Citrus tristeza virus revisited: Quick decline and seedling yellows—the cost of sour orange resistance gene(s). Proc. Int. Soc. Citricult. 2: 963-965.
5. Bar-Joseph, M., S. M. Garnsey, and D. Gonsalves
   1979. The closteroviruses: a distinct group of elongated plant viruses. Adv. Virus Res. 25: 93-168.
6. Bar-Joseph, M., D. J. Gumpf, J. A. Dodds, A. Rosner, and I. Ginzburg
   1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology 75: 195-198.
7. Bar-Joseph, M. and R. Hull
   1974. Purification and partial characterization of sugar beet yellows virus. Virology 62: 552-562.
8. Bar-Joseph, M., G. Loebenstein, and J. Cohen
   1972. Further purification and characterization of particles associated with citrus tristeza virus. Virology 50: 821-828.
9. Boyko, V. P., A. V. Karasev, A. A. Agranovsky, E. V. Koonin, and V. V. Dolja
   1992. Coat protein duplication in a filamentous RNA virus of plants. Proc. Natl. Acad. Sci. USA 89: 9156-9160.
10. Che, X., D. Piestun, M. Mawassi, G. Yang, T. Satyanarayana, S. Gowda, W. O. Dawson, and M. Bar-Joseph
    2001. 5'-coterminal subgenomic RNAs in *Citrus tristeza virus*-infected cells. Virology 283: 374-381.
11. Dawson, W. O., T. Satyanarayana, S. Gowda, M. Mawassi, M. R. Albiach-Martí, M. A. Ayllón, and S. Rabindran
    2000. Development of a genetic system of the largest known plant virus, citrus tristeza virus. Proc. Int. Soc. Citricult. 2: 969-971.
12. Deng, Z., S. Huang, P. Ling, C. Yu, Q. Tao, C. Chen, M. K. Wendell, H. B. Zhang, and F. G. Gmitter, Jr.
    2001. Fine genetic mapping and BAC contig development for the citrus tristeza virus resistance gene locus in *Poncirus trifoliata* (Raf.). Mol. Genet. Genomics 265: 739-747.
13. Dodds, J. A. and M. Bar-Joseph
    1983. Double-stranded RNA from plants infected with closteroviruses. Phytopathology 73: 419-423.
14. Dolja, V. V., A. V. Karasev, and E. V. Koonin
    1994. Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. Annu. Rev. Phytopathol. 32: 261-285.
15. Fawcett, H. S. and J. M. Wallace
    1946. Evidence of the virus nature of citrus quick decline. Calif. Citrogr. 32: 88-89.
16. Goelet, P., G. P. Lomonosoff, P. J. G. Butler, M. E. Akam, M. J. Gait, and J. Karn
    1982. Nucleotide sequence of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA 79: 5818-5822.
17. Gowda, S., T. Satyanarayana, M. A. Ayllón, M. R. Albiach-Martí, M. Mawassi, S. Rabindran, S. M. Garnsey, and W. O. Dawson
    2001. Characterization of the cis-acting elements controlling subgenomic mRNAs of *Citrus tristeza virus*: production of positive- and negative-stranded 3'-terminal and positive-stranded 5'-terminal RNAs. Virology 286: 134-51.
18. Karasev, A. V.
    2000. Genetic diversity and evolution of closterovirus. Annu. Rev. Phytopathol. 38: 293-324.
19. Karasev, A. V., V. P. Boyko, S. Gowda, O. V. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. C. Cline, D. J. Gumpf, R. F. Lee, S. M. Garnsey, D. J. Lewandowski, and W. O. Dawson
    1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511-520.
