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Further characterization studies on *Citrus Tristeza Virus* (CTV) isolates collection from Mediterranean countries and their interaction with citrus viroids

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Tesi di Dottorato

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A vous chers parents, à mes sœurs et mon frère, vous avez toujours vu en moi un certain potentiel, vous ne m'avez pas laissé le choix, et je vous en remercie.

A mon grand père qui nous a quitté et que je n'ai pas eu l'occasion de revoir, qu'ALLAH bénisse son âme.

" C'est celui qui s'égare qui découvre de nouveaux chemins "

Nils Kjær

" En cherchant à hâter les choses, on manque le but.

La poursuite de petits avantages fait avorter les grandes entreprises"

Confucius

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Abstract:

The biological properties of a collection of citrus tristeza virus (CTV) isolates from 13 Citrus-growing countries mostly Mediterranean, an area for a long time free from severe CTV isolates, were investigated. The study confirmed the presence of such isolates in this area, as a lot of biologically indexed isolates were able to produce even stem pitting on Madam vinous Sweet Orange (SwO) considered specific for the most dangerous strains.

The symptoms expressed by 27 CTV isolates on the standard set of CTV indicator plants; Mexican lime (M.L), Duncan grapefruit (Gft), sour orange (SO) seedlings, Madam vinous SwO seedlings and Madam vinous SwO grafted on sour orange enable the establishment of the Biogroups that are present in the Mediterranean Area ranging from the severe Biogroups 4 and 5, mostly in the Middle East region, to the Mild Biogroup 1 in other regions.

Interestingly, all the isolates belonging to the Biogroups 4 and 5 gave a positive reaction with the MCA13 MAbs, produced to detect severe CTV isolates in Florida, but being sometimes ineffective to detect others elsewhere. Such rapid tests are of important concern and are needed to identify isolates that have potential economic impact in commercial citrus groves and to adopt a sound CTV management strategy.

But discriminative analysis needed have to be, as rapid as reliable, and this was another aim of this work; finding potential molecular markers that could be used for sensitive and quick identification of virulent CTV isolates through the study of a Mediterranean CTV collection.

For this purpose the symptom expression and biogroups obtained for the 27 studied isolates were compared with conventional Single Strand Conformation Polymorphism (SSCP) and Capillary Electrophoresis CE-SSCP results, for four 3′ terminal genes (p18, p20, p27 and p23), and with genetic variation by sequencing.

The conventional SSCP is a relatively robust and easy to perform procedure, and is a good primary molecular differentiation for CTV isolates, but it has as main

limitation the non quantification of profile differences in terms of the genetic distance between the corresponding DNA fragments providing therefore only a qualitative picture of the variation.

CE-SSCP, as alternative to gel slab techniques has become a promising alternative to gel based SSCP methods, presents the advantages of high sensitivity, high specificity and is easy to perform. The technique was able to distinguish between a set of already known different sequences and the repeatability of the results within and between different runs was also evaluated.

A great diversity of SSCP profiles was observed among isolates for the genes studied for both methods and some isolates of the same biogroups displayed the same haplotype, with both conventional and CE-SSCP analysis, mostly for the p20 gene, even if they were from different geographical origin.

Other field CTV isolates have to be phenotyped and subjected to other Conventional and Fluorescent SSCP tests so that the proposed models can be verified, confirmed or modified.

Another part of the work, aiming to study the interaction between CTV and Citrus Viroids, demonstrated the encapsidation of viroid particles by CTV coat protein. This phenomenon was more likely to happen when the conditions were favorable for CTV multiplication, and was confirmed for the available CTV strains in the collection and for the three most economically important citrus viroids; *citrus cachexia viroid* (CVd-II), *citrus exocortis viroid* (CEVd) and *citrus dwarf viroid* (CDVd). This phenomenon can allow to viroid particles to be transmitted by citrus aphids.

Riassunto:

Le proprietà biologiche di una collezione di isolati del virus della tristeza degli agrumi (CTV) afferenti a 13 paesi produttori di agrumi, prevalentemente Mediterranei, un area per molto tempo indenne da severi isolati di CTV, sono stati indagati. Lo studio ha confermato la presenza di tali isolati nella zona, visto che molti isolati saggiati biologicamente sono stati in grado di indurre anche la butteratura del legno sull'Arancio dolce Madam vinous considerato sintomo specifico indotto dai ceppi più pericolosi del virus.

I sintomi espressi da 27 isolati d CTV sul set standard delle piante indicatrice; Limetta messicana, Pompelmo duncan, Arancio amaro da semenzale, l'Arancio

dolce Madam vinous e l'Arancio dolce innestato su Arancio amaro ha consentito la definizione dei Biogruppi che sono presenti nel Mediterraneo che variano dai severi Biogruppi 4 e 5, per lo più nella zona del Medio Oriente, al Biogruppo di ceppi blandi nelle atre zone.

È interessante specificare che tutti gli isolati appartenenti al Biogruppo 4 e 5 hanno dato una reazione positiva con l'anticorpo monoclonale MCA13, prodotto per individuare i ceppi severi del CTV in Florida, ma che si è rivelato a volte inefficace per rilevarne altri altrove. Tali test diagnostici rapidi sono di grande importanza e sono necessari per identificare gli isolati che hanno un potenziale impatto economico in agrumeti commerciali e per adottare una strategia di lotta adatta ai ceppi di CTV ritrovati in campo.

Ma le analisi discriminatorie devono essere quanto più rapide che affidabile, e questo era un altro obiettivo di questo lavoro, trovare potenziali marcatori molecolari che potrebbero essere utilizzati per un identificazione sensibile e veloce di isolati virulenti di CTV attraverso lo studio di una collezione di CTV del Mediterraneo.

A tal fine, l'espressione dei sintomi e i Biogruppi ottenuti per i 27 isolati studiati sono stati confrontati con i risultati ottenuti con il metodo convenzionale del Polimorfismo di Conformazione dei Singoli Filamenti (SSCP) e con l'Elettroforesi Capillare del Polimorfismo di Conformazione dei Singoli Filamenti (CE-SSCP) per i quattro geni (p18, p20, p23 e p23) e con le variazione genetiche mediante il sequenziamento.

La SSCP convenzionale è una procedura relativamente robusta e facile da eseguire ed è una buona differenziazione molecolare primaria tra gli isolati di CTV, ma presenta come limitazione principale la non quantificazione delle differenze di profili ottenuti in termini di distanza genetica con i frammenti corrispondenti di DNA fornendo quindi solo una rappresentazione qualitativa della variazione.

La CE-SSCP, in alternativa alla tecnica con il gel di polyacrylamide, è diventata una promettente alternativa al SSCP convenzionale e presenta i vantaggi di alta

sensibilità, alta specificità ed è facile da eseguire. La tecnica è stata in grado di distinguere tra una serie di sequenze già note come differenti e la ripetibilità dei risultati è stata valutata sia per la singola corsa che tra le diverse corse elettroforetiche.

Una grande diversità dei profili di SSCP è stata osservata fra gli isolati per i geni studiati con entrambi i metodi ed alcuni isolati degli stessi Biogruppi hanno esibito lo stesso aplotipo sia con la SSCP convenzionale che con la capillare, principalmente per il gene p20, anche se provenivano da aree geografiche differenti.

Altri isolati da CTV da campo devono essere sottoposti ad analisi del fenotipo e altre prove di SSCP convenzionale e fluorescenti, in modo da poter verificare, confermare o modificare i modelli proposti.

Un'altra parte del lavoro che aveva come scopo studiare l'interazione tra CTV e i viroidi che colpiscono gli agrumi, ha dimostrato l'incapsidazione dei viroidi dalla proteina di rivestimento del CTV. Questo fenomeno si è rivelato più probabile quando le condizioni sono favorevoli per la moltiplicazione del CTV, ed è stato confermato per i ceppi disponibili nella collezione di isolati e per i tre viroidi economicamente più importanti degli agrumi; il viroide della cachessia (CVd-II), il viroide dell'exocortite (CEVd) e il viroide del nanismo degli agrumi (CDVd). Questo fenomeno può permettere alle particelle del viroide di essere trasmesse da afidi vettori di CTV in agrumeto.

CHAPTER 1: BIBLIOGRAPHIC REVIEW

Citrus production is adversely affected by a number of diseases caused by viruses and viroids that had major impacts on citrus production.

Citrus tristeza virus (CTV) alone has caused the loss of millions of trees worldwide. The virus originated in Asia and has spread by man to most citrus producing areas causing variable losses depending on the virus strains predominant in each citrus region.

CTV isolates often differ in biological characteristics, such as symptom intensity in different citrus species. The slow decline is very frequent on the Mediterranean area, but more severe strains exist and can be found elsewhere.

Changing rootstocks is of common use by growers in response biotic and abiotic stress that can limit citrus production.

To overcome the CTV 'quick decline' on sour orange and also the Phytophthora root rot affecting trees grafted on sweet orange or rough lemon root stocks, the trifoliate orange [Poncirus trifoliate (L.) Raf.] has been used as

rootstock, but some cultivars on this latter were dwarfed and unthrifty, with obvious bark scaling near the graft union demonstrating to be sensitive to viroid infection. Also known as "exocortis", these bark scaling symptoms signal the presence of CEVd in the latently infected scion.

