Fifty Years of IOCV, 1957 to 2007: From Graft-Transmitted Citrus Agents to Viroids, Viruses and Endogenous Bacteria

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INTRODUCTION

In November, 1957, the well-known Citrus Experiment Station (CES) of the University of California at Riverside, USA, celebrated its 50th anniversary. This was a good opportunity to hold the first international conference on so-called "virus" diseases of citrus, of which many had been studied in California, if not in Florida. Maladies such as Tristeza, Psorosis, Concave Gum and Blind Pocket, Crinkly leaf and Infectious variegation, Stubborn, Xyloporosis and Cachexia, Exocortis, and Vein enation had been shown to be transmissible by graft-inoculation. and were, for this reason, thought to be of viral nature, but not a single causal agent had yet been identified, mechanically transmitted, purified, or even seen in the electron microscope.

Two historically important diseases were not covered at the 1957 citrus "virus" disease conference because they were not known by the scientific community. The first deals with "Infectious chlorosis of Citrus", studied by L. C. Trabut in Algeria in the 1900s and 1910s (5). He transmitted the disease agent by graft inoculation (see 1). This is the first recorded graft-transmission of a citrus disease. Trabut is also known for having noted and selected the Clementine mandarin in Father Clement Rodier's garden near Oran, Algeria. The second topic concerns also transmission: L. K. Lin was able to transmit Huanglongbing by graft-inoculation in southern China in the 1950s, and thus proved the infectious nature of the disease (4).

In view of the importance of graft-transmissible diseases and the need for international cooperation, the International Organization of Citrus Virologists (IOCV) was founded during the 1957 meeting in Riverside (Fig. 1). Every 3-4 yr since, IOCV has met in different locations in the six continents where citrus is grown. At the 9th conference in Argentina in 1993, the 25th anniversary of the IOCV was celebrated and reviewed by Garnsey (2), and Lee and Garnsey (3) updated progress at the 13th Conference.

The 50th anniversary of the foundation of IOCV in Riverside in 1957 was celebrated in October 2007 in Adana, Turkey, where the IOCV held its 17th conference. During these first 50 yr of IOCV, research on graft-transmissible diseases of citrus, mostly by members of the IOCV community, has led to the discovery that many of these diseases were indeed due to viruses, but others were found to be caused by, or associated with, new agents that were unknown in the 1950s, namely viroids and endogenous bacteria. One of us (JMB), who witnessed the foundation of IOCV in 1957 and has attended all but one of the 17 IOCV conferences, has brought back to our memories, in the June 2008 IOCV newsletter, some of the organizational and social highlights of these conferences as well as their pre- and/or post-conference tours. Here, we present a summary of the scientific achievements accomplished during the last 50 yr in the study of graft-transmissible diseases of citrus.

Fig. 1. Delegates attending the "Citrus Virus Diseases" conference in Riverside, CA in 1957 at which the IOCV was founded.

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I. VIROIDS

Discovery of viroids: PSTVd and CEVd. Viroids were discovered through the study of two diseases: potato spindle tuber (PST) and citrus exocortis (CE). CE emerged as a problem associated with Tristeza-Quick decline (TQD). When it was learned that sour orange was responsible for TQD, control of the disease involved replacing sour orange by TQD-compatible rootstocks. CE was described in 1948 as a bark scaling disorder affecting trifoliate orange rootstocks used to control TQD (15). In Australia, the bark-scaling disease on trifoliate orange was called "Scaly butt" and was shown to be transmissible by graft-inoculation (4, 5). A similar disease was reported on Rangpur lime rootstocks in Brazil and was considered to be the same as CE (25). Major developments occurred when Etrog citron was shown to be a sensitive indicator plant for CE (7, 41, 42), and when the CE agent was transmitted by means of dodder (*Cuscuta subinclusa*) and/or mechanically to petunia and other herbaceous plants (52, 53, 54, 55, 56). These herbaceous hosts, as they showed high titers of the disease agent, made it easier for Semancik & Weathers (45) to characterize the CE agent. Indeed, using *Gynura aurantiaca* plants, they found that the agent was an infectious, naked, low molecular weight RNA, similar to the potato spindle tuber (PST) agent studied by Diener (11) who coined the name "viroid" for the new agent.

The name "viroid" was adopted for these small RNA molecules, and the CE agent became known as the CE viroid or CEVd. As the result of the work of Diener and Semancik, viroids were soon recognized as a new type of plant pathogen with the following characteristics. They are covalently closed (circular) single-stranded RNA molecules. They are small RNA molecules with only 246 nucleotide residues for the smallest viroid, and 401 for the largest one. They do not code for proteins. They replicate, using the host-cell machinery, and are considered to be fossils of a pre-cellular world.

CEVd is only one of several citrus viroids. Three developments were essential for the discovery of additional citrus viroids: (i) the use of citron, a less selective host than gynura (12, 13, 14), as the source of viroid RNAs; (ii) the use of sequential Polyacrylamide Gel Electrophoresis (sPAGE) (38), and (iii) the use of silver staining to detect the viroid RNA bands on the gel, as developed for tRNAs (18). Under these conditions, when nucleic acid preparations of citron plants inoculated with different field sources were analyzed by sPAGE, all sources from both California and Spain were found to contain several viroids with distinct physical and biological properties (12, 13, 14). The total number of different viroids amounted to five: CEVd, CVd-I, CVd-II, CVd-III, and CVd-IV. The International Committee on Taxonomy of Viruses has adopted the terms *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CBVd) and *Citrus bark cracking viroid* (CBCVd) for CVd-I, CVd-III and CVd-IV, respectively. When citrons were inoculated with single viroids, some distinct symptoms were observed. Similar results were also obtained by others (16, 20, 22). An additional viroid initially termed CVd-OS was also reported in Japan (19) and recently renamed *Citrus viroid VI* (CVd-VI).

Cachexia is also caused by a viroid. Reichert and Perlberger (35) described a disease of sweet lime in Palestine to which they gave the name "xyloporosis". Childs (8) described a disease on Orlando tangelo that resembled xyloporosis and gave it the name "cachexia". He also showed cachexia to be graft-transmissible (9).

Roistacher et al. (40) suggested that cachexia might be caused by a viroid because of the similarity in transmission

properties between the cachexia agent and CEVd. Semancik et al. (46) confirmed this hypothesis in 1988. They analyzed by sPAGE the nucleic acids extracts from citron tissues only infected with severe isolates of Cachexia and detected an RNA of about 300 nucleotides not observed in healthy extracts (46, 47). The RNA had all the properties of viroids. In addition, when inoculated first to citron and next from citron to Parson's special mandarin, the indicator plant for cachexia (39), typical cachexia symptoms were obtained. Furthermore, the cachexia viroid could be identified with CVd-II, and more specifically with CVd-IIb (also termed CCaVd for citrus cachexia viroid), CVd-IIa and CVd-IIb being two variants of CVd-II (13, 14). Only the fast migrating variant, CVd-IIb, induced symptoms when inoculated on Parson's Special mandarin indicator plants or on field grown Orlando tangelo and Alemow. Later, sequencing analysis showed that a five-nucleotide motif located in the variable "V" domain allowed discrimination between the pathogenic, cachexia-inducing variant CVd-IIb and the non-pathogenic CVd-IIa variant (33, 34, 36).

The cachexia viroid is a variant of the Hop Stunt Viroid (HSVd). Another interesting development occurred when it was shown that CVd-II (IIa and IIb) hybridized with HSVd-specific cDNA probes, thus showing that the cachexia viroid CVd-IIb was a variant of the hop stunt viroid (1, 10). Similar conclusions were also obtained and confirmed by sequence comparisons (2, 23, 43, 44). As a consequence, the cachexia viroid is now officially named HSVd.

Cachexia and xyloporosis are one and the same disease. Reanwarakorn and Semancik (37) have shown the cachexia-inducing variant of HSVd to cause not only cachexia on Orlando tangelo but also xyloporosis on Palestine sweet lime. Koch's postulates have been fulfilled for both diseases.

Effect of single and multiple citrus viroids. The major results of an extensive experimentation carried out in Corsica to study the effect of single and multiple viroids on Clementine trees grafted on trifoliate orange were as follows (50, 51). (i) CEVd induced exocortis symptoms on trifoliate orange. Surprisingly, this is probably the first time that Koch's postulates for CEVd were carried out, even though the association of CEVd with exocortis symptoms on trifoliate orange was well known previously. (ii) Only the cachexia variant of HSVd induced cachexia symptoms on the Clementine scion. (iii) CEVd, HSVd, or CVd-IV (CBCVd) induced bark-cracking symptoms on the trifoliate orange rootstock. (iv) Antagonism was observed between CEVd and CVd-IV (CBCVd) for bark-scaling and bark-cracking symptoms on trifoliate orange. (v) Synergisms were noticed; for instance between CVd-I (CBLVd) and CVd-III (CDVd), they resulted in exocortis-like scaling symptoms on trifoliate orange in the absence of CEVd; an exocortis-like reaction was also observed on citron. In Japan, multiple citrus viroids have also produced exocortis-like symptoms on citron (20). In Cyprus, exocortis and cachexia viroids affected growth, yield and fruit quality of lemon trees grafted on sour orange (21).

The gummy bark agent: viroid or not? First reported as phloem discoloration of sweet orange (27), gummy bark (GB) on sweet orange (29) in Eastern Mediterranean, North-African, Near East and Middle East countries has symptoms very similar to those of cachexia on mandarin. GB being reported as graft transmissible (27, 28, 29) and Cachexia being caused by a viroid, the idea that the GB agent was also a viroid gained popularity. The cachexia viroid being a variant of HSVd, the possibility existed that the putative GB agent might be an additional variant of HSVd (30). However, molecular characterization of HSVd

variants present in GB sources present in Turkey did not allow identification of such a variant (31). Several GB sources from the Sultanate of Oman were also studied (6). In addition to HSVd, all samples contained CEVd, CVd-III (CDVd), and CVd-IV (CBCVd), and novel variants of CEVd and CVd-III (CDVd) were identified in all the GB sources. These results as well as previous data ruled out CVd-I (CBLVd) as the causal agent of GB, but there were no clues to entertain or reject the possibility that CVd-IV (CBCVd) or the new variants of CEVd and CVd-III (CDVd) may be involved.