20. Kitajima, E. W., D. M. Silva, A. R. Oliveira, G. W. Müller, and A. S. Costa
    1964. Thread-like particles associated with tristeza disease of citrus. Nature 201: 1011-1012.

21. Mawassi, M., A. V. Karasev, E. Mietkiewska, R. Gafny, R. F. Lee, W. O. Dawson, and M. Bar-Joseph
1995. Defective RNA molecules associated with the citrus tristeza virus. Virology 208: 383-387.
22. Mawassi, M., E. Mietkiewska, M. E. Hilf, L. Ashoulin, A. V. Karasev, R. Gafny, R. F. Lee, S. M. Garnsey, W. O. Dawson, and M. Bar-Joseph
1995. Multiple species of defective RNAs in plants infected with citrus tristeza virus. Virology 214: 264-268.
23. Mawassi, M., E. Mietkiewska, R. Gofman, G. Yang, and M. Bar-Joseph
1996. Unusual sequence relationships between two isolates of citrus tristeza virus. J. Gen. Virol. 77: 2359-2364.
24. Mawassi, M., T. Satyanarayana, S. Gowda, M. R. Albiach-Martí, C. Robertson, and W. O. Dawson
2000. Replication of heterologous combinations of helper and defective RNA of *Citrus tristeza virus*. Virology 267: 360-369.
25. Mawassi, M., T. Satyanarayana, M. R. Albiach-Martí, S. Gowda, M. A. Ayllón, C. Robertson, and W. O. Dawson
2000. The fitness of *Citrus tristeza virus* defective RNAs is affected by the lengths of their 5'- and 3'-termini and by the coding capacity. Virology 275: 42-56.
26. Meneghini, M.
1946. Sobre a natureza e transmissibilidade do doença "tristeza" dos citrus. O Biológico 12: 285-287.
27. Mullis, K. B. and F. A. Faloona
1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. 155: 335-350.
28. Pappu, H. R., A. V. Karasev, E. J. Anderson, S. S. Pappu, M. E. Hilf, V. J. Febres, R. M. G. Eckloff, M. McCaffery, V. Boyko, S. Gowda, V. V. Dolja, E. V. Koonin, D. J. Gumpf, K. C. Cline, S. M. Garnsey, W. O. Dawson, R. F. Lee, and C. L. Niblett
1994. Nucleotide sequence and organization of eight 3' open reading frames of the citrus tristeza closterovirus genome. Virology 199: 35-46.
29. Rosner, A. and M. Bar-Joseph
1984. Diversity of citrus tristeza virus strains indicated by hybridization with cloned cDNA sequences. Virology 139: 189-193.
30. Rosner, A., I. Ginzburg, and M. Bar-Joseph
1983. Molecular cloning of complementary DNA sequences of citrus tristeza virus RNA. J. Gen. Virol. 64: 1757-1763.
31. Satyanarayana, T., S. Gowda, V. P. Boyko, M. R. Albiach-Martí, M. Mawassi, J. Navas-Castillo, A. V. Karasev, V. V. Dolja, M. E. Hilf, D. J. Lewandowski, P. Moreno, M. Bar-Joseph, S. M. Garnsey, and W. O. Dawson
1999. An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. Proc. Natl. Acad. Sci. USA 96: 7433-7438.
32. Satyanarayana, T., M. Bar-Joseph, M. Mawassi, M. R. Albiach-Martí, M. A. Ayllón, S. Gowda, M. E. Hilf, P. Moreno, S. M. Garnsey, and W. O. Dawson
2001. Amplification of *Citrus tristeza virus* from cDNA clone and infection of citrus trees. Virology 280: 87-96.
33. Sekiya, M. E., S. D. Lawrence, M. McCaffery, and K. Cline
1991. Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristeza virus. J. Gen. Virol. 72: 1013-1020.
34. Webber, H. J.
1925. A comparative study of the citrus industry in South Africa. Union S. Afr. Dept. Agric. Bull. No. 8, 106 pp.
35. Yang, G., M. Mawassi, L. Ashoulin, R. Gafny, V. Gaba, A. Gal-On, and M. Bar-Joseph
1997. A cDNA clone from a defective RNA of citrus tristeza virus is infective in the presence of the helper virus. J. Gen. Virol. 78: 1765-1769.
36. Yang, Z. N., X. R. Ye, S. Choi, J. Molina, F. Moonan, R. A. Wing, M. L. Roose, and T. E. Mirkov
2001. Construction of a 1.2-Mb contig including the citrus tristeza virus resistance gene locus using a bacterial artificial chromosome library of *Poncirus trifoliata* (L.) Raf. Genome 44: 382-393.
37. Zeman, V.
1930. Una enfermedad nueva en los naranjales de Corrientes. Physis 19: 410-411.