The propagation of viroid-infected trees has caused significal economic losses in citrus industry and is a major threat in regions where susceptible rootstocks are used.

Although viroids cause several different diseases in citrus [e.g., cachexia and xyloporosis (HSVd) in addition to exocortis (CEVd)], it is important to note that viroid infection can also be beneficial to citrus production. For example, both CEVd and Citrus dwarfing viroid (formerly known as Citrus viroid III) have been used to dwarf citrus, thereby allowing a predictable degree of tree size control and the use of higher planting densities (Hutton *et al.*, 2000).

Citrus as all woody perennial crops should have a long productive life during which they can be challenged with other pathogens as CTV that is readily aphid transmitted. While interaction of viroids with other plant pathogens (fungal, viral) has been reported in some cultures as grapevine and potato, nothing is known about what can induce this natural and non-pathogenic viroid variant in the presence of another disease pressure.

Once trees are infected, the disease agents cannot be eliminated. This is of particular economic importance, since citrus trees should have a long productive life. Thus the best control measures would be to prevent the introduction of the disease or propagate plants that are disease resistant.

Characterization of CTV family and virions

Characterization of the family: *Closteroviridae*

The plant virus family *Closteroviridae* is comprised of viruses with flexuous rod-shaped virions of 1250 to 2200 nm in length (Alkowni *et al.,* 2004). These viruses all contain a positive sense single-stranded RNA genome that approaches 20 kb (Alkowni *et al.,* 2004). Initially, when established in 1998 the family consisted of just two genera, Closterovirus and Crinivirus. The major

differentiating trait of the two genera was the possession of monopartite and bipartite genome, respectively (Martelli *et al*., 2002). Karasev (2000), argued that Closteroviruses should be classified by the type of insect vector rather than by the number of genomic RNAs. He proposed a genus named Vinivirus, but the International Committee on Taxonomy of Viruses (ICTV) study group on Closteroviruses and Allied viruses changed it to Ampeloviruses (from ampelos, Greek for grapevine) to prevent confusion with the genus Vitivirus (Martelli *et al*., 2002). The revised version of the family was approved by the ICTV in July 2002. The family consists of 3 genera: Closterovirus, Crinivirus and Ampelovirus, with the differentiating trait being the family of the insect vector (Fig. 1) (Martelli *et al*., 2002).

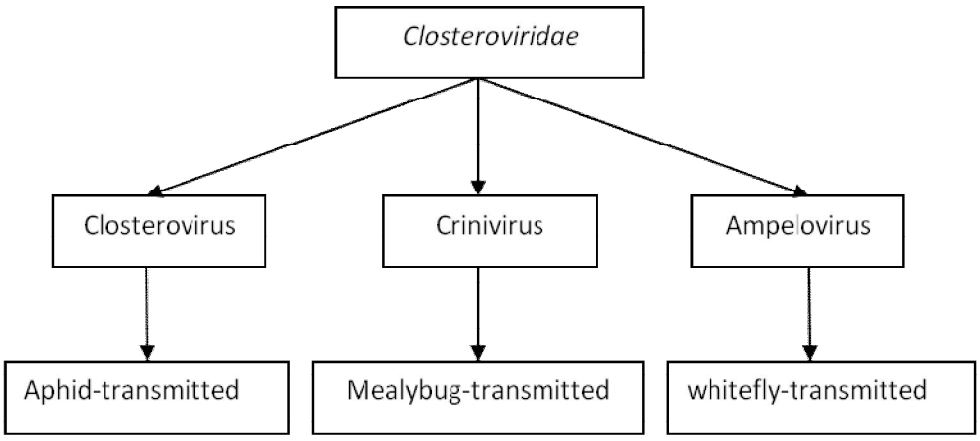


Fig. 1 The three genera of the family *Closteroviridae* with its associated insect vector.

The ancestral virus in the evolution of the Closteroviruses was most likely a monopartite virus. This suggestion is based on the phylogenetic clustering of all the whitefly transmitted closteroviruses and the great uniformity in their genome organization. This also indicates a recent origin of the bipartite genome that had not yet had enough time to significantly evolve (Karasev, 2000).

Phylogenies inferred from the amino acid sequences of the closterovirus HEL, RdRp and the HSP70 suggests that the closterovirus co-evolved with their insect vectors. This co-evolution probably took place over a considerable period of time for the great diversity to arise. Thus evolution within the family *Closteroviridae* most probably followed the three families of insects: aphids, mealybugs and whiteflies (Karasev, 2000).

CTV genome

As with other members of the family Closteroviridae, CTV virions are bipolar and are coated with separate coat proteins 25 kDa (CP) and 27 kDa (CPm), designated as major and minor CPs that encapsidate about 97 and 3% of the virion length, respectively (Febres *et al.*, 1996; Satyanarayana *et al.*, 2004). Its virions are flexuous filaments of about 2000 x 11 nm, with about 6% RNA content, that are helically constructed with a basic pitch of about 3.7 nm (Bar-Joseph *et al.*, 1972).

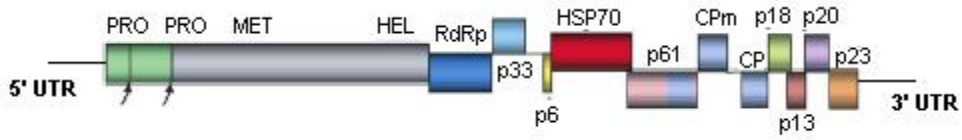


Fig. 2. Genomic organization of citrus tristeza virus, showing the relative position of the 12 ORFs and their expression products; **PRO:** papain-like proteases, **MT:** methyltransferase, **HEL:** helicase, **RdRp:** RNA-dependent RNA polymerase, **p6:** hydrophobic protein, **HSP70** heat-shock proteins, **CPm:** minor coat protein, **CP:** major coat protein, **UTR:** Untranslated Terminal Regions.

The ~19.3-kb genomic RNA (gRNA) of CTV is single-stranded and positive sense, and its complete nucleotide sequence has been determined in at least eleven distinct isolates (Albiach-Martí *et al.*, 2000; Harper *et al.*, 2009; Karasev *et al.*, 1995; Mawassi *et al.*, 1996; Pappu *et al.*, 1994; Ruiz-Ruiz *et al.*, 2006; Suastika *et al.*, 2001; Vives *et al.*, 1999, 2005; Yang *et al.*, 1999).

The CTV gRNA is organized into 12 open reading frames (ORFs) (Fig. 2), potentially encoding at least 17 protein products, and two untranslated regions (UTRs) of about 107 and 273 nt at the 5′ and 3′ termini, respectively. ORFs 1a

and 1b, encoding proteins of the replicase complex, are directly translated from the gRNA, 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, 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.

p23, p20 and p25 genes

The three proteins P23, P20 and P25 have been found to act as RNA silencing suppressors in *Nicotiana tabacum* and *Nicotiana benthamiana* plants (Lu *et al.*, 2004).

The only CTV protein with no homologue in other closteroviruses is P23. The molecular determinants of CTV induced symptoms are presently unknown, although transgenic Mexican limes (*Citrus aurantifolia*) over-expressing the p23 protein display symptoms identical to those caused by CTV in this host, and symptom appearance is associated with p23 accumulation (Ghorbel *et al*., 2001). Therefore, the accumulation of p23 rather than its origin seems to determine the intensity of the symptoms in transgenic limes.

In citrus protoplasts, its sgRNA is the most abundant at the beginning of the infection and the second most prevalent in later stages, suggesting a role of p23 in early steps of viral replication or transcription (Hilf *et al.*, 1995; Navas-Castillo *et al.*, 1997). However, p23 accumulates at low levels in citrus-infected plants (Pappu *et al.*, 1997). *In vitro,* p23 has the ability to bind RNA in a non-sequence-specific manner, and mutations affecting the cysteine and histidine residues of a zinc finger domain conserved in different isolates, increase the dissociation constant of the p23-RNA complex (López *et al.*, 2000). Additionally, p23 is involved in regulating the synthesis of plus and minus strands during RNA replication, with the zinc finger domain and an adjacent basic region being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana *et*

al., 2002). Furthermore, transgenic Mexican lime plants constitutively expressing p23 of the severe CTV strain T36 display alterations resembling the symptoms induced by CTV in this host, with their intensity being associated with p23 accumulation (Ghorbel *et al.*, 2001). This strongly suggests that this protein is an important pathogenicity factor, a view supported by recent experiments showing that p23 is a potent suppressor of local silencing in *N. tabacum* (Lu *et al.*, 2004).