Recent work with several GB samples from the Sudan has given additional data (24). (i) Similar to the results from Turkey and Oman, only CVd-IV (CBCVd) was found in all GB sources from the Soudan. (ii) An HSVd variant as the causal viroid of GB had to be ruled out since two Sudanese sources, one with severe GB and one with mild GB, were free of HSVd. (iii) CEVd and CVd-III (CDVd) , found in all sources from Turkey and Oman, were not present in two sources of the Sudan. Therefore, CVd-IV (CBCVd) remains the only candidate for a putative viroid etiology of GB.

CVd-V: a new viroid in *Atalantia citroides*. Plants of *Atalantia citroides* grafted on rough lemon rootstocks were graft-inoculated in the *Atalantia* scion with the five citrus viroids (CEVd, CVd-I (CBLVd), CVd-II (HSVd), CVd-III (CDVd), and CVd-IV (CBCVd)) (3). Three years later, all five co-inoculated viroids were detected in the rough lemon rootstocks, but none in the *Atalantia* scions. However, a new viroid, CVd-V was detected in the *Atalantia* scions in which it replicated and accumulated to detectable titers. Infectivity of CVd-V was demonstrated by graft- and/or slash-inoculations to citron. Partial sequencing has shown that CVd-V contains two segments corresponding to the upper and lower strands of the Conserved Central Region (CCR) of members of the genus *Apscaviroid*.

This work raises several interesting questions. The fact that the five viroids inoculated in the *Atalantia* scions could not be detected in these scions, but were present in the rough lemon rootstocks, suggests that the long-distance transport of viroids functions in *Atalantia*, but that the infection process via cell-to cell movement via plasmodesmata is impaired. However, even though the five inoculated viroids did apparently not replicate in *Atalantia,* a new viroid, CVd-V, was able to replicate in *Atalantia* as well as in citron. CVd-V has been recently characterized as a new member of the genus *Apscaviroid* (49).

Viroids as dwarfing agents. Several publications have been devoted to using viroids as dwarfing agents for high density plantings. Hutton et al. (17) is an excellent example of such work. Among the viroids tested in long-term field assays, CVd-III (CDVd) has been recognized as the most promising viroid to control tree size without undesirable effects. Several variants of this viroid were initially recognized by their distinct mobilities in sPAGE analysis (13, 14) and later characterized as three distinct sequence variants (CVd-IIIa, CVd-IIIb, CVd-IIIc) differing in size by as much as 18 nucleotides (34, 48). Further characterization of 33 field isolates recovered from different hosts and different locations showed that most variants were highly similar to CVd-IIIa or to CVd-IIIb, whereas variants related to CVd-IIIc were rather unusual (26). These variants act as true strains with different levels of severity on the Etrog citron indicator (26) but only limited information is available regarding their effect as dwarfing agents on field grown trees (50).

Conclusion. Viroids were unknown in 1957 when IOCV was founded. The work on citrus exocortis disease was essential for the discovery of viroids in general, and citrus viroids in

particular (45). By 2007, 50 yr after the foundation of IOCV, seven citrus viroids have been characterized: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd, ex-CVd-I), *Hop stunt viroid* (HSVd, ex-CVd-II), *Citrus dwarfing viroid* (CDVd, ex-III), *Citrus bark cracking viroid* (CBCVd, ex-CVd-IV), CVd-V and *Citrus viroid VI* (CVd-VI, ex-CVd-OS) (3, 12, 13, 14, 19, 46, 47). They belong to four genera in the *Pospiviroidae* family (Table 1).

TABLE 1

LIST OF CITRUS VIROIDS

Genus	Species
Pospiviroid	CEVd
Hostuviroid	HSVd (ex CVd-II)
Cocadviroid	CBCVd (ex CVd-IV)
Apscaviroid	CBLVd (ex CVd-I)
Apscaviroid	CDVd (ex CVd-III)
Apscaviroid	CVd-V
Apscaviroid	CVd-VI (ex CVd-OS)

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II. VIRUSES

In 1957, when IOCV was founded, several citrus diseases were known to be infectious, because the agents associated with these diseases had been transmitted by graft-inoculation. In those days, the only agents known to be graft-transmissible in plants were the viruses. Therefore, all graft-transmissible diseases of plants were thought to be virus diseases, even though the putative virus had never been seen or purified. The "virus" hypothesis turned out to be true for several diseases, such as tristeza, psorosis, leprosis, infectious variegation, or satsuma dwarf, for which the causal viruses were eventually isolated, but not for other diseases, such as exocortis and cachexia, which turned out to be caused by viroids, as seen above, or stubborn and huanglongbing [ex-greening], where bacterial agents were found (see below Endogenous bacteria). Here only major developments in virus diseases of citrus since 1957 will be considered. Early historical developments of these diseases can be found in references 1 and 2.

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TRISTEZA

Citrus tristeza virus (CTV) (genus *Closterovirus*, family *Closteroviridae*) is the causal agent of devastating epidemics that changed the course of the citrus industry. Adapted to replicate in phloem cells of a few species within the family *Rutaceae* and to transmission by a few aphid species, CTV and citrus probably coevolved for centuries at the site of origin of citrus plants. CTV dispersal to other regions and its interaction with new scion varieties and rootstock combinations resulted in three distinct syndromes named (i) tristeza (quick decline), (ii) stem pitting, and (iii) seedling yellows. The first, inciting decline of varieties propagated on sour orange, has forced the rebuilding of many citrus industries using tristeza-compatible rootstocks. The second, inducing stunting, stem pitting and low bearing of some varieties, causes economic losses in an increasing number of countries. The third is usually observed by biological indexing, but rarely in the field (162). Over the last several years, our understanding of CTV has grown considerably and review articles on various aspects of tristeza have appeared (26, 27, 139, 162, 204). It is hoped that the following lines will reflect these remarkable achievements.

The causal agent. The infectious nature of the disease, named tristeza by Moreira in Brazil in 1942 (154), was established in 1946 by Fawcett and Wallace (71) in California when they induced the decline of sweet orange on sour orange by graft inoculation, and by Meneghini (152) in Brazil when he transmitted the disease by *Toxoptera citricida*. Thread-like particles associated with tristeza were purified and seen in the electron microscope for the first time by Kitajima et al. (130, 131) in Brazil. The work was reported in 1963 in São Paulo city at the third IOCV conference, and tristeza became the first citrus disease, whose infectious nature was supported by the presence of associated virus particles in the infected plants. The thread-like or flexuous particles had a diameter of 10 to 12 nm and were as long as 2000 nm. Further purification in Israel led to the identification of a ~28 kDa coat protein in 1970-1972 (19, 20), and in 1985 to the characterization of a single stranded RNA genome of 6.5 x 10^6 Da (24), a value in agreement with a double stranded RNA replicative form of 13.3 x 10^6 Da observed previously (59). These RNA values suggested a genomic size of about 20 kb, thus giving CTV the largest RNA genome of known plant viruses. In the meantime, mechanical transmission of CTV by the stem-slash technique was demonstrated and allowed Koch's postulates to be fulfilled (78, 82, 169). Taxonomically, CTV was found to be a semipersistently aphid-transmitted closterovirus associated with phloem tissue in infected citrus (22). Eventually, from 1995 to 2006, the complete nucleotide sequences of biologically distinct CTV isolates were achieved through the efforts of many investigators in several laboratories (5, 129, 144, 213, 232, 244, 252). The positive-stranded genomic RNA (gRNA) of Florida CTV strain T-36 consisted of 19,296 nt (129) and the Israeli CTV strain VT had 19,226 nt (144). The genomes of the two strains had similar organizations and encompassed 12 open reading frames (ORFs) and two untranslated regions (UTRs) at both ends of the gRNA. As known today (162), ORFs 1a and 1b, encoding proteins of the replicase complex, are directly translated from the genomic RNA, and together with the 5'- and 3'-UTRs are the only regions required for RNA replication. The remaining ORFs, expressed

via 3'-coterminal subgenomic RNAs (sgRNAs) (117), encode proteins required for virion assembly and movement (p6, p65, p61, p27 and p25), asymmetrical accumulation of positive and negative strands during RNA replication (p23), or suppression of post-transcriptional gene silencing (p25, p20 and p23), with the role of proteins p33, p18 and p13 as yet unknown. The latter three proteins have been shown to be prescindible for systemic infection of at least some citrus hosts (234).

The major coat protein, CP or p25, was found to encapsidate about 97% of the genomic RNA while the minor coat protein, p27 or CPm, a diverged copy of CP, covered the rest of the genome at its 5' end (72, 225).

Replication and expression of the CTV genome produces multiple subgenomic and defective RNAs. As with other RNA viruses replication of the CTV genome requires the synthesis of a negative-stranded complementary RNA that serves as template for the synthesis of new positive-stranded gRNA molecules. Expression of genes in the 3' moiety of the gRNA leads to production of positive- and negative-stranded 3' co-terminal sgRNAs, the first being about 40-50 times more abundant than the second (224). Production of these sgRNAs is regulated independently both in amount and in timing (117, 181) by individual controller elements (12, 101). Also, a set of less abundant 5′-coterminal positive-stranded sgRNAs is generated, likely by premature termination of the gRNA at those controller elements (101). Finally, two abundant positive-stranded 5′-coterminal sgRNAs of about 800 nt (LMT1 and LMT2) are produced by different mechanisms (13, 45, 102, 104). Therefore, infected cells accumulate more than 30 different sgRNA species. In addition to the genomic and subgenomic RNAs (117), plants infected with CTV were shown to contain multiple species of defective RNAs (dRNAs) (142, 143). These dRNAs are small molecules derived from the parental viral genome and consist of the 5' and 3' terminal segments of the gRNA, with extensive internal deletions. Their formation in infected cells occurs after template switching of the RNA polymerase following different mechanisms (9, 142, 143, 251) and it can strongly influence the virus life cycle (replication, accumulation, symptoms). Mawassi et al. (142, 143) showed that at least some d-RNAs are encapsidated by the p25 coat protein.Generation of so many positive- and negative-stranded RNAs (genomic, subgenomic and defective) in infected cells leads to the presence of abundant double stranded RNAs (dsRNAs) in CTV-infected plant extracts, as described by Dodds and Bar-Joseph (59). The presence of these dsRNAs was exploited as a diagnostic tool and a way of discriminating between CTV isolates before the nature and characteristics of sgRNAs and d-RNAs was discovered (60, 61, 62, 109, 156). Ds-RNA analysis was also used to characterize changes in the viral population after aphid or graft transmission to new hosts (110, 156, 157, 158, 159) or after interference between CTV isolates in cross protection experiments (3, 161). It was later found that some of the discriminating dsRNAs were in fact defective dsRNAs.

CTV-encoded proteins p20, p23, and p25 act as silencing suppressors in tobacco. Plant hosts use two RNA silencing systems as defense mechanisms against virus infections: intracellular silencing and intercellular silencing. In citrus hosts, genes involved in post-transcriptional gene silencing have been found in many citrus gene-libraries (31). For viral infection to take place, these host RNA-silencing defense mechanisms have to be suppressed. It has been shown that the CTV genome encodes at least three proteins, which suppress RNA silencing in *Nicotiana*

benthamiana and *N. tabacum* (137). Protein p23 suppresses intracellular silencing, protein p25 (coat protein) targets intercellular silencing, and protein p20 inhibits silencing at both levels. It has been suggested that the simultaneous suppression of intracellular and intercellular silencing antiviral defense by CTV proteins may explain, in part, why CTV causes the most destructive viral disease in citrus worldwide. As with silencing suppressor proteins encoded by other viruses, at least p23 has been shown to be a pathogenicity determinant involved in the expression of CTV-specific symptoms like vein clearing in different hosts (69, 87) and seedling yellows in sour orange or grapefruit (6).CTV diagnostic and strain differentiation.

After evidence that tristeza disease was a transmissible disease (71, 152) and further description of the lime disease in Gold Coast (122), indexing on Mexican lime seedlings was firmly established as a reliable diagnostic of tristeza, quick decline and stem pitting diseases, respectively in Brazil (49), California (247) and South Africa (145). Similarly, the seedling yellows syndrome described in Australia (75) and South Africa (150), was also associated with tristeza symptoms in Mexican lime. Indexing on Mexican lime was a major achievement that allowed associating the three syndromes caused by CTV, namely decline, stem pitting and seedling yellows (146, 147, 148), even if for years it was accepted that CTV would be a complex pathogen with at least two separable components. This hypothesis was abandoned after demonstration that an infectious cDNA clone of the isolate T36 from Florida was able to induce the three syndromes (222, 223). For 30 yr, indexing on Mexican lime was the only method available for reliable diagnostics of CTV, and later it has been used as the reference for developing new serological and molecular detection procedures and for strain characterization purposes. However, some isolates causing symptomless infection on Mexican lime have been described, that require alternative methods for their detection (1, 35).

Quick diagnostic methods based on CTV detection were developed along the years for different purposes. Observation of the CTV filamentous flexuous virions in plant extracts by electron microscopy was used to diagnose tristeza infection in the eradication program launched in Israel (18, 21). Light microscope observation of CTV-induced inclusion bodies in freehand petiole or shoot sections cut with a razor blade and stained with Azur A, was proposed as simple diagnostic procedure that required no lab equipment (79, 81). However, after CTV purification and development of the first antibodies (80, 92), it was the application of immuno-enzymatic techniques (46) to CTV detection (23, 39, 84) that produced a major breakthrough in diagnostic of CTV diseases. Availability of ELISA detection was critical to advance our knowledge on CTV incidence and epidemiology and to improve control procedures by quarantine, eradication and certification programmes (25, 93, 95, 97, 100, 132, 180). ELISA detection has been massively used with CTV, probably more than with any other plant virus. Additionally, development of monoclonal antibodies specific to different epitopes allowed using ELISA to discriminate between CTV isolates (42, 111, 182, 235, 242). The most widely used monoclonal antibodies include 3DF1 and 3CA5, recognizing epitopes conserved in most CTV isolates (41, 42), and MCA13 that recognizes an epitope largely conserved in virulent isolates (42, 191, 192).

When partial or full-length sequences of the CTV gRNA became available, diagnostic procedures based on specific

detection of the viral RNA were developed. These included molecular hybridization with different types of probes (28, 172, 208) and several RT-PCR amplification-based methods (184, 189). The highest sensitivity for CTV detection was recently achieved using real-time RT-PCR protocols that also allow quantification of genomic RNA copies in infected citrus tissues or in viruliferous aphids (32, 214, 221). However, rather than for diagnostic purposes, the best contribution of these sequence-based detection techniques has been for CTV strain characterization. Variations in biological characteristics of CTV isolates, particularly in the type and intensity of symptoms induced in different cultivar or scion-rootstock combinations had been observed since the initial epidemics, and the need for procedures to reliably describe and characterize isolates has been stressed in most IOCV conferences and in CTV research in general (8, 14, 15, 16, 17, 35, 47, 76, 83, 115, 123, 148, 163, 165, 166, 167, 187, 204, 206, 216, 217, 236). Also evidence for the presence of different strains or variants in CTV isolates, which could be separated after aphid transmission or host change (107, 115, 195), was available before the concept that RNA viruses are usually a population of genetic variants was firmly established (65).

For years, characterization of CTV isolates was based exclusively on symptom expression upon inoculation on different indicator plants. However, differences in the indicators and the environmental conditions used to perform indexing made results obtained in different laboratories difficult to compare, even if a standard indicator set and optimised incubation conditions were proposed (83, 85; 199, 243). Availability of different monoclonal antibodies enabled further characterization of CTV isolates and monitoring cross-protection experiments using differences in ELISA reaction patterns (42, 95, 127, 128, 183, 191, 192, 235, 236, 255, 258). Later, when the genomic sequence of distinct CTV genotypes became available, sequence differences were exploited to discriminate between isolates or groups of isolates, to monitor cross protection experiments or to characterize the structure of viral populations and their changes in the aphid- or graft-transmission processes to new hosts. The techniques used for these purposes included molecular hybridisation with specific probes (2, 3, 4, 135, 172, 208, 226, 228), restriction fragment length polymorphism analysis (90, 91, 230), single-strand conformation polymorphism analysis (55, 56, 125, 138, 209, 210, 211, 212, 218, 220, 230), and different RT-PCR protocols based on genotype-specific primers (11, 44, 118, 119, 120, 219, 220). More recently, real-time RT-PCR with genotype-specific probes allowed specific detection and quantification of different sequence variants present in the viral population of field CTV isolates (7, 215).

The search for a quick diagnostic to reliably identify severe and mild CTV isolates for control purposes was the aim of this impressive panoply of isolate characterization techniques. Since the genetic basis for CTV virulence is still largely unknown, they are based on molecular markers more or less conserved in isolates of either type; however, the complex nature of many CTV populations and the wide presence of recombinant sequences (113, 141, 144, 151, 211, 244, 245, 248, 250) frequently jeopardizes assignment of unknown isolates to biologically characterized groups, even using several markers (228). There is a clear need of mapping the genetic determinants of the different CTV-induced syndromes, which implies the use of a proper genetic system based on the use and manipulation of infectious cDNA clones of the CTV

genomic RNA. In this regard, there were important advances in the last 10 yr (103, 222, 223) that led to locating the genetic determinant of the seedling yellows syndrome in a 3'-terminal region including the gene *p23* and the 3' UTR (6).

Transmission and epidemiology of CTV. CTV dispersal in nature occurs via different aphid species depending on the world region. While *Toxoptera citricida*, the most efficient CTV vector, was well established for years in Asia including the Indian subcontinent, Australia, sub-Saharan Africa and South America (50, 149), in the nineties of the past century the aphid reached Venezuela, Central America and different Caribbean countries including Cuba, Dominican Republic and Florida (29, 112, 134, 197, 256, 257). Recently, it has been detected in back yard citrus trees in northern Spain and Portugal (126), far from the important citrus-producing areas. *Aphis gossypii*, that is about 6-25 times less efficient than *T. citricida* in transmitting CTV (256), was reported as the main vector in the Mediterranean basin and areas of North America (43, 58, 114, 140, 194, 254). *A. spiraecola* and *T. aurantii* were found less efficient CTV vectors than *A. gossypii* (114, 185, 254); however, *A. spiraecola* might play an important role in CTV dispersal in some citrus areas due to the large populations it builds up in comparison with *A. gossypii* (114). *Myzus persicae*, *A. craccivora* and *Uroleucon jaceae* have been reported as CTV vectors only in India (240, 241). CTV transmission is considered to occur in a semipersistent mode, with viruliferous aphids being able to transmit the virus for at least 24 h, but infectivity being lost within 48 h after acquisition (194). The ratio of aphids carrying CTV in the field ranges from 19 to 27%, as detected by nested RT-PCR amplification of CTV RNA from individual aphids (140). The viral and aphid factors involved in CTV transmission are presently unknown, and the need for a helper factor as in other plant viruses (233) has not yet been demonstrated for CTV. This lack of knowledge on the transmission mechanism derives from the difficulty to aphid-transmit CTV after *in vitro* acquisition by the aphids from purified preparations (116). Inefficiency of this process is probably due to fragility of CTV virions that break easily during the purification process.

Availability of quick diagnostic procedures, mainly ELISA, allowed determining the spatio-temporal patterns of CTV dispersal in regions growing citrus varieties under distinct climatic conditions and with different aphid populations (40, 93, 94, 95, 96, 97, 98, 100, 108, 121, 155). In locations where *T. citricida* was predominant, CTV incidence was found to increase from 5 to 95% infected trees in only 2-4 yr; disease increase was essentially continuous; aggregates of infected trees were common, and new infections frequently occurred in trees immediately adjacent to existing infections. In contrast, in areas where *A. gossypii* was predominant the same disease increase occurred in 8-15 yr; it followed a stair-step line; limited aggregation of infected trees was observed, and new infections usually did not occur close to existing infected trees, but rather several tree spaces away. The biology and feeding habits of both vector species might be the cause of these distinct spread patterns (94, 96).