Sambade *et al.*, (2003) discriminate between mild and severe CTV isolates using a specific region in gene p23. In fact, they found that there were 43 polymorphic amino acid positions (approximately 20%); however, in three p23 regions, CTV isolates of the mild group had the same amino acid sequence that differed from the sequence of other isolates. Those regions included positions 24–29, 50–54 and 78–80. In the first, isolates of the mild group had Lys^{24}, Glu^{26} and Lys^{29}, whereas isolates of the severe group had Glu^{24}, Lys^{26} and Val^{29}. These changes result in a strong modification of the isoelectric point of this p23 region. Amino acid changes in the second region left the isoelectric point essentially unaffected: isolates of the mild group had Val^{50}, Thr^{53} and Asn^{54}, and isolates of the severe group had Ile^{50}, Asn^{53} (except for VT that also had Thr^{53}) and Ser^{54}. Finally, in the third region, the isolates of the mild group had Ala^{78}, Leu^{79} and Lys^{80}, whereas isolates of the severe group had Ala^{78}, Ser^{79} and Arg^{80}. The isolates of the atypical group had Gly^{78}, Leu^{79} and Lys^{80}, except for T36 that had Arg^{80}. The two latter regions are located in the RNA binding domain of p23 identified previously, which includes several basic residues between positions 50 and 67, and a putative zinc finger motif (positions 68–86). Interestingly, the residues involved in RNA-binding (the basic residues, and the Cys and His coordinating the Zn ion), were conserved in all isolates with differences affecting only certain positions in their close vicinity.

P20 accumulates in amorphous inclusion bodies within infected cells (Gowda *et al.*, 2000). p20 has been shown to be one of the three proteins involved in suppression of RNA silencing (Lu *et al.*, 2004). Suppressors of RNA silencing have been shown to be required for the systemic infection of plants (Cronin *et al.*,

1995; Ding *et al*., 1995; Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Kasschau and Carrington, 2001; Bayne *et al*., 2005; Schwach *et al*., 2005).

It was also found that both CTV p20 and CP can interfere with the systemic spread of silencing, while p23 can only suppress the local silencing (Lu *et al*., 2004). Thus, CTV evolved a complex system of RNA silencing suppression with three components targeting distinct facets of RNA silencing response.

Using the polymerase chain reaction (PCR) Mawassi *et al.,* (1993) and Pappu *et al.,* (1993b) determined the nucleotide sequences of the p25, the coat protein (CP) gene, of diverse CTV strains and found that the sequences were conserved at 90% and that there was a relationship between the sequences and the symptoms caused by the CTV strains. While Gillings *et al.,* (1993) developed a restriction fragment length polymorphism (RFLP) assay to differentiate CTV strains using the *Hin f1* restriction enzyme that digests PCR products of the CP gene produced in seven different characteristics patterns that associated with specific biological activities.

Cevik *et al.* (1996) analyzed in detail the CP gene sequences of many biologically and geographically diverse strains of CTV. They grouped the strains by known biological activity and found minor but consistent differences in the nucleotide sequences for several groups of CTV strains.

The development of the MCA13 MAbs allowed the detection of severe CTV isolates in Florida by ELISA test. The MCA 13 isolates reactivity is conferred to the presence of the amino acid phenylalanine (F) at the position 124 of the coat protein amino acid sequence (Pappu *et al.,* 1993a,b). This epitope is conserved among severe CTV isolates that cause either decline, stem pitting or seedling yellows.

CTV strains

Depending on virus strains and on the species or scion–rootstock combinations, CTV may cause three distinct syndromes named tristeza, stem pitting (SP) and seedling yellows (SY). Tristeza disease is a decline syndrome

caused by CTV infection of different citrus species [sweet oranges, mandarins, grapefruits (*Citrus paradisi* Macf.), kumquats or limes (*Citrus aurantifolia (*Christm.) Swing.)] propagated on rootstock species such as sour orange or lemon [*C. limon* (L.) Burn. f.]. Its most dramatic expression is quick decline (Fig.3), a syndrome in which a tree with normal appearance starts showing wilt symptoms and completely collapses in a few weeks. Commonly, affected trees show dull green or yellow thin foliage, leaf shedding and twig dieback, small chlorotic leaves resembling the effects of nitrogen deficiency, and small pale-coloured fruits that are unmarketable (Moreno *et al*., 2008). CTV induces obliteration, collapse and necrosis of sieve tubes and companion cells close to the bud union, producing an excessive amount of non-functional phloem (Schneider, 1959). This causes progressive reduction of the root system with deficient supply of water and minerals, which results in wilting, chlorosis and dieback symptoms. As this specific interaction does not occur with many other citrus species, the tristeza syndrome can be avoided using decline tolerant species as rootstocks.

SP disease (Fig.3) is probably initiated by interruption of meristematic activity at limited areas of the cambium that results in irregular radial growth with local depression at the inactivated points (Schneider, 1959). Extensive pitting may limit radial growth and produce stunting, thin foliage with small yellow leaves, low bearing and small fruits with low juice content that are unmarketable. Citrus cultivars sensitive to SP are affected regardless of whether they are a seedling, or used in a grafted combination as a rootstock or a scion. Acid limes show the highest sensitivity, grapefruits and some sweet orange varieties intermediate sensitivity, and mandarins the highest tolerance (Duran-Vila and Moreno, 2000; Timmer *et al.*, 2000). Contrasting with tristeza, the SP syndrome usually does not cause tree death, but unthrifty growth and chronic yield reductions also cause high cumulative economic losses. Moreover, areas invaded by SP isolates may suffer permanent limitations to production by sensitive varieties.

The third CTV-induced syndrome, SY (Fig.3), is characterized by stunting, production of small pale or yellow leaves, a reduced root system and sometimes a complete cessation of growth of sour orange, grapefruit or lemon

seedlings (Fraser, 1952; McClean, 1960). Sometimes plants showing SY recover and produce a new flush with normal leaves. Moreover, field isolates from grapefruit or lemon trees usually do not induce SY. These and other observations led to the interpretation that SY was caused by a component associated with CTV that eventually could be 'filtered off' by sour orange, grapefruit or lemon plants (McClean, 1963; Wallace and Drake, 1972). However, the finding that a clonal CTV population obtained from an infectious cDNA clone still produced SY similar to the original isolate indicated that this syndrome is induced by specific pathogenicity determinants in the CTV genome (Satyanarayana *et al.*, 1999, 2001).

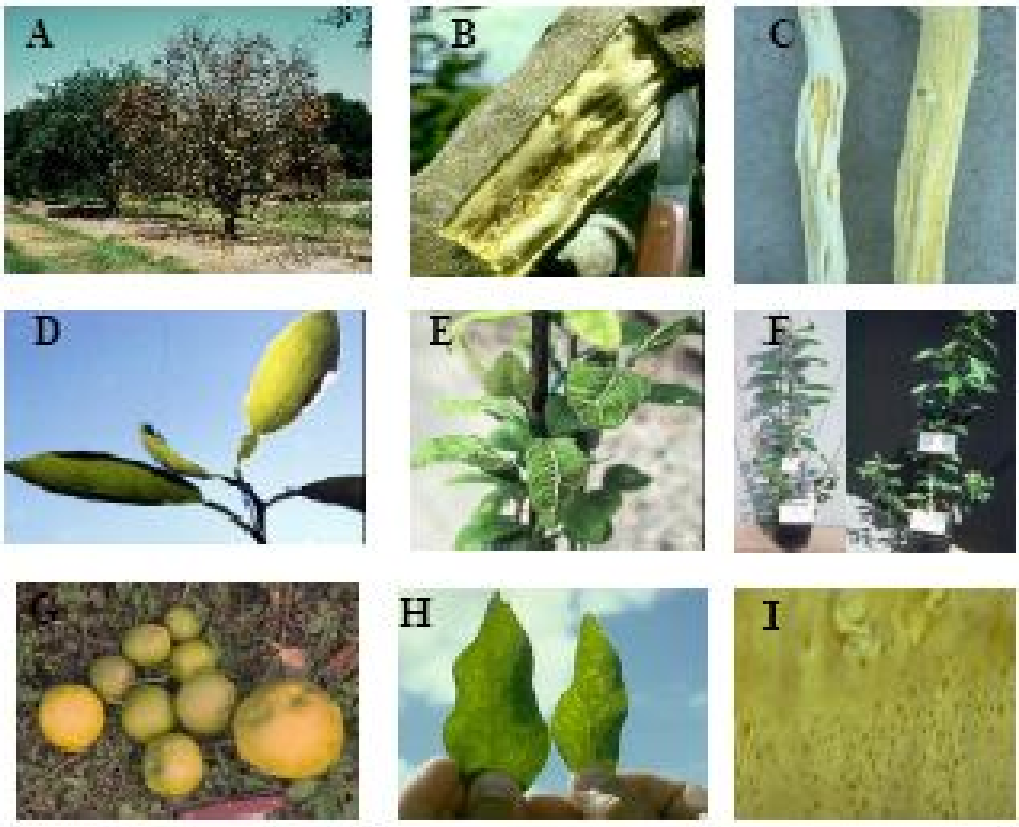


Fig. 3 Symptoms caused by *citrus tristeza virus*. (A) Sweet orange tree on sour orange rootstock undergoing tristeza decline (Lee, R.F.); (B) Stem pitting on Pera sweet orange, occurring in Brazil (Lee, R.F.); (C) Stem pitting on grapefruit in Venezuela (Lee, R.F.); (D) Cupping of the leaf in Mexican (Roistacher, C.N.); (E) Vein corking symptoms on leaves of a Mexican lime seedling inoculated with a very severe seedling-yellow tristeza isolate (Roistacher, C.N.); (F) Seedling yellows reaction on grapefruit (left) and sour orange (right) seedlings in the greenhouse (Roistacher, C.N.); (G) Grapefruit collected from a Marsh grapefruit tree on rough lemon rootstock in Colombia which was affected by stem pitting strains of tristeza (Lee, R.F.); (H) Vein-clearing symptoms in the leaf of a Mexican lime seedling (Lee, RF.); (I) Pinholes in the bark,

caused by bristles in the wood, cause honeycombing on the back side of the bark patch over the sour orange rootstock (Lee, R.F.). *Photographs presented in this figure were downloaded from www.ecoport.org. The author of the photograph is given in the parenthesis.*

CTV diversity in the Mediterranean Region

The finding of Toxoptera citricida, the most efficient CTV vector, in Portugal and Spain (Ilharco *et al.*, 2005), let all mediterranean countries think seriously about CTV threat, especially that the area is not particularly preserved regarding destructive CTV strains as it was previously thought. Massive efforts has been done to know more about the local strains.