CTV control: inoculum exclusion and suppression; shoot-tip grafting. The most efficient control measure for virus diseases is inoculum exclusion from non-affected areas. For the pathosystem CTV-citrus this can be achieved by launching sanitation, quarantine and certification programs. In early times, CTV-free budwood was obtained by growing nucellar plants, a very slow process that could not be applied to monoembryonic varieties, or by

thermotherapy of infected varieties, a treatment that was inefficient with some CTV isolates (38, 199, 200, 201). Development of shoot-tip grafting *in vitro* (174, 175) was a major breakthrough that facilitated elimination of most graft-transmissible citrus pathogens (176, 179) and opened the way to improve quarantine procedures (177, 179) and to launch certification programs in which pathogen-free local varieties or imported ones could be safely propagated by citrus growers (86, 173, 178, 180, 227, 229, 249, 259).

With some plant viruses, disease spread can be reduced by controlling vector populations and roguing infected plants to reduce inoculum sources. In the case of CTV, available data suggest that control of aphids does not significantly reduce the rate of disease increase (99, 160). However, suppression of infected trees to slow down disease spread was performed in several areas with low CTV incidence, including Israel (21, 25), Cyprus (133) and the Central Valley of California (63, 64, 100). Obviously, suppression activities had to be coupled with budwood certification programs to avoid efficient CTV dispersal via infected budwood. In areas where disease incidence was high and/or CTV dispersal by aphids was too active, the only way to avoid tristeza damage was by using CTV compatible rootstocks instead of sour orange to propagate new citrus plantings.

CTV control: cross-protection/ pre-immunization against stem pitting. As early as the 1950s, it became known that the decline and death of citrus trees grafted on sour orange roots could be prevented by the use of compatible rootstocks (33, 34, 48, 105, 186), but this was by no means a solution to control the stem pitting syndrome caused by CTV isolates in certain species and varieties such as *Citrus aurantifolia* (Mexican, West Indian, or Galego lime), grapefruit, and even certain sweet orange varieties such as Pera, which, when infected with severe CTV isolates, showed stem pitting whatever the rootstock or even when grown as seedling trees (88). For stem pitting, the solution has come from "cross-protection", a phenomenon in which infection of a sensitive plant with a protective (mild) isolate of a virus protects the plant against post-infection of a severe isolate of that virus (77). Early observations on citrus cross-protection with mild CTV isolates were reported in the 1950-1960s (51, 89, 106, 186, 190, 231). In successful "pre-immunization", an application of cross-protection, virus-free mother trees of a valuable citrus variety are inoculated with a protective CTV isolate; nursery trees produced from this "pre-immunized" variety, when planted in the field, will be protected against severe CTV isolates naturally propagated by the local CTV aphid-vectors. Brazil, severely affected by tristeza since 1937, and with CTV isolates, such as the Capão Bonito isolate, inducing stem pitting not only on Galego lime, but also on grapefruit and sweet orange, as well as Rangpur lime and Caipira sweet orange used as rootstocks, was one of the initial leaders in pre-immunization, and Gerd Müller (Instituto Agronomico, Campinas, SP., Brazil) devoted his career to citrus pre-immunization.

Brazil. In Brazil, existence of mild (protective) and severe isolates was demonstrated in the 1950s (51, 106) and reviewed in 1980 (207). The severe Capão Bonito isolate was described in 1968 (166). Identification and isolation of protective CTV isolates was of course essential for success. To that purpose, outstanding trees with only mild symptoms were identified in severely affected orchards of Galego lime, grapefruit, and Pera sweet orange varieties, with conspicuous symptoms of CTV on most trees. Such mildly affected trees were selected as putatively protective virus

sources. Forty five of these sources were used to inoculate nucellar clones of Galego lime, Pera sweet orange, and Ruby Red grapefruit for pre-immunization tests in the field and the greenhouse. It was found that several protective isolates from Galego lime sources, were not only protective for Galego lime, but also for sweet orange and grapefruit. However, three protective isolates from Pera sweet orange sources, while protecting sweet orange and grapefruit, did not protect Galego lime (165). Next, five of the best Galego lime isolates were further tested on Galego lime. In 1969, the trees were 6 yr old and had been exposed to natural infection in the field for 4.5 yr. The pre-immunized Galego limes had grown satisfactorily under conditions where non pre-immunized control limes declined rapidly (167). On the basis of these results, pre-immunized Galego lime clones were distributed to growers for further evaluation. Similarly, nucellar clones of Pera sweet orange were pre-immunized with the above three mild Pera sweet orange sources. By 1968, the best combination of pre-immunized Pera sweet orange clone (#66) became available for field testing by interested growers. By 1980, ten million pre-immunized Pera sweet orange trees were present in nurseries, young orchards and production groves, most of the Pera budwood being derived from the original pre-immunized clone (52, 164). By 1997, the number of Pera #66 trees reached 80 million and almost no breakdown in protection had been observed. More recently, however, there have been a few cases where orchards from the protected Pera clone had a great number of trees with severe stem pitting (171). This indicated that there was a need for new and better pre-immunizing isolates as well as for deeper characterization of these isolates at the biological and molecular levels. RFLP and SSCP analyses of the coat protein gene have shown that changes have occurred between the protective CTV isolate "Pera IAC" present in (i) symptomless 20-yr-old Pera sweet orange mother trees and (ii) 3 to 4 yr-old daughter trees showing severe stem pitting symptoms, suggesting that breakdown of cross-protection had occurred (230).

Today, all Pera sweet orange trees in São Paulo State are from pre-immunized budwood and represent the largest pre-immunized crop in the world. In 1980, good protection of sweet orange had also been observed against the very severe Capão Bonito isolate of CTV (164). However, 6 yr later, the results were not clear cut (170). Similarly, numerous mild CTV isolates from California failed to cross protect against severe stem pitting isolates (205), even though, previously, successful cross-protection against seedling yellow and stem pitting isolates in sweet orange and grapefruit was reported, using protective CTV isolates obtained in the green house by four different methods (202, 203).

Australia. In Australia, stem pitting almost wiped out the grapefruit industry in the 1950s. Trials with Marsh grapefruit to assess the protective value of various CTV isolates against natural infection by severe strains have been in progress for 30 yr under two different climates: in a hot and dry inland site, and a milder and more humid coastal site. The protective CTV isolates were selected from vigorous and productive grapefruit trees in orchards severely debilitated by stem pitting. An acceptable degree of protection was obtained at both sites, but the protection was better at the inland site, benefiting from a hotter climate and having less abundant populations of *Toxoptera citricida*, the predominant CTV vector (36, 53, 76). PCR-amplification of the CTV coat protein gene, followed by RFLP analysis of the resulting cDNA was developed to distinguish the mild cross-

protecting isolates used to control grapefruit stem pitting, from all other Australian isolates (91). Also, difficulties experienced with the protective isolate PB61 in pre-immunization of red grapefruit trees but not white and pink grapefruit trees (37) have led to investigate whether uneven distribution of CTV could be a contributing factor to breakdown of cross protection. Indeed, in early autumn, CTV isolate PB61 was detected only sporadically in the mature spring flush of red grapefruit (Star Ruby and Rio Red), but by late autumn, CTV was easily detectable in all tissues of red grapefruits. Thus, budwood collected in early autumn, but not in late autumn, might have lacked the protective CTV isolate (260). These findings have resulted in changes in the distribution dates within the Australian Citrus Budwood Scheme to ensure that all budwood is effectively pre-immunized with PB61.

Japan. In Japan, protective isolates to control stem pitting on Morita navel sweet orange have been obtained by various methods: (i) from outstanding field trees (pummelo hybrid, nucellar Valencia, Hassaku); (ii) by heat treatment of a Morita navel orange infected with a severe isolate of CTV-seedling yellows, and (iii) by *T. citricida* transmission from CTV-infected trees to Mexican lime seedlings (123, 124, 132). Groups of virus-free nursery plants of Morita navel grafted on trifoliate orange rootstock were graft-inoculated each with one of the eight protective isolates selected. Budwood from the plants infected with the protective strains was propagated on potted trifoliate orange seedlings and let to grow to a height of ~ 30cm. Half of these potted pre-immunized plants were then challenge-inoculated, each with 5 to 20 feeding *T. citricida* aphids infected with the severe CTV isolate carried originally by the original Morita navel trees. Plants were later transplanted to a field closely adjacent to citrus trees of several varieties, infected with severe CTV isolates. Analysis of the trees at 4, 9, and over 10 yr after challenge inoculation showed that protection against the severe CTV isolate was effective for 7 to 9 yr after challenge inoculation, thereafter the cross-protection ability was lost. In the early years, the pre-immunized trees were more vigorous, fruit size was larger, and yield was higher, when compared to the trees inoculated only with the severe isolate. Protective isolates M-15A and M-16A, obtained through *T. citricida* transmissions, gave better protection than the other protective isolates (124).

South Africa. In South Africa, pre-immunization with mild CTV isolates started in 1982 in the frame of the 1981 Citrus Improvement program (CIP), in which all selected citrus material was submitted to shoot-tip grafting for elimination of all graft-transmissible agents, including CTV. As CTV is endemic and spread by *T. citricida* in southern Africa, the CTV- free citrus material from shoot-tip grafting had to be cross-protected by protective CTV isolates before being released in the field and becoming the target of natural infection, possibly with severe CTV isolates. The single protective CTV isolate used for cross-protection was the "Nartia" isolate, later called GFMS 12, from a 50-yr-old Nartia (Marsh-type) grapefruit in Western Cape province (246). Of the commercial cultivars grown in southern Africa, grapefruit is the most sensitive to stem pitting. Very severe CTV stem pitting decline was found in 1979 and 1980 to affect young Redblush and Marsh grapefruit trees in Natal and Western Cape provinces of South Africa, while sister trees from the same budwood batches, but growing in Transvaal and Eastern Cape provinces, did not develop these severe symptoms, indicating an influence of environment on symptom expression (54). Like all other

selected material in the CIP, grapefruit selections were also pre-immunized with the GFMS 12 isolate. However in 1993, 6-yr-old pre-immunized Star Ruby grapefruit trees were found with various degrees of stem pitting and variable fruit size (239). It was later found that isolate GFMS 12 was not a single CTV strain. Indeed, when single aphid transmissions of CTV from GFMS-12 were performed, nine different sub-isolates (12-1 to 12-9) were identified on the basis of biological and molecular characterizations (238). For instance, stem pitting of grapefruit was significantly less in plants inoculated with sub-isolates 12-2, 12-5, and 12-8 than in those inoculated with the original isolate GFMS-12, while with sub-isolate 12-3 stem pitting was more severe. Hence, in the frame of the CIP, isolate GFMS-12 was officially replaced by isolate GFMS 35 for the pre-immunization of all red grapefruit, including Star Ruby. Indeed, GFMS-35, from a Redblush grapefruit, had been found, over a 12-yr period, to perform better than GFMS-12 for protection of Marsh grapefruit (238). Over the years, isolates GFMS-12 and GFMS-35 as well as several additional CTV isolates have been further evaluated and characterized. Fifty single aphid transmissions from isolate GFMS-35 resulted in only two sub-isolates, 35-1 and 35-2. Like sub-isolate 12-3 from GFMS-12, sub-isolate 35-2 produced significantly more stem pitting on Marsh grapefruit test plants than the original GFMS-35 isolate, but the isolate could still be classified as mild. Single-strand conformation polymorphism (SSCP) analysis of CTV gene fragment p27B was able to differentiate between isolates GFMS-12 and GFMS-35 as well as between sub-isolates of these isolates (138). SSCP was also used to show that strain prevalence of GFMS 12 and GFMS 35 in four different grapefruit varieties was altered (153). Finally, a field plot of Star Ruby grapefruit trees on Swingle citrumelo was established at Nelspruit to study their response to pre-immunization with isolates GFMS-12, GFMS-35, sub-isolates 12-2 (mild stem pitting), 12-3 (more severe stem pitting), as well as other isolates from outstanding Star Ruby grapefruit trees in various regions of South Africa. Results on growth, production and disease rating were collected when the trees were 7-yr-old. The data showed that trees pre-immunized with isolate GFMS-35 gave the best results, followed by isolate GFMS-78 (derived from a 10-yr-old planting in Malelane) and sub-isolate 12-2. Isolate GFMS-35 continues to be recommended for use as a pre-immunizing isolate for Star Ruby grapefruit in the southern Africa citrus industry (239).

Peru. In Peru, in the 1970s, the commercial production of sweet oranges, and in particular the Washington navel oranges, came practically to an end as the result of severe CTV stem pitting on the orange tree scions. The causal CTV isolates were introduced into Peru in the 1950s with Satsuma budwood from Japan. Native CTV isolates for cross-protection were from surviving Washington navel and Mexican lime trees selected in the 1980s. Also, CTV isolates derived from passage through *Passiflora* spp. (168, 202) were introduced from California. Some of these native and introduced isolates have been able to protect citrus under open field conditions. Washington navel and Mexican lime trees carrying the protective isolates have been planted with commercial success since the early 1990s. Certain California CTV isolates derived from passage through *Passiflora* have successfully protected citrus in Peru under severe Peruvian inoculum pressure. This suggests an alternative method for developing protective isolates relatively rapidly, rather than waiting for orchards to die and searching for surviving trees (30).

Transgenic citrus expressing CTV proteins. L. Peña and his colleagues in Spain have transformed citrus hosts with CTV genes. Transgenic Mexican lime plants expressing the coat protein gene (*p25*) of CTV were obtained by *Agrobacterium*-mediated transformation, and protection against graft-inoculated CTV was demonstrated (66, 68). This is the first demonstration of pathogen-derived resistance in transgenic plants against a *Closterovirus* in its natural host. Furthermore, Mexican lime plants were transformed with the *p25* gene under selective and non-selective regeneration conditions, ***i.e.*** with and without selection for *nptII* (resistance to Kanamycin) and *uidA* (GUS or β-D-glucuronidase marker). More than 30% of the transgenic limes regenerated under non-selective conditions had silenced transgenes, and in all cases silencing affected all three transgenes incorporated (67).

Experiments have also been conducted with the CTV *p23* gene. As indicated above, *p23* is one of the three CTV genes, which suppress the host silencing defence mechanism. First, gene *p23* from a severe CTV strain (strain T36) was used to transform Mexican lime, a very sensitive CTV host. Most interestingly, the transgenic Mexican lime plants, free of CTV, but expressing protein p23, exhibited symptoms such as leaf vein-clearing, very similar to those induced by CTV itself (87). Next, the same results were obtained when *p23* came from a mild CTV strain (strain 317). Symptoms were correlated with accumulation of p23 protein, irrespective of the source of the *p23* gene, CTV strain T36 or T317. Furthermore, citrus plants other than Mexican lime were also transformed with the *p23* gene. Sweet orange and sour orange, two susceptible citrus hosts, and CTV-resistant trifoliate orange also led to CTV-like leaf symptoms. These symptoms did not develop when plants were transformed with a truncated version of *p23*. In these transgenic citrus plants other than Mexican lime, p23 was barely detectable, but symptom intensity correlated with levels of *p23* transcripts. Finally, with plants, such as *Nicotiana* spp., which are non-hosts for CTV, expression of *p23* led to accumulation of p23 protein, but no symptoms were obtained, indicating that p23 interferes with plant development only in citrus species and relatives (69).

In the above experiments with the *p23* gene, the transgenic Mexican limes expressing *p23* exhibited leaf symptoms characteristic of CTV. However, other lines of *p23* transgenic Mexican limes have been obtained, which, on the contrary, had normal phenotypes and did not show symptoms! Interestingly, these asymptomatic lines displayed features typical of post-transcriptional gene silencing: multiple copies of the transgene, low levels of the corresponding mRNA, methylation of the silenced transgene, and accumulation of *p23*-specific small interfering RNAs (siRNAs). When propagations of these silenced lines were graft- or aphid-inoculated with CTV, they showed no symptoms and did not accumulate virions or viral RNA, indicating that post-transcriptional silencing of *p23*, conferred resistance to CTV in the silenced Mexican lime lines (70). Additional transgenic limes were obtained using the 3'-terminal 549 nt of the CTV gRNA in sense, anti-sense and intron-hairpin formats. While only a single sense-line plant with a complex transgene integration pattern was resistant, nine of the 30 intron-hairpin lines showed CTV resistance, with 9%–56% of bud-propagated plants, remaining uninfected on graft inoculation. CTV resistance was correlated with low accumulation of the transgene-derived transcript rather than with high

accumulation of transgene-derived siRNAs (136).

Finally, transgenic Rio Red grapefruit trees expressing CTV genes are under testing for CTV resistance in Texas (253).

CTV as a virus vector for systemic expression of foreign genes in citrus. The CTV vector for expression of foreign genes in citrus as engineered by W. O. Dawson and his colleagues in Florida has been the result of several lines of work: (1) development of a full-length infectious CTV cDNA clone; (2) *in vitro*-production of RNA transcripts from the cDNA clone; (3) infection of *Nicotiana benthamiana* protoplasts with the RNA transcripts and production of virus of the cDNA clone (recombinant virus); (4) amplification of the virus by successive passages in protoplasts using virions in crude sap as inoculum; (5) by the third to seventh passages in protoplasts, maximal amounts of recombinant progeny virus were produced, which were used for inoculation of small citrus trees by slashing stems in the presence of virion preparations (196). A relatively high percentage of plants became infected with the recombinant virus from the protoplasts, resulting in the first defined pure culture of CTV in plants. The comparative biology of the pure culture of recombinant CTV with that of the parental population *in planta* demonstrated that the recombinant virus retained through all of the recombinant DNA manipulations the normal functions of replication, movement, and aphid transmissibility, and had a symptom phenotype indistinguishable from that of the parental population (222, 223). Several strategies were examined to develop a CTV-based vector for transient expression of foreign genes in citrus trees using the green fluorescent protein (GFP) as a reporter. Engineered vector constructs were examined for replication, encapsidation, GFP expression during multiple passages in protoplasts, and for their ability to infect, move, express GFP, and be maintained in citrus plants. The most successful vectors based on the 'add-a-gene' strategy have been unusually stable, continuing to produce GFP fluorescence after more than 4 yr in citrus trees (73). One of these vectors has been useful to compare CTV distribution in the phloem of different citrus species (74).

The CTV vector and control of huanglongbing. In 2004 and 2005 huanglongbing (HLB) was detected respectively in Brazil and Florida. There is a general consensus that the citrus industry, because of HLB, cannot survive in the absence of trees resistant to HLB. As such trees do not exist to date, they have to be produced. Since the causal agent of HLB is, most-likely, the bacterium *Candidatus* Liberibacter asiaticus, citrus trees carrying and expressing the gene for an anti-microbial peptide (AMP) killing or inhibiting the liberibacters, would be resistant to HLB. To that purpose, Dawson's CTV-based vector comes in most appropriately. The CTV-vector allows insertion of one, two or three AMP genes into the viral genome and expresses the extra gene(s) as it multiplies and spreads throughout the trees. The foreign gene is expressed systemically in both shoots and roots. Most interestingly, after having been amplified within an initial citrus tree, the vector can be transmitted by graft-inoculation to other citrus trees of any size or variety. Since it is generally acknowledged that expression from a viral vector is not permanent, but transient, the CTV-vector was first used for introducing AMP genes into citrus and screening them against bacterial diseases such as citrus canker or HLB. As however the vector has continually expressed foreign genes in citrus for six years, it is very likely that a high percentage of the trees will retain the foreign

peptide gene for ten years or more, and therefore the vector is now used towards the production of HLB- and/or canker-resistant trees (57). Data have already been produced which show that two to three AMP peptides could be expressed from the same vector. Thus, it would be possible to express from the same vector, for instance, two different proteins against HLB and another against citrus canker. A vector has already been developed that will allow re-application of anti-HLB protein or peptide when the first vector has lost the gene. Alternatively, a second application of something better could be added. It has been suggested that the CTV-vector could be graft-inoculated into selected rootstock species (able to multiply CTV) so that scions would become infected through transfer of the vector across graft unions from rootstock to scion. Essentially, any gene could be used in the vector, whether its product directly interacts with the liberibacter, induces the plant to become more resistant, induces the plant to tolerate the bacterium, induces the plant to reduce movement of the bacterium, or other means.