The Mediterranean countries where CTV has caused extensive tree losses include Israel and Spain. In the remaining countries CTV is confined to few isolated foci (Djelouah and D'Onghia, 2001).

CTV-VT was originally isolated in 1970 from a declining sweet orange (Citrus sinensis) cv. Valencia tree grafted on the sour orange rootstock in the Hibatt Zion area, Israel (Bar-Joseph and Loebenstein, 1973).

Variation in natural CTV populations in eastern Spain was studied by comparing the SSCP pattern of two gRNA regions, p20 and segment A located within ORF1a, in randomly selected trees at various locations (D'Urso *et al.*, 2003).

CTV was detected on the Cyprus island in the 1980´s (Kyriakou et al., 1992), a study conducted in 2007 by Papayiannis *et al.,* on the Cypriot isolates demonstrated high nucleotide diversity and highlighted the presence of isolates inducing stem pitting on branches of grapefruit and sweet orange. The Cypriot isolates clustered with a large universal isolates, including the severe isolates T36 and T3 from Florida, B246 from South Africa, B-CTV from India and the mild isolate 28C from Portugal.

These strains were also found in Croatia (Cerni *et al.*, 2005), 443-4 (AY791844) and 446-6 (AY791842) are the accession numbers of some sequenced CPG of Croatian isolates.

CTV has been detected in Corsica in 1981, the isolate found B192 in the Kumquat K123 (Bové *et al.*, 2002) is from a major interest as it gives mild or no symptoms on Mexican lime, no vein clearing or stem pitting even if it reacted positively with MCA13, subsequent infections were found in 1994 and 1997 in commercial orchards.

In Italy, CTV was reported at the beginning of 2001 in nurseries on numerous trees imported illegally from abroad and also sporadically on isolated trees in the field. During spring 2002 and 2003, surveys carried out in citrus groves of the Ionian coast of Apulia (south-east Italy), established on sour orange, disclosed the presence of large-sized foci of tristeza. The infected trees were located in two commercial orchards of the Taranto province. In the first focus (Castellaneta) infected Navelina orange trees, approximately 20 years old, were symptomless or stunted and pitted, with heterogenous fruits in size and ripening. In the second focus (Massafra), typical decline with necrosis at the bud union was observed on Navelina orange and Clementine Mandarin trees, approximately 15 years old (Birisik, 2003; Birisik *et al.*, 2004).

Surveys undertaken in some libyan orchards and nurseries revealed the presence of CTV infection and local isolates clustered in the Mediterranean group (Abukraa, 2008).CTV was officially reported in Syria in 2006 (Abou Kubaa *et al.*, 2009).

In Morocco, cv. ‘Meyer’ was introduced and grafted onto sour orange during the 1930s (Cassin, 1963), this material was suspected to carry CTV, and this was confirmed some years later by biological tests on Mexican lime (Chapot and Delucchi, 1964). Other cases of CTV have been reported on ‘Meyer’ and other citrus trees in Morocco and were eradicated on several occasions up to the 1990s (Nadori and Zebzami, 1992).

Lbida et al. studied the biological, serological and genomic diversity of three CTV isolates from various geographical regions isolate P1 isolated from lemon cv. ‘Meyer’ in a field near Marrakech in 1983, and isolates P2 and R1 detected in imported Spanish Clementine germplasm by the Moroccan NPPO in 1998 and 2000. P1 induced severe vein clearing on Mexican lime and grapefruit,

mild stem pitting on Mexican lime and moderate stem pitting on grapefruit and reacted positively with the monoclonal antibody MCA-13. P2 and R1 only induced mild vein clearing on Mexican lime. The coat protein amino-acid sequence of P1 clones clusters close to severe strains CB3–104 and FL7, respectively from Brazil and Florida (Group 5), whereas the sequences from P2 and R1 cluster close to typical strains 25–120 from Portugal and T30 from Florida (Group M).

Diagnosis, characterization and differentiation of CTV isolates

Because CTV isolates induce such different disease phenotypes and severities, efforts have been made to develop molecular techniques that rapidly identify CTV isolates as well as molecular markers related to CTV-induced symptoms.

Biological indexing was performed for years for the diagnosis of CTV infection. Mexican lime (Citrus aurantifolia) seedlings is still a very powerful tool that upon CTV infection show typical symptoms of vein clearing in young leaves, leaf cupping, short internodes and stem pitting in greenhouse under relatively cool conditions (Roistacher, 1991).

Apart from Mexican lime, Duncan grapefruit, Eureka lemon, Madam vinous, and Sour orange can be used for detection and biological characterization of CTV isolates (Chang-yong, 1996).

Comparison of CTV isolates to provide a pathogenicity profile has been and still is done by indexing on this standard panel of citrus indicator species (Garnsey *et al.,* 1991; 2005) allowing the classification of CTV isolates into several biogroups (table 1), but this biological characterization is a slow and expensive procedure that cannot be used for routine identification.

Table.1. CTV biogroups (Garnsey *et al.*, 2005)

Biogroups	LR	Dec	SY	GSP	OSP
0	-	-	-	-	-

1	+	-	-	-	-
2	+	+	-	-	-
3	+	+	+	-	-
4	+	+	+	+	-
5	+	+	+	+	+
6	+	+	+	-	+
7	+	+	-	+	+
8	+	-	-	+	+
9	+	+	-	+	-
10	+	-	-	+	-

LR: foliar and stem pitting symptoms in Mexican lime; **DEC**: chlorosis and stunting on sweet orange/sour orange combination; **SY**: indicates a seedling yellows reaction in sour orange seedlings; **GSP**: indicates stem pitting in Duncan grapefruit seedlings; **OSP:** stem pitting in Madam Vinous sweet orange seedlings.

After CTV purification different antisera and monoclonal antibodies to the coat protein having good specificity were obtained allowing CTV detection by SDS-immunodiffusion tests (Garnsey *et al*., 1979), then an ELISA test was set up (Bar-Joseph et al., 1979) improving the efficiency of mass CTV detection, its availability was a critical tool to expand research in areas such as CTV epidemiology, virus movement or isolate characterization, and to improve efficiency of eradication programmes (Bar-Joseph *et al.*, 1989; Gottwald *et al.*, 1996a,b, 1998, 2002; Kyriakou *et al.*, 1996).

Finally, after the complete nucleotide sequence of the CTV gRNA was available, a variety of diagnostic procedures based on specific detection of viral RNA were developed, including molecular hybridization with cDNA or cRNA probes (Barbarossa and Savino, 2006; Narváez *et al.*, 2000; Rosner and Bar-Joseph, 1984) and several RT-PCR amplification-based methods (Nolasco et al., 1993; Olmos *et al.*, 1999). Real-time RT-PCR protocols have greatly improved sensitivity of detection and allowed quantification of genomic RNA copies in infected citrus tissues or in viruliferous aphids (Bertolini *et al.*, 2007; Ruiz-Ruiz *et al*., 2007; Saponari *et al.*, 2007).

Single-Strand Conformation Polymorphism (SSCP)

Firstly described by Orita and co-workers (1989 a, b), SSCP is a powerful structural analysis in which DNA fragments of the same length can be separated based on their sequence.

The theory behind SSCP is that any given single-stranded DNA (ssDNA) fragment may form a sequence-specific tertiary structure. Double stranded DNA (dsRNA) may be separated by denaturation to form two complementary ssDNA. If ssDNA is allowed to re-nature without re-annealing to the complementary strand, intra-strand base pairing within the single strands occurs, allowing tertiary structures to form. The conformation of these structures is dependent on the primary sequence of the DNA.

It has been demonstrated that small changes in the sequence may alter the conformation of the ssDNA and therefore their electrophoretic profile. DNA molecules with differences between their primary sequences will show polymorphic banding patterns when electrophoresed in non denaturing flat polyacrylamide gels.

When compared to nucleotide sequencing, it is much less expensive. Because SSCP does not provide information about the exact number of base changes, or the location of the changes, it cannot replace nucleotide sequencing or be used for phylogenetic analyses. Its primary advantage is that it can be used to process large numbers of samples rapidly; thus SSCP has potential for use as a screening technique. Isolates that are identified as variants by SSCP can then be selected for further study.

Compared with these other methods, SSCP is simpler to perform, may be more sensitive, and is amenable to processing large numbers of samples. One of the advantages of SSCP, when compared to the other molecular techniques is that no further manipulation of the PCR product is needed; the DNA obtained from the PCR reaction is subjected to SSCP without purification or enzymatic manipulation. Because the conformations of the single strands cannot be

predicted, the conditions for SSCP must be determined empirically and may differ based on the size of the DNA fragment being tested.