In the CTV-vector approach, the HLB-resistant trees to be produced are not transgenic trees, the foreign gene is not inserted in the citrus genome and there will be no gene dispersion through pollen to weeds or different plants, including citrus. The most efficient vector of CTV, *Toxoptera citricida*, is present in Florida since 1995. If the CTV-vector is aphid-transmissible, it will be naturally transmitted to other citrus trees, which will become theoretically HLB-resistant. This might be a favorable situation regarding resistance to HLB, but it might not be accepted by regulatory agencies or the public at large. If so, the CTV-vector can probably be engineered so as to become non-transmissible by the aphids.

Tristeza: conclusion. Control of quick decline tristeza through replacement of sour orange by compatible rootstocks was essentially developed in the 1940-1950s and saved the citrus industry. One of the major achievements of the scientific community in favour of the citrus industry over the last 50 yr has been the use protective CTV isolates to control stem pitting by pre-immunization of Pera sweet orange in Brazil, Morita sweet orange in Japan, white and red grapefruit varieties in Australia and South Africa, as well as many other varieties in many other countries (125, 188, 255). Initially, protective (mild) strains were collected from surviving or good-looking trees in severely affected and declining orchards. Selection of protective isolates was empirical and time consuming. The putative protective strains were characterized and differentiated from one another by the comparison of symptoms, mild or severe, that developed in inoculated indicator plants in the greenhouse or in experimental trees in the field. Such biological characterizations of CTV isolates have remained indispensable (193), but have been complemented by molecular techniques in recent years, when it became known that, as with other RNA viruses, CTV isolates do not contain a unique genomic sequence. They have a population of sequence variants usually clustered around one or more master or consensus sequences (10) whose composition affects their biological properties. Characterization of this population structure is crucial to understand the biology and evolution of CTV isolates. SSCP (210, 212) has been found well suited to assess the population structure of CTV isolates, and several examples of such SSCP analyses have been given above. Finally; the future will tell if CTV, as a vector for expression of foreign genes in citrus, will contribute to save the citrus industry from huanglongbing.

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II 2. OTHER VIRUSES

In 1957, citrus diseases, which were graft- or arthropod-transmissible were presumed to be caused by viruses, as they were the only infectious agents of plants known in those days. Indeed, the phloem- and xylem- restricted bacteria were only discovered from 1967 on, and the viroids, from 1971 on. Chapter I has been devoted to the viroid diseases of citrus. Section II 1, reviewed separately above, deals with one of the major virus diseases of citrus: tristeza. It was not the first citrus virus to be purified - putative particles of *Citrus tristeza virus* were seen in the electron microscope in 1963 (96), and partial purification was reported in 1965 (179). In 1963, icosahedral particles were purified from plants infected by *Satsuma dwarf virus*. Since then, several more viruses in citrus have been detected and characterized to varying degrees, and

the following lines review these developments. Much of this information has been presented at IOCV conferences. The final section (III) deals with diseases whose agents turned out to be endogenous phloem- or xylem-restricted bacteria.

***Satsuma dwarf virus* (SDV).** Satsuma dwarf was first reported from Japan in 1950 (208, 209), and was subsequently reported from Turkey (9), China (33, 215), and Korea (100). On Satsuma, SDV causes stunting and boat-shaped leaves. It can infect all citrus types and many close relatives, but it is most severe in mandarins. Several non-citrus rutaceous species have been shown to support SDV infection (89). One non-rutaceous natural host has been identified, China laurestine (*Viburnum odoratissimum*), which is symptomless (99), and SDV has been mechanically transmitted to cowpea, sesame, and several *Nicotiana* spp. amongst others (94, 184, 187). No vector has been identified, but SDV does move naturally slowly in orchards in concentric circles; a soil inhabiting vector is suspected (101). Transmission is enhanced where China laurestine is interplanted with citrus (101).

In 1963 icosahedral virions measuring 26 nm were isolated from infected tissue (170). Electron microscopy of SDV-infected leaf tissues revealed spherical viruslike particles in the cytoplasm and in membrane-bound tubules (169). The virus can be resolved by sucrose gradient centrifugation into three components, one of which is composed of empty capsids. The capsid is composed of two coat proteins of 42 kDa and 22 kDa respectively (183), and the genome consists of two single-stranded positive-sense RNA molecules, namely RNA1 (7.0 kb) and RNA2 (5.4 kb) (86). It was initially considered as a tentative member of the *Nepovirus* genus in the *Comoviridae* family (87).

Four closely related viruses, Citrus mosaic virus (CiMV), Navel orange infectious mottling virus (NIMV), Natsudaidai dwarf virus (NDV) (186) and Hyuganatsu virus (HV)(82) were also reported in Japan. The first three are serologically related to SDV and each other, and share over 75% amino acid sequence identity (87). CiMV and NIMV virions have similar morphology to SDV, although NIMV is slightly smaller (185).

The nucleotide sequences of the CiMV coat protein gene on RNA2 (83) and the RNA dependent RNA polymerase (RdRp) gene on RNA1 (84) show little similarity to viruses in the *Comoviridae* family. The nucleotide sequence of SDV has also been determined (85, 88), and it was concluded that SDV was sufficiently distinct from the *Comovirus, Fabavirus* and *Nepovirus* genera in the *Comoviridae* family, and should belong to a separate genus. It was later shown that by comparing SDV with CiMV, NIMV and NDV, they can be placed into three species, namely SDV, CiMV (including NDV) and NIMV (86), and that they are separate from the *Comoviridae* and *Sequiviridae* families (91).

It has since been proposed to name *Satsuma dwarf virus* as the type member of a new genus called *Sadwavirus*, and that CiMV, NDV, NIMV and HV are isolates of SDV (104). *Strawberry mottle virus* is also a member of this genus. No family has been designated so far.

Control is primarily by the use of virus-free budwood. SDV can be eliminated by heat treatment (80) and shoot tip grafting. The possibility of providing protection via transgenic resistance has been investigated. Trifoliate orange lines transformed with the capsid polyprotein gene from CiMV were obtained and showed varying degrees of susceptibility and tolerance; one line had an infection rate of only 7% 60 days after inoculation (90).

Citrus variegation virus (CVV)* and *Citrus leaf rugose virus (CLRV). Infectious variegation (IV) on lemon was first reported in California in 1939 (51). The disease was transmitted by graft-inoculation to lemon and sour orange seedlings, causing mosaic-like variegation, crinkling, flecking and distortion. Shortly before this in 1936, a disorder called crinkly leaf (CL) psorosis was described (48). It was soon recognized IV and CL were barely distinguishable (50), CL having essentially crinkle leaf symptoms but no variegation symptoms. Later it was found that both syndromes are incited by strains of the same virus, *Citrus variegation* virus (CVV). In addition to California, IV has been reported in Florida (68) and several Mediterranean countries (117). In Florida, a disease called citrus leaf rugose was described in 1975 which shared some characteristics with IV and CL (62). IV and CL were initially thought to be part of the citrus psorosis complex, but as progress was made in virus characterization, it became clear that they are not related to psorosis; psorosis did not provide cross protection against IV (32), and virions similar to those purified from CVV-infected plants were not present in psorosis-infected trees (189). For concave gum, blind pocket, crinkly leaf, infectious variegation, leaf rugose, cristacortis and impietratura to be unrelated to psorosis. [See also "Concave Gum – Blind Pocket", Roistacher & Bové (2009), in: Citrus Diseases, www.ivia.es/iocv].

In 1960, mechanical transmission of IV was achieved, the first citrus virus to be transmitted in this way (68). Purification of virions was reported shortly thereafter (31, 37, 42, 121, 211). Both IV and CL were associated with three components that could be separated by gradient centrifugation, but only the bottom component was infectious on its own. The particles are mostly spherical, measuring 25-30 nm. CLRV was purified in 1974, its virions having the same characteristics as CVV (62). CVV was shown to be serologically related to several ilarviruses including Tulare apple mosaic, Elm mottle and asparagus viruses IIP and IIS (192). Calvert et al. (23) used CVV RNA to prepare a library of cDNA clones, which they used to characterize the homology between two CVV isolates and one CLRV isolate.

CVV and CLRV are now classified as members of the genus *Ilarvirus* in the family *Bromoviridae.* The nucleotide sequence of CLRV RNA1 consists of 3,404 nucleotides and contains one open reading frame (ORF) which encodes a putative translation product of 1,051 amino acids (118 kDa) (177). RNA2 consists of 2,990 nucleotides and contains one ORF which encodes a deduced translation product of 832 amino acids (95 kDa) (66). The coat protein gene is encoded on RNA3, and comparisons between CVV and CLRV indicate that CVV may be more closely related to other ilarviruses than to CLRV (14).

Lovisolo (110) speculated that since ilarviruses were first found in citrus in North America and Europe, they may have been introduced into citrus there via infected pollen, or thrips.

Citrus tatter leaf virus (CTLV). Citrus tatter leaf was first described in California by Wallace and Drake (204) in *Citrus excelsa* which developed foliar symptoms after graft inoculation from apparently healthy Meyer lemon. Troyer citrange also developed symptoms, and Wallace and Drake (205, 206) attributed these symptoms to a second virus which they called citrange stunt virus (CSV). Attempts to separate tatter leaf from citrange stunt by successive graft inoculation passages through various citrus species during 22 yr were unsuccessful, and the conclusion was drawn that only one virus

was involved: *Citrus tatter leaf virus* (CTLV) (156, 159).

In Japan in 1962, satsuma trees on trifoliate orange rootstocks were observed with scion swellings above the rootstock and bud-union crease (181), and indexing studies on these trees using *C. excelsa* and citrange resulted in tatter leaf symptoms (132). A later study showed that trees in Japan with bud-union crease are consistently infected with CTLV (130). A 25% reduction in yield of CTLV-infected Ponkan mandarin on trifoliate orange compared to healthy trees has been reported (182).

CTLV has been reported in Meyer lemons from several countries including South Africa (34), Australia (20), and the USA states of Florida (61) and Texas (188). In South Africa, a budunion-crease of Shamouti orange on Swingle citrumelo was attributed to CTLV (119). Also in China, mandarin and orange trees on trifoliate orange displaying budunion crease were found to be infected with CTLV (92).

CTLV infects most Citrus species symptomlessly (133). Chlorotic leaf lesions are produced in Mexican lime, *C. excelsa*, citranges and citrumelos. The virus can be mechanically transmitted to many herbaceous plants. In cowpeas it causes necrotic local lesions and variable systemic necrosis, and in *Chenopodium quinoa*, chlorotic local lesions appear on inculated leaves, followed by a systemic mottle. The virus has also been isolated from wild lily (*Lilium longiflorum*) in Japan with yellowing symptoms (81).

Semancik and Weathers (178) partially purified from cowpeas flexuous rod-shaped particles measuring 650 x 19 nm, and Miyakawa and Matsui (132) observed 600-700 x 15 nm particles in leaf-dip preparations from citrus and cowpea. Later, Nishio et al. (150) purified the virus and determined by electron microscopy that the flexuous particles were 600-650 x 13 nm, with conspicuous criss-cross patterns when stained with uranyl acetate. The coat protein is a single species of 27 kDa, and the virus possesses a single RNA species of 6,496 nt (151). CTLV is serologically related to *Apple stem grooving virus* (ASGV), a *Capillovirus* in the family *Flexiviridae*. Comparing the sequence of the 3'-terminal end nucleotides of the CTLV genome to that of the ASGV genome, Yoshikawa et al. (210) concluded that CTLV is a capillovirus closely related to ASGV. Magome et al. (114) compared sequences of several ASGV isolates from apple, Japanese pear and European pear with those of CTLV from citrus, and concluded that CTLV should be considered an isolate of ASGV.

Evidence for CTLV strain variability has been presented on the basis of (i) symptom expression with some isolates failing to induce budunion crease (134), (ii) serology where ELISA with both CTLV and ASGV antisera gave variable results (75), and (iii) nucleotide sequencing (114).

CTLV is readily transmitted mechanically (61). Seed transmission has been reported in both *L. longiflorum* and *C. quinoa* plants (81), but not in kumquat (149). Control is achieved by using virus-free budwood. CTLV is not eliminated by standard shoot tip grafting (161), but thermotherapy of 40°/30°C for more than 60 days is effective (22, 131, 158). Because satsuma budwood is intolerant to the temperatures used for thermotherapy, Koizumi (99) developed a method of subjecting a CTLV-infected satsuma with new shoots to shorter heat treatment and using shoot tip grafting thereafter. This procedure resulted in CTLV-free plants. Also, when Valencia or Washington navel orange budsticks were grown at 32°C and new shoots with three leaf primordia were grafted *in vitro*, about 42% of CTLV-free plants were obtained (142).

Citrus leprosis viruses (CiLV). Leprosis was originally thought by both Floridian and South American researchers in the early 20th century to be a fungal disease (46), but spray trials in Florida in 1950 provided evidence against this theory (98). The association of leprosis with false spider mites (*Brevipalpus* spp.) led to the virus theory (15, 48). Knorr (97) showed that grafting symptomatic bark tissue into young citrus stems resulted in a localized spread of the leprosis agent into the receptor host as witnessed by localized lesion development. These results provided evidence of transmission of an infectious agent, and were confirmed by Rossetti et al. (163) who demonstrated that mites from leprosis-free areas were unable to transmit leprosis unless they had access to affected tissues.

Citrus leprosis has spread to several South and Central American countries (71, 155), and most recently into Mexico (170). It has not been reported in Florida since 1968 (26). On leaves, local lesions begin as chlorotic patches which can develop a necrotic center with a chlorotic halo (163). Older, larger lesions may contain concentric brown rings sometimes containing gum. Fruit symptoms start as flat yellow patches, and as the fruit matures the lesions enlarge, turn black or brown and become sunken. Lesions on twigs and branches start as small chlorotic flat patches which coalesce into raised, brown areas. Extensive lesion formation causes dieback, and psorosis-like bark scaling develops (155). Leprosis can be mechanically transmitted to several herbaceous plant species (111, 112).

In 1972, Kitajima et al. (95) observed by electron microscopy short, rod-like particles measuring 100-200 nm x 40 nm in leprosis- infected sweet orange tissue, with some particles apparently budding through the nuclear membrane ("nuclear" type virus). Later, EM studies revealed non-enveloped particles (120-130 x 50-55 nm) in the endoplasmic reticulum (28, 111), sometimes with dense viroplasm-like masses in the cytoplasm ("cytoplasmic" type virus) (155). Both types of virus were described as rhabdovirus-like, with the cytoplasmic form much more common than the nuclear one (71).The first report of partially purified virions from leprosis-infected tissue came in 2000, with the publication of electron micrographs showing 80-120 x 45-50 nm rod shaped particles (29); unfortunately, the authors did state whether this was the cytoplasmic or nuclear form.

The discrepancy between the nuclear and cytoplasmic forms of leprosis virus has now been largely resolved. Guerra-Moreno et al. (71) constructed a cDNA library from an RNA extract of the cytoplasmic virus. They identified two RNA species (RNA1 and RNA2), which did not hybridize with the nuclear types, suggesting that two distinct viruses were involved. The complete nucleotide sequence of both RNAs of the cytoplasmic form was published in 2006 and while there were some sequence similarities with genera such as *Tobamovirus, Bromovirus, Furovirus and Tobravirus*, and no similarity with the *Rhabdoviridae*, it was concluded that the cytoplasmic form of the virus be considered the type member of a new genus, *Cilevirus,* in an unassigned family (106). The nuclear type virus remains unclassified.

***Citrus psorosis virus* (CPsV).** Citrus psorosis bark scaling symptoms were first observed in Florida in 1896 (180), but the graft-transmissibility and hence the infectious nature of the causal agent was only demonstrated 30 yr later, first by the induction of leaf flecking symptoms in graft-inoculated seedlings (47) and then in the reproduction of bark scaling (49). The widespread occurrence of psorosis in California and Texas was the stimulus of the first budwood registration program (154).

Because leaf flecking symptoms of various types were also induced when seedlings were inoculated from trees with crinkly leaf and concave gum diseases, these were referred to as psorosis-type diseases, but cross protection studies did not support this conclusion (32, 65) and later virus protein studies established that they were not related (35). For concave gum, blind pocket, crinkly leaf, infectious variegation, leaf rugose, cristacortis and impietratura to be unrelated to psorosis. [See also "Concave Gum – Blind Pocket", Roistacher & Bové (2009), in: Citrus Diseases, www.ivia.es/iocv].

The report of a new disease of citrus named citrus ringspot in 1968 (206) actually led to the purification of *Citrus psorosis virus* (CPsV). It was found to be mechanically transmissible to some herbaceous plants (65), which aided determining which fractions during purification were infectious (41, 58, 147). Infected plants were found to contain a 48 kDa protein, and antiserum to this was used to trap particles for electron microscopy (41). The particles were of two lengths, and appeared to be spiral in shape. Psorosis strains of different origins were shown to have associated similar spiral particles and capsid protein (35, 146). An elegant EM study determined that the filamentous particles occur in both open circular form and closed linear forms (59, 128). It was proposed that the name *Ophiovirus* be adopted. It is now the accepted genus name, CPsV is the type species, and a new family, *Ophioviridae*, has been designated (129). The genome of two different CPsV isolates were completely sequences, and in both cases it was found to consist of three separately encapsidated genomic RNAs, two of them associated with the smaller particles and one with the larger (125, 137, 138, 172, 173, 175). Genomic variation of CPsV isolates from different countries was analyzed (126) and at least three distinct groups have been recognized. Phylogenetic analyses also indicated that exchange of genomic segments may have contributed to CPsV evolution (126).

Monoclonal and new polyclonal antibodies to CPsV were developed in different laboratories (6, 43, 107) that enabled quick detection of the virus by several ELISA procedures (6, 7, 44, 45, 60, 107, 123, 213). After sequencing the CPsV genomic RNA, detection of the virus was also achieved by molecular hybridization and RT-PCR techniques (108, 109, 60, 124). Although Koch's postulates have not yet been fulfilled for psorosis disease, detection of CPsV by ELISA, molecular hybridization, RT-PCR and EM was closely associated with psorosis diseases as diagnosed by field symptoms, biological indexing and cross protection against severe psorosis B isolates (124).

No vector has been identified, but there is evidence of natural spread in orchards (13, 190), and *Olpidium* zoospores, known to carry other ophioviruses, obtained from the roots of psorosis infected trees contain CPsV RNA (153). In regions where no natural spread was observed, the disease has been controlled by appropriate sanitation, quarantine and budwood certfication procedures (140, 142, 143). For regions with natural disease spread, additional measures may be necessary. For this purpose, transgenic plants showing pathogen-derived resistance have been obtained (212).

Citrus vein enation virus (CVEV). Citrus vein enation was first described in California on rough lemon and Mexican lime and shown to be caused by an aphid-transmitted pathogen (202, 203). Woody gall was first described on rough lemon in Australia (54, 55), and it was soon demonstrated that these two symptoms were caused by the same agent (203). Vein

enation/ woody gall has been reported from many countries, and probably has almost world-wide distribution. It is a non-destructive disease, and the only report of reduced growth is in young trees with extensive woody gall symptoms in Peru (12).