SSCP analysis can be limited by the formation of single and double stranded fragment, and multiple conformations of the same fragment as the same genetic sequence may fold to form multiple conformations each with a different migration time.

Several works used SSCP for the CTV polymorphism detection, based on the coat protein gene p25 (Cerni *et al.* 2005), ; Corazza-Nunes *et al.,* 2006), the minor coat protein gene p27 (Iglesias et al., 2008), the p20 gene (D'Urso et al., 2003), the p23 gene (Iglesias *et al.,* 2008).

Capillary Electrophoresis

Capillary Electrophoresis (CE) is a separation technique carried out in a buffer-filled capillary tube that extends between two reservoirs containing platinum electrodes. Separations depend on the rates at which charged analytes migrate under an electric field. The migration rate of a species is determined by its charge to size ratio. CE yields rapid, high resolution separations with very small sample volume. CE has been applied to a variety of applications including inorganic anions and cations, amino acids, drugs and explosives. Most notably, CE was used for the human genome to aid in determining the complete sequence for human DNA.

Capillary Electrophoresis Single-Strand Conformation Polymorphism (CE-SSCP):

The introduction of automated multiple capillary electrophoresis instruments allowed the throughput of SSCP to increase substantially (Munnely *et al*., 1998). In fact, CE-SSCP is a powerful analysis technique that separates heat

denaturated DNA fragments of the same length according to their sequence. This technique presents very high sensitivity, detecting differences up to one base pair.

The use of Capillary Electrophoresis (CE) as alternative to gel slab techniques has become a promising alternative to gel based SSCP methods.

There are many optimization parameters that can be adjusted when developing CE-SSCP. An increase of capillary length will increase the detection power, but also will lengthen polymer fill time and analysis run time.

By labelling the PCR fragments with fluorescent dyes (either using dye-labelled primers or post-PCR end labelling) it is possible to detect ssDNA structure using Capillary Electrophoresis (CE-SSCP).

CE-SSCP is a qualitative assay and is dependent on a range of compositions and conditions. First, assay temperature is an important parameter. It is well known that the conformation of DNA is highly temperature dependent. The question is then if two different conformations can be distinguished equally well at any given temperature. The answer to that has obviously been no far a long time, and most labs have therefore routinely performed SSCP assay at two or more temperatures in order to obtain high sensitivity. Many studies show that sensitivity of CE-SSCP is higher at ambient or sub-ambient temperatures; this may often cause a problem as not all CE-instruments have cooling capacity. However, this should not be a hindrance for performing SSCP at higher temperatures.

The length of the PCR fragments is also an important parameter in SSCP analysis. In gel based systems it was shown that the sensitivity of SSCP was drastically reduced when fragments longer than 400bp. However, generally mutation detection up to 400 bp can be performed, which is acceptable as most exons that are analyzed rarely extend.

Polymer and buffer composition are very important determinants of good CE-SSCP results. A number of different polymers have been suggested for the assay. Commercial polymers supplied by the manufacturers are available, but are not always the best choice.

The use of the POP™ conformation analysis polymer (CAP) on the Applied Biosystems 3130/3130xl Genetic Analyzers is an efficient, convenient, and cost-effective solution for performing SSCP (Applied Biosystem application note, 2006).

The sensitivity of CE-SSCP has been found to be between 96% and 100% (Andersen *et al.*, 2003). Apart from the high sensitivity, the advantages of the method are also a high specificity and the method is very easy to perform reproducibly. The main disadvantages are the need for a PCR fragment labelled with a fluorescent dye. In addition, the PCR fragments should not exceed 500 bp

.

Main citrus phytosanitary problems in the Mediterranean area

Among graft transmissible diseases that have been reported in the Mediterranean countries, Citrus tristeza disease, Citrus psorosis disease and viroid disease are of the most serious diseases and remain the most spread diseases (Whiteside *et al.*,1988; Roistacher, 1991).

Psorosis disease is caused by citrus psorosis ophiovirus (Derrick *et al.*, 1988). In infected trees a scaly bark symptoms on the trunk (Fig. 4), staining of interior wood of branch and gummy as well as shortened leaf internodes and mottling patterns on leaves. Poor fruit quality and decreased yield were recorded (Roistacher, 1991).

Fig.4. Bark scaling symptoms on the trunk due to psorosis infection

Viroids

Viroids are unique infectious agents that are restricted to the plant kingdom, and among the broad spectrum of plant pathogens viroids are the smallest and the simplest form of RNA-based infectious agents. They infect several crop plants, causing symptoms of differential severity, which range from mild effects such as hardly visible growth reduction, up to deformation, necrosis or chlorosis and severe stunting (Singh *et al.,* 2003). Some viroid strains do not cause symptoms at all and seem to behave as simple RNA replicons rather than pathogens. However, symptoms depend very much on environmental conditions and may change during infection as has also been found with plant viruses (Semancik, 2003).

Naturally, viroids are single-stranded and covalently closed circular RNAs. The viroid RNA genome lacks any encapsidation (Flores *et al.,* 1997) or any other form of a protective coat. Thus, the viroid is an infectious 'naked' RNA. Moreover, viroids do not encode proteins and this absence of any functional open reading frame classifies them as non-coding RNAs, unlike viral RNAs. Nevertheless, the viroid genome has the genetic information necessary for a replicon, as it copies itself and proliferates autonomously without the support of a helper virus; it provides information for host specificity and movement from cell to cell and long distance; and finally, it is able to induce a specific pathogenic effect in its host. The viroid genome must interact directly with host-encoded factors to accomplish these functions.

Viroids can be classified into two major families, the Pospiviroidae [type species potato spindle tuber viroid RNA (PSTVd)] and the Avsunviroidae [type species avocado sunblotch viroid (ASBVd)]), which are subdivided into several genera that are phylogenetically related and for which online information is available. Most viroids belong to Pospiviroidae; there are five genera and 24 species. The Avsunviroidae comprise two genera, with three species in total.

Viroid localization is either nuclear (family Pospiviroidae) or chloroplastic (family Avsunviroidae), where they replicate with the aid of host-encoded DNA-dependent RNA polymerases. Viroids can therefore be considered as parasites of the transcriptional machinery of the organelles (nucleus or chloroplast), in contrast to most plant RNA viruses, which replicate in the cytoplasm and can be therefore regarded as parasites of the translational machinery of the cell.

Pospiviroidae family

All representatives of *Pospiviroidae* are characterized by their specific rod-like secondary structure, nuclear localization and asymmetric mode of replication. Based on comparative sequence analysis a model was proposed that divides the rod-like secondary structure into five domains, namely the central conserved region (CCR), flanked by pathogenic (P) and variable (V) domains and two terminal domains left (TL) and right (TR), respectively (Keese and Symons, 1985). In the beginning it was suggested that each domain is responsible for a particular function. Later on the experiments showed that the situation is more complex and more than one domain can take part in one function.

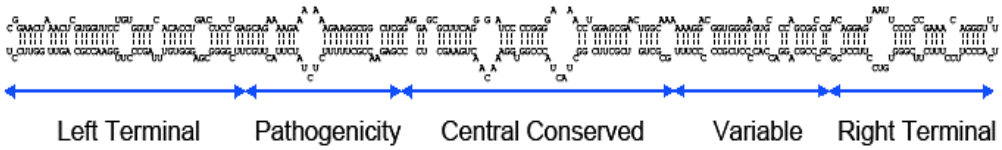


Fig. 5. The five domainsorganization in viroid structure

Citrus Viroids

Citrus are the hosts where the highest number of viroids have been recovered, all of which belong to the family Pospiviroidae (Duran-Vila *et al.*,1988; Flores *et al.*, 2004). *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV) (table.2). In addition, two other citrus viroids (*Citrus viroid*

original source, CVd-OS reported in Japan with 68% homology with CVd-III, and Citrus viroid V, CVd-V) have been proposed as tentative species of the genus Apscaviroid (Ito *et al.*, 2001; Serra *et al.*, 2008a).

They vary in size from 275 to 375 nucleotides and were originally classified in five different groups on the basis of: (i) electrophoretic mobility on 5% sequential polyacrylamide gels (sPAGE); (ii) sequence similarity determined by molecular hybridization against specific DNA probes; (iii) host range; and (iv) reaction on Etrog citron (*Citrus medica* L.) indicator (Duran-Vila *et al.*, 1988). All of them are now referred to five species of family Pospiviroidae.

Citrus viroid III (CVd-III), recently renamed Citrus dwarfing viroid (CDVd) is a member of the genus Apscaviroid that induces, in *Citrus medica* L., stunting and a characteristic 'leaf dropping pattern' caused by the moderate epinasty resulting from petiole and mid-vein necrosis (Rakowski *et al.*, 1994; Semancik *et al.*, 1997).

Several CDVd variants were initially recognized by their distinct mobilities in sequential polyacrylamide gel electrophoresis (sPAGE) (Duran-Vila N et al., 1988), and these were later characterized as three distinct sequence variants (CVd-IIIa, CVd-IIIb and CVd-IIIc) . These variants differ in size by as much as 18 nucleotides located in the left and right regions flanking the CCR, but limited information is available regarding whether or not these changes are associated with distinct biological properties.

Citrus bent leaf viroid (CBLVd) initially described as Citrus viroid I (CVd-I) induces moderate epinasty and point necrosis of the mid vein of Etrog citron (Duran-Vila *et al.*, 1986) and mild dwarfing in trees grafted on trifoliate orange rootstock (Poncirus trifoliata (L.) Raf.) (Semancik *et al.*, 1997). CBLVd is an apscaviroid and appears to be a chimera containing parts of central domain (C) of Apple scar skin viroid (ASSVd) and the pathogenicity (P) and terminal left (TL) domains of CEVd (Ashulin *et al.*, 1991). Two strains of CBLVd (namely CVd-Ia and CVd-Ib) with distinct electrophoretic mobilities were identified in citrus (Duran-Vila *et al.*, 1988). Hataya *et al.*, (1998) have suggested that CVd-Ia

arose by partial sequence duplications involving the right terminal region from CVd-Ib.

The recently described Citrus viroid V (CVd-V) has a rod-like conformation and induces, in Etrog citron, mild stunting and very small necrotic lesions and cracks, sometimes filled with gum (Serra *et al.*, 2008a, b). Moreover, as Etrog citron plants co-infected with CDVd and CVd-V show synergistic interactions manifested in enhanced leaf symptoms and very pronounceddwarfing (Serra *et al.*, 2008a), these host–viroid combinations provide a convenient model to identify the pathogenicity determinants in members of the genus Apscaviroid.

Table2. Citrus viroids Family, genus species and disease caused.

Family	Genus	Species	Citrus disease
Pospoviroidae Central conserved region (CCR)	Pospoviroid	CEVd	Exocortis
	Apscaviroid	CVd-I (CBLVd)	
	Hostuviroid	CVD-II (HSVd)	Cachexia
	Apscaviroid	CVd-III	
	Cocadviroid	CVd-IV	

At present, Koch's postulates have only been fulfilled for two well-known citrus diseases, exocortis caused by CEVd and cachexia of which the causal agent is HSVd (variants CVd-IIb and CVd-IIc).

Other citrus viroids (CVd-I, CVd-II, CVd-III) have been identified as part of the graft-transmissible dwarfing complex (GTDC) (Duran-Vila *et al.*, 1988; Gillings *et al.*, 1991; Bar-Joseph, 1993).

Exocortis is characterized by bark scaling and splitting in sensitive species, such as trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and most of its hybrids, Rangpur lime (*Citrus limonia* Osb.) and Palestine sweet lime (*Citrus limettioides* Tan.). Most citrus species grown commercially, such as sweet orange, grapefruit, and mandarin are tolerant to CEVd. Therefore, these species can act as symptomless carriers and, when infected and propagated on sensitive rootstocks,

develop the stunting and bark scaling symptoms characteristic of the exocortis disease.

Cachexia induces wood pitting and gumming on the trunk, above or below the bud-union according to the position of the sensitive species [mandarin, mandarin hybrids, kumquats, alemow (*Citrus macrophylla* Wester), rough lemon (*Citrus jambhiri* Lush) and *Rangpur lime*], as well as stunting, chlorosis and tree decline.

The Group III citrus viroids have been poorly described due to a host range exclusive to citrus and the absence of a causal relationship to any citrus diseases. CVd-III viroids dwarf citrus plants grafted on specific rootstocks but do not cause any detrimental effects, apart from some reports of growth abnormalities observed under some conditions. The CVd-IIIb variant is distributed in all the citrus areas of the world and has been investigated as a graft transmissible dwarfing agent in order to obtain high density plantings.

CVd-III was initially described as several independently transmissible viroids, CVd-IIIa and CVd-IIIb, migrating as distinct bands in sPAGE analysis (Duran-Vila *et al.,* 1988b) with a relatively narrow size range of 280 to 292 nucleotides. Further analysis by hybridization with specific cDNA probes demonstrates that they shared sequence homology (Semancik and Duran-Vila, 1991) and sequencing demonstrated that they are highly homologous variants of a single viroid (Rakowsky *et al.,* 1994). The sequence of the terminal regions of CVd-III appears to be derived from the conserved regions of the Potato Spindle Tuber Viroid (PSTVd) and the Apple Scar Skin Viroid (ASSVd) (Stasys *et al.*, 1995). Specific symptoms are induced by CVd-III in *Citrus medica* characterized by leaf-drooping resulting from petiole bending (Duran-Vila *et al.*, 1988b).

Types of interactions between micro organisms

The occurrence of more than one virus species in a single plant is not uncommon especially in perennial crops, and when two or more viruses co-infect a plant they may influence each other in several ways. They compete for host

resources but, however, there are few reports indicating that unrelated viruses suffer a disadvantage during mixed infection (Poolpol and Inouye, 1986).

Often, one virus may assist a second, co-infecting virus, leading to increased titres and more severe symptoms and this phenomenon is referred to as viral synergism (Goodman and Ross, 1974; Vance *et al.,* 1995; Pruss *et al.,* 1997). This occurrence, that virus accumulation differs for one or both viruses when in a mixed infection relative to singly infected plants, appears to be a common result of mixed virus infections.

In some cases, the two viruses may benefit from the co-infection (Scheets 1998; Fondong *et al.,* 2000). Synergism has also been known to occur between viruses and their satellite virus or RNA (Rodriguez-Alvarado *et al.,* 1994; Sanger *et al.,* 1994; Scholthof, 1999), or even between viruses and viroids (Valkonen, 1992).

The mechanisms behind synergism may vary. In some cases the helper virus may aid another virus in movement (Barker, 1989), thereby enabling it to invade tissues it otherwise could not. In other cases, viral replication and accumulation are enhanced (Savenkov and Valkonen, 2001).

Antagonistic interactions are sometimes observed, in which the unrelated viruses suppress the infection of each other (Poolpol and Inouye, 1986). However, this is different from cross-protection that takes place between closely related viruses or virus strains (Fraser, 1998).

Viroids were also found to induce tolerance. In fact, Solel *et al.,* (1995) reported that *Citrus medica* and *Rangpur lime* seedlings both infected by CEVd diplayed no severe defoliation when challenged with *Phoma tracheiphila* the fungus responsible of mal secco disease comparing with viroid free seedling.

CEVd seems to reduce the systemic advance of the mycelium from the leaves into the branches (Solel *et al.,* 1995). The mechanism by which this occurs is not known, but may involve the production of host pathogenesis-related (PR) proteins, as in tomato in which CEVd induced the expression of two PR proteins associated with hypersensitive responses and implicated in host defense against

fungal infections (Hadidi, 1988; Belle *et al.*, 1989; Gracia Breijo *et al.*, 1990; Vera and Conejero, 1989).

Virus-Viroid interaction

Case of Grapevine Coinfection of plants with two or more several unrelated viruses often results in a more severe disease than the sum effect of infection with each of the viruses alone and is known as viral synergism.

The Vein-banding disease which can be devastating to grapevines with up to 80 % fruit loss in sensitive varieties (Martelli and Savino, 1988) has been hypothesized to be either a late season expression associated with fanleaf degeneration caused by grapevine fanleaf virus (GFLV) (Martelli and Savino, 1988) or that these leaf symptoms associated with vein banding disease are the response to grapevine yellow speckle viroids (GYSVd-1, GYSVd-2) intensified by co-infection with fanleaf virus (Krake and Woodham, 1983).

Later in 1995, Szychowski *et al.*, demonstrate that expression of the vein banding disease is induced by an unique synergistic reaction between a viroid, GYSVd-1 and a virus, GFLV.

Characteristic vein-banding symptoms became visible on the mature leaves of vines which were dual infected with grapevine viroids and GFLV during the third leafing season (Fig. 6).

Fig. 6. The vein band disease symptoms on grapevine leaves induced by the mixed infection with GYSVd and GFLV.

Symptoms of yellow speckle are ephemeral, most evident at the end of the summer and consist of a few to many chlorotic spots on leaves.

This unusual interaction between viroids and viruses extends the biological potential of viroids.

Case of potato: Synergistic reaction occurs also between Potato Spindle Tuber Viroid (PSTVd) and Potato virus Y (PVY), both were isolated from plants of Kennebec cultivar with severe necrotic symptoms in the field (Singh and Somerville, 1987). The same symptoms were reproduced in the greenhouse only when potato plants were infected either simultaneously with PSTVd and PVY, or with PSTVd prior to PVY infection. The study was extended on other cultivars, and eight cultivars out of thirteen developed necrotic responses similar to cv. Kennebec.

Infection of potato by PSTVd and PVY produces thus a synergistic response resulting in severe necrosis not observed with either pathogen alone and PVY concentration was found to be significantly higher in doubly infected plants compared with those infected with PVY alone (Singh and Somerville, 1987).

Valkonen (1992), found that whereas PVY couldn't be detected by ELISA in plants of Solanum brevidens infected solely with this virus, its accumulation was increased c. 1000-fold in plants doubly infected with PSTVd or tobacco mosaic virus (TMV).

Syller and Marczewski (2001), reported that the reaction of potato plants to the combined secondary infection with PSTVd and Potato Leafroll luteovirus (PLRV) strikingly differed from those caused by either pathogen alone. The plants were extremely stunted and dwarfed, their leaves being rugose and uprisen, and showing greatly pronounced chlorosis. A synergistic reaction was found in all doubly infected potato plants.

Furthermore, serious disturbances in sprout emergence were observed in the double infection (Syller and Marczewski, 2001).

In contrast, when tomato plants cv. Rutgers were challenged with PSTVd and PLRV, no combined effects could be observed (Syller and Marczewski, 2001).

Potato spindle tuber viroid (PSTVd) can decrease tuber yield and quality in the potato (*Solanum tuberosum* L.) (Beemster and de Bokx, 1987). It also reduces pollen viability in infected tomato (*Lycopersicon esculentum* Mill.) (Hooker *et al.*, 1978) and potato (Grasmick and Slack, 1986).

Salazar *et al.* (1995) reported that the aphid M. persicae readily transmitted PSTVd to potato, Physalis floridana and Datura stramoniumplants from the source plants doubly infected with the viroid and potato leafroll luteovirus (PLRV) but not from plants infected with PSTVd alone.

Case of Citrus: Van Vuuren and Graça, 1996, challenged combination Delta Valencia on Yuma Citrange rootstock with ultra-mild, mild, intermediate and severe CTV isolates and a citrus viroid (CVd) isolate.

None of the CTV isolates caused stem pitting in sweet orange and they were all free from the seedling yellows components. The CVd isolate gave a mild reaction on Etrog citron, and according to sPAGE results, belongs to Group III of citrus viroids.

Tree volume measurements were made annually, and all fruits were harvested, sized and weighed. The presence of CVd isolate in combination with CTV isolates reduced tree size in general by 26% comparing with those challenged by CTV alone (Van Vuuren and Graça, 1996). Also, the production of the CVd infected trees was equal to the uninfected trees, due to the 21% higher production efficiency (kg/m3 canopy) of CVd infected trees.

CHAPTER 2. OBJECTIVES

The first objective of the thesis was to evaluate whether the Mediterranean CTV collection was infected with other pathogens or not, in order to:

1- Achieve information on geographical distribution of virus diseases in the Mediterranean basin,

2- Monitor the presence of other risky introduction of viruses/viroids in our country,
3- Ascertain if the reaction of bioindicators or the SSCP profiles are eventually influenced by any mixed infection,
4- Build up a storage collection of different strains/isolates of citrus virus/viroids of the Mediterranean.

The characterization of CTV isolates was performed with the biological indexing on the universal indicators for the determination of the biogroups, and the sequencing.

The second objective of the thesis was to evaluate the variability in four regions of the CTV genome using the conventional SSCP and the Capillary Electrophoresis-SSCP using the Genetic Analyzer. The purpose was:

1- to better evaluate the differences in the collection of CTV isolates,
2- to achieve a broad information on CTV isolates in the Mediterranean,
3- to study if mixed infections play a pressure on CTV genome.

Four regions of the genome; p18, p20, p23 and p25 are studied considering their potential involvement in symptom production, and their predominant sequence variants were compared in the RNA population of those isolates with the conventional SSCP first then with the CE-SSCP.The profiles obtained will be compared with the results of the biological indexing and the gene sequencing in view of developing SSCP markers.

Another aim of this study was to investigate on CTV interaction with one of the citrus viroids in a mixed infection.

CHAPTER 3. MATERIAL AND METHODS

The CTV collection

CTV sources were collected in citrus orchards during surveys conducted by the Mediterranean Agronomic Institute of Bari (MAIB) in several countries, mostly from the Mediterranean area. These sources grafted onto different

rootstocks (Sour orange, Citrange troyer or Rough lemon) were assigned with an "IAMB-Q" number and maintained under insect-proof screenhouse (Fig. 7).

Globally, these CTV sources included countries such Albania, Algeria, China, Croatia, Cyprus, Egypt, Iran, Italy (Apulia and Sicily), Lebanon, Montenegro, Morocco, Palestine, Syria and Trinidad.

Biological indexing

CTV strain discrimination was carried out by graft-inoculation (Roistacher, 1991) of five specific woody indicators: Mexican lime, sour orange, sweet orange, Duncan grapefruit and sweet orange/sour orange combination as reported by Garnsey *et al.,* (1991) for CTV biogroups establishment.

Three indicators, about 1 year old, were chip budded using 2 blind buds from each selected CTV source. After sealing the graft with parafilm, the inoculated plants were labelled with isolate code and all with the positives and negatives controls were placed in an aphid proof greenhouse at cool temperatures (22-24°C). Budding and pruning equipment was disinfected in dilute bleach between treatments.

Observations were carried out after 2 weeks for graft success and after first flushing for symptom development (Roistacher, 1991).Plants were fertilized as well as sprayed when needed to control insect or mite infestations.The evaluation

of the presence of CTV-syndromes was performed taking into account that vein-clearing and leaf-cupping can be induced by all CTV strains in Mexican lime (universal indicator); CTV-“stem pitting” can provoke pittings in grapefruit and rarely in sweet orange, whereas “yellows” reaction in sour orange and grapefruit seedlings are induced by CTV-SY strain.

Symptom severity was quantified by the response in each host and rated as mild or severe. CTV-SP symptom evaluation consists on the observation of pits in grapefruit and sweet orange; in the latter only severe CTV isolates can induce pitting and, eventually gumming. Moreover, the presence of vein corking in the leaves of Mexican lime and even in the sweet orange is a marker of isolate severity.

In order to verify the success of the graft inoculation into the specific indicators, Direct Tissue Blot Immunoassay (DTBIA) (Djelouah and D’Onghia, 2001), was carried out for all the indexed plants. Five tender shoots from each indicator were cut transversely with a sterile razor and the sections were pressed carefully on the nitrocellulose membrane. After blocking with 1% bovine serum albumin (BSA), the membrane was incubated with the Mabs 3DF1+3CA5 mixture conjugated with alkaline phosphatase (PlantPrint). Membranes were developed by using the BCIP-NBT (Sigma fast tablets), then read under a light microscope at 10x and 20x magnification. The positive reaction was revealed by the presence of purple–violet blots in the region of phloem tissue cells.

Serological detection

The plant sources were preliminarily tested for CTV infection by DAS ELISA as reported by Bar Joseph *et al.,* (1979), using two commercial kits; i.e. one polyclonal antiserum (PAbs) from Agritest (Italy) and one mixture of Monoclonal antibodies 3DF1+3CA5 from Ingenasa (Spain) which is known for its reaction with most CTV isolates and which is specific to the two highly preserved epitopes of CTV coat protein (Garnsey *et al.,* 1989).

Plates were coated with polyclonal antibodies diluted 1:250 in coating buffer (Annex 1) and incubated for 2 h at 37°C. After washing three times the

plates with washing buffer (Annex 1), samples were grinded in extraction buffer (Annex 1) at 1/10 concentration, using bark or petiole tissue. Two wells were filled with 100µl of extract of each sample, and of positive and of negative controls. Plates were then incubated overnight at 4°C.

After the same washing as described above, 100µl of alkaline phosphatase linked antibodies diluted 1:250 in conjugate buffer (Annex 1), were added to each well and the plates were incubated for 2 h at 37°C.

Plates were dried after the last wash, then 100µl of P-nitrophenyl phosphate prepared with 1mg/1ml in substrate buffer (Annex 1), were added to each well. The plates were incubated at room temperature and an absorbance reading was done up to 2h in a conventional ELISA plate reader at 405nm.

CTV sources were considered positive if the OD405 values were more than 2,5 times above the values of healthy extracts.

TNA extraction and RT-PCR reaction

Total RNA was extracted from finely trimmed leaf tissue (50 mg) using TRIzol® reagent (Life Technologies), that contains phenol and guanidinium isothyocyanate, further purified following the manufacturer's instructions. Briefly, after tissue homogenization in 1ml TRIzol®, an incubation of 5' at RT for the complete dissociation of nucleoproteic complex, addition of 1/5 V of Chloroform, hand agitation for 15'', incubation at RT for 3', centrifugation at 12000g for 15' at 4°C, 0,5 ml of isopropanol is added then to the surnatant and incubated for 10' at RT, then centrifuged at 12000g for 10' at 4°C, the pellet is then washed with 1ml of 75% ethanol, vortexed and centrifuged at 7500g for 5' at 4°C, the pellet is then dried and finally resuspended in sterile distilled water.

For cDNA synthesis, 2 µl of the RNA extract was heat denatured at 94°C for 2 min and chilled on ice. Single step RT-PCR was performed in a 25 µl reaction volume containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 3mM MgCl2 0.4mM dNTPs, 1µM of each primer (Table 3), 4 units of RNaseOut¨, 20 units of SuperScript II reverse transcriptase, and 2 units of Taq DNA polymerase (Life Technologies).

Thermocycling conditions were: 1 cycle of 45 min at 42°C for reverse transcription, 1 cycle of 2 min at 95°C for inactivation of reverse transcriptase, 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, and a final extension of 10 min at 72°C.

RT-PCR products were analysed in a 1% agarose gel stained with ethidium bromide.

Tab.3 . Primers used for CTV amplification by the RT-PCR

CTV Genome part	Primer sequence (5' to 3')	Fluorescence	Fragment size
p20	F: ACAATATGCGAGCTTACTTTA R: AACCTACACGCAAGATGGA	6-FAM NED	540bp
p23	F:GGTTGTATTAACTAACTTTAATTC R:AACTTATTCCGTCCACTTCAATCA	6-FAM VIC	594bp
p25	F: ATGGACGACGAAACAAAGAA R: ATCAACGTGTGTTGAATTTCC	NED VIC	415bp
p18	F: TTCTATCGGGATGGTGGAGT R: GACGAGATTATTACAACGG	6-FAM NED	425bp

Single strand conformation polymorphism analysis (SSCP)

The test was performed for the 4 CTV genes amplified by RT-PCR (p20, p23, p25 and p18) aiming a primary molecular differentiation between isolates prior to cloning and sequencing. It is performed directly on the RT-PCR products; for each sample, 1.5 to 3µl of RT-PCR product were taken and the denaturing buffer (95% formamide, 20mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol) was added until a final volume of 10 µl (the volume of each

sample varies with the concentration of the PCR products). Samples were then heated at 95°C for 5 min, and quickly transferred to an ice box for 5 min.

Denatured products were separated by electrophoresis at 4°C in 8% non-denaturating polyacrylamide gel using TBE buffer (Annex 2) and a constant voltage of 200V for 3h. The gels were silver stained by fixation in acetic acid solution (10% for at least 20 min), wash with distilled water 3 times for 1 min, Incubation in 1% nitric acid for 3 min, wash again with distilled water 3 times for 1 min, incubation in silver nitrate solution (Annex) for 30 min and wash with distilled water for 20 sec.

Developing solution was then added and incubated until the appearance of the bands; the reaction was stopped by quick gels immersion in 10% acetic acid for 10 min.

Viroid detection

Biological indexing: the procedure was as for the biological indexing for CTV, with the difference of using *Citrus medica* as indicator plant, and the indexed plant were kept in the greenhouse at 35°C.

RNA extraction and fractionation

Leaf tissue of *C. medica* (5 g) were homogenized in an extraction buffer containing 40 ml of water-saturated phenol and 10ml of Tris buffer (125mM Tris–HCl, pH 8.9, 15mM EDTA, 0.8% (w=v) SDS, 0.8% (v=v) b-mercaptoethanol), and the total nucleic acids were partitioned in 2M LiCl. The preparations were further purified by non-ionic cellulose chromatography. Specifically, the preparations were resuspended in 37ml STE buffer (50mM Tris–HCl, pH 7.2, 100mM NaCl, 1mM EDTA) containing 35% ethanol and mixed with 1.25 g of non-ionic cellulose CF-11 (Whatman).

The cellulose was washed three times with STE containing 35% ethanol, and the RNAs bound to the cellulose were eluted with STE and concentrated by ethanol precipitation.

RNA analysis and purification of Viroid circular forms

Aliquots of the nucleic acid preparations were analyzed by two sequential rounds of polyacrylamide gel electrophoresis (sPAGE), the first under non-denaturing conditions and the second under denaturing conditions. For preparative purposes, the denaturing gel was stained with ethidium bromide and the segment containing circular viroid forms was frozen, crushed and mixed with phenol: chlorophorm: isoamyl alcohol and elution buffer (Tris–HCl, pH 8.9, 1mM EDTA, 0.5% (w=v) SDS). The extracted RNAs were recovered by ethanol precipitation. For analytical purposes, the denaturing gel was stained with silver.

RT-PCR amplification

Retrotranscription and PCR amplification was performed on purified circular viroids (10 ng) using two specific oligonucleotides of opposite polarity as reported in table. Primer CEVd-R1 was annealed in buffer (10mM Tris–HCl, pH 8.5, 20mM KCl) at 95°C for 2min. First-strand cDNA was synthesised with 25 U of avian myeloblastosis virus reverse transcriptase (AMV-RT), using dNTPs (1mM each) in RT buffer (50mM Tris–HCl, pH 8.5, 8mM MgCl2, 30mMKCl,1mM DTT). The reaction mixture (20 ml final volume) was incubated at 42°C for 45 min. Second-strand cDNA synthesis and PCR amplification (50 ml final volume) was performed using 4 ml of the first-strand cDNA reactionmixture, 2.5U Pfu DNA polymerase (Stratagene), respective primers (0.5 mg each), dNTPs (1mM each), BSA 0.1mg=ml in PCR buffer (20mM Tris–HCl, pH 8.8, 10mM KCl, 10mM (NH4)2SO4, 2mM MgSO4, 0.1% Triton X-100). PCR parameters consisted of 30 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 1min, with a final extension at 72°C.

Table 4. Primers used for viroid RT-PCR

Viroid	Primer sequence (5’ to 3’)
CDVd	H:CTCCGCTAGTCGGAAAGACTCCGC C: ACTCTACCGTCTTTACTCCA
HSVd	C: GGCTCCTTTCTCAGGTAAG H:CCGGGGCAACTCTTCTCAGAATCCA
CEVd	H:GGA AAC CTG GAG GAA GTC GAG G C: CCC GGG GAT CCC TGA AGG ACT TC

Isolating, cloning and sequencing

Amplified products from RT-PCR are ligated into a plasmid vector System and transformed into competent E.coli cells using standard procedures and plated according to Sambrook *et al,* (1989) protocols.

The white transformed colonies harboring the recombinant plasmid were selected on the plate supplied with X-gal and ampicilin; picked and used directly in PCR reactions, with the same primers used for the RT-PCR step, to confirm the presence of the specific insert, then were analysed by SSCP to help in the choice of clones to sequence.

Ligation of the IC/RT-PCR product with pGEM -T easy vector

Following electrophoresis of the RT-PCR products in 1% agarose gel, the DNA bands were excised, and the gel slice was then dissolved, binded to minicolumn, washed and eluted with sterile water using (Wizard SV Gel and PCR Clean-Up System; Promega, USA), according to the manufacturer’s instructions. Two µl of elution products were inserted in the commercial plasmid vector, pGEM-T Easy Vector System using the original TA Cloning Kit (Promega, USA) (Fig. 8), by a ligation reaction; 5 µl of 2x ligation buffer (Promega) were mixed with pGEM-T vector (50ng/µl), 0,3 Units T4 DNA ligase (Promega) and 1µl of sterile water. The ligation mixture was incubated overnight at 4°C.

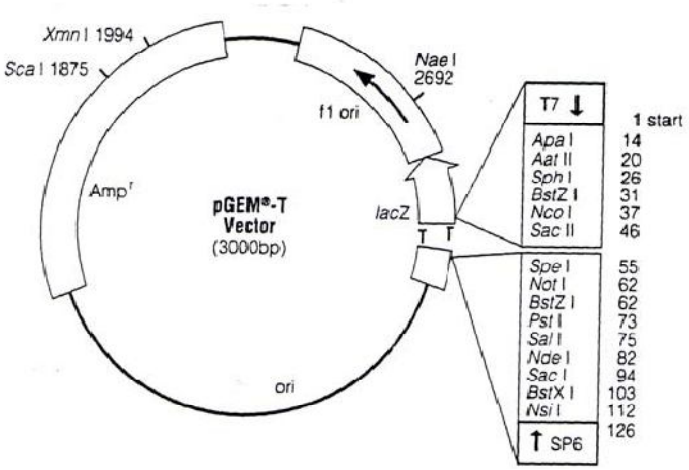


Fig. 8 Site map of the pGEM-T cloning vector.

Preparation of competent cells

All the steps in this procedure were done aseptically. Escherichia coli INVaF cells (Invitrogen, USA), were streaked across the surface of Luria Bertani (LB) solid plate (Annex) and incubated at 37°C for 16h.

A single colony was then incubated into 2 ml of LB liquid (Annex) medium and incubated overnight at 37°C with shaking at 250 rpm. Additional growth of bacteria cells was performed by inoculating 50 µl of bacterial culture in 10 ml of LB liquid and incubating them at 37°C for 3h with shaking at 250rpm. The culture was chilled in ice bath for 10 min to stop growth and then, was harvested by centrifugation at 6000 rpm for 10 min at 4°C. supernatant, the bacterial pellet was gently resuspended in 0.6 ml cold 0.1 M CaCl2 then kept in ice for at least 2h before transformation. Aliquots were prepared and stocked at -80°C.

Transformation of competent cells

One hundred µl of competent cells suspension were added to the 10 µl ligation mixture in a sterile microfuge tube and were kept in ice for 30 min.

The tubes were incubated in a water bath at 42°C for 2 min, and then cooled immediately in an ice bath for another 2 min. The cells were mixed with

600 µl with LB liquid then incubated for 30 min at 37°C followed by another 30 min at 37°C with agitation at 150rpm.

Screening of the colonies

After centrifugation for 2 min and elimination of the major part of the remaining liquid, the bacterial suspension was spread onto plated agarized LB medium supplied with X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) (Sigma) and ampicilin (50mg/ L). Inverted plates were incubated overnight at 37°C.

Using sterile toothpicks, white colonies harboring the recombinant plasmid were selected on the plate then re-inoculated and numbered in another plate.

Identification of the bacterial colonies carrying recombinant DNA

Plasmids were extracted from bacteria cells using the “boiling method” (Sambrook et al., 1989). The transformed colonies were “picked” and inoculated