Enations are initiated as cytological abnormalities of phloem fiber primordial cells adjacent to protophloem sieve tubes (78). They enlarge by hyperplasia of the affected fiber primordials. The mesophyll and epidermal tissue on the abaxial side of leaf veins divide less prolifically and their growth ceases as leaves mature. Woody galls develop from affected cells of procambial tissue between metaxylem and metaphloem of vascular bundles, and contain large amounts of abnormal xylem tissue. Gall growth is indeterminate.

Rough lemon, Volkamer lemon, Rangpur lime and Mexican lime develop galls. Lime and sour orange develop conspicuous vein enations in the field, while sweet orange, lemon, mandarin and rough lemon develop enations under cool greenhouse conditions. Enations are sometimes present in infected Palestine sweet lime and kumquat. In India, enations have been observed on grapefruit, *C. amblycarpa, C. macroptera, C. latipes* and *C. pennivesculata* (118). No non-rutaceous host has been reported.

There is a reported synergistic effect between CVEV and the yellow vein pathogen resulting in a marked enhancement of the yellow vein symptom in Etrog citron, rough lemon and Mexican lime (207). In Japan, CVEV was shown to cross-protect against *Citrus tristeza virus* in some hosts (102). Vein enations are suppressed in Mexican lime and Pineapple sweet orange by CTV T30 isolates, but not by other isolates, and are not suppressed in sweet orange by viroids (193).

CVEV is transmitted in a persistent manner by several species of aphids, namely *Myzus persicae, Aphis gossypii, Toxoptera aurantii* (73) and *T. citricida* (116), which are also semi-persistent vectors of CTV. There is a latency period of 2-3 days. There is no evidence for seed transmission.

Virus-like particles were observed by electron microscopy of enations from rough lemon leaves and in the salivary glands of viruliferous aphids (115). Isometric particles have been purified , and based on morphology and disease characteristics, it was suggested that CVEV might be a luteovirus (36). It was then demonstrated that positive ELISA results could be obtained for CVEV using some commercial Barley yellow dwarf virus kits (27). Attempts to amplify cDNA using luteovirus primers have not been successful, thus the luteovirus proposal remains unconfirmed.

Citrus yellow mosaic virus (CYMV). In 1993, a graft-transmissible mosaic disease of pummelo was reported from India (2). Bacilliform particles were detected in extracts from infected leaf tissue, and it was suggested that the associated virus was a rhabdovirus. Subsequently, the disease, which was named citrus yellow mosaic, was found causing losses in sweet orange (4). Partial characterization of the virus showed that it was not a rhabdovirus, but that it possesses a dsDNA genome, and via sequence determination it was found to be a *Badnavirus* (4, 5). Serological studies showed it to be serologically related to sugarcane bacilliform badnavirus (4). Subsequent DNA sequencing and phylogenetic analysis has shown CYMV to be most closely related to *Cacao swollen shoot virus* (10, 79). The complete nucleotide sequence has been determined to be 7,559 bp in length and to contain six putative open reading frames (79).

Electron microscopy of CYMV-infected leaves revealed the presence of

aggregations of free virions in cells, as well as inclusions or viroplasms in the cytoplasm, similar to other badnaviruses (18).

Transmission from citrus to citrus by the citrus mealybug, *Planococcus citri,* has been shown (64). The virus has been experimentally transmitted to three non citrus hosts, *Canna indica*, sorghum and maize (8).

Indian Citrus ringspot virus (ICRSV). A psorosis-like ringspot was discovered in India on sweet orange in 1989, but field trees did not develop bark scaling (1). Filamentous rod-shaped particles resembling capilloviruses were purified from infected leaves with modal dimensions of 640 x 15 nm (21). There was no serological reaction to antisera against Florida citrus ringspot virus, now known to be a strain of CPsV. Further characterization of the virus has shown it to have a ssRNA genome of 7.5 kb, and a coat protein of 34 kd (166). Several isolates of ICRSV from different parts of India have been described and analysis showed some variation near the N terminus of the coat protein but conservation in the core region (76). Comparisons showed some isolates had 98-99% homology, while others showed only 84-85% homology (166, 168). All were serologically related.

It has been proposed that ICRSV is the type member of a new genus, *Mandarivirus*, in the family *Flexiviridae* with *Potexvirus* being the closest related genus (166, 168).

It can be mechanically transmitted to *Chenopodium quinoa, C. amaranticolor,* soybean, cowpea and French bean cv. Saxa (167). No natural vector has been identified. Virus-free plants have been generated through shoot tip grafting (77).

Citrus leaf blotch virus (CLBV). A new graft transmissible agent was reported in 1984 in Nagami kumquat from Corsica showing a bud union crease on Troyer citrange (141). In a host range study, the agent caused three types of symptoms: (i) vein clearing in sweet orange, sour orange, Troyer citrange, grapefruit, Dweet tangor, Orlando tangelo, alemow, but not Mexican lime amongst others; (ii) stem pitting in Etrog citron, and (iii) graft incompatibility on Troyer citrange. Some 800 nm flexuous particles were observed in leaf dip preparations. Many years later, partial purification studies resulted in the detection of 900 x 14 nm particles containing a ssRNA genome of 8,747 nt and a coat protein of 41 kDa (56, 194, 195). The virus was named Citrus leaf blotch virus and suggested to be the type species of a new genus (196). It has been detected in citrus in Australia, USA and Japan (195). Sequence comparisons between isolates from different countries showed very low genetic variability (197), thus allowing reliable detection of CLBV by molecular hybridization and RT-PCR (57, 195). A cDNA clone of the CLBV genomic RNA was shown to be infectious in *Nicotiana occidentalis*, *N. benthamiana* and in citron. Virions produced from this clone were indistinguishable from the wild type and induced characteristic leaf blotching in Dweet tangor and stem pitting in citron, but not bud union crease in plants propagated on citranges, suggesting that an additional agent was present in the original CLBV isolate (198).

In 1968, routine biological indexing of citrus in California showed the presence of a graft-transmissible agent in Cleopatra mandarin which caused chlorotic blotching in Dweet tangor. The agent was named Dweet mottle virus (DMV) (159). After the partial characterization of CLBV, two sources of DMV were compared with CLBV (199). DMV induced leaf blotch in Dweet tangor and stem pitting in Etrog citron, but no symptoms in other species. Nucleotide sequencing showed that there was 96-98%

homology (depending on the gene) between CLBV and DMV, suggetsing that CLBV is actually the causal agent of Dweet mottle disease (198). A low rate of seed transmission of CLBV has been reported (72), thus forcing a change in quarantine and certification procedures related to citrus seed.

Citrus yellow vein clearing virus (CYVC). A survey of citrus in Pakistan reported in 1988 described a disease of lemons and sour orange with variegation/ringspot symptoms which were negative for CVV in ELISA (24). Bové (16) described the same symptoms which he called 'yellow vein clearing'. In a further survey conducted by Catara et al. (25), symptoms were not found in sweet orange, mandarin, grapefruit or limes in the field. Graft transmissibility was demonstrated, with typical symptoms on lemon and sour orange, and a mild mottle in sweet orange. Then, Grimaldi and Catara (69) detected flexuous particles in partially purified preparations, with modal length of 670-700 nm. Electron microscopic examination of infected tissue revealed low numbers of filamentous particle aggregates in phloem cells. Yellow vein clearing has also been reported from Turkey (152).

Miscellaneous viruses. There is a number of citrus diseases whose etiology remains undetermined. There are some for which viruses have been associated with, but their role in the disease is still unknown. **Citrus blight**, which was first reported in Florida in the 1890s, received a lot of attention in the latter part of the 20th century, but the causal agent remains elusive; an idaeovirus was reported from infected trees (39), and there has been a suggested role for unusual strains of CTV found in roots of blighted trees (40). A newer disease in Brazil, **citrus sudden death** (CSD) (17, 67, 135), has epidemiological (11) and anatomo-pathological (162) similarities to CTV. A *Marafivirus*, as well as CTV, which is endemic in Brazil, have been detected in all CSD infected trees (112). CSD is graft-transmissible, and an aerial vector is involved (30). The respective roles of the *Marafivirus* and CTV in CSD are not known. Another marafivirus-like agent was reported in Texas, apparently causing a symptomless infection (74).

In 1972, ***Tobacco necrosis virus* (TNV)**, a *Necrovirus*, was detected in citrus leaves infected with either psorosis or concave gum plus cristacortis (212). Back inoculation of purified TNV to several citrus species resulted in local necrotic lesions**.** ***Olive latent virus 1***, a *Necrovirus* in the *Tombusviridae* family, has also been detected in citrus in several Mediterranean countries - it does not appear to cause any disease symptoms (53, 121).

In 1983, a report at the 9th IOCV conference presented evidence of flexuous particles in association with a new, graft-transmissible disease of Satsuma trees in Japan called **citrus yellow mottle** (191). Unfortunately, no further work has been reported. A latent virus was detected in a symptomless navel selection which was mechanically transmitted to several herbaceous hosts (63). Flexuous particles were observed in extracts of both the citrus and non citrus hosts.

Rod-shaped virus particles were isolated from a psorosis isolate in California (105), and a cucumovirus from a tatter leaf source (120). They are presumably latent viruses co-infecting with CPsV and CTLV respectively.

Diseases of Unknown Etiology. While the few viruses mentioned above may not all cause diseases, there are several other citrus diseases for which no pathogen of any sort has so far been detected. These include **concave gum and blind pocket** (52), **cristacortis** (200), **abnormal bud union** of

rough lemon with sweet orange scions (70, 127, 143), **impietratura** (165), **yellow vein** (207), **leathery leaf** (3), **yellow ringspot** (135, 144, 145) and similar diseases designated by other names (19, 38), **fatal yellows** (175, 176), **Bahia bark scaling** (148), **chlorotic dwarf** (93), **measles** (103) and several others. New technologies are being developed which will probably identify some of these pathogens; there is still much work to be done.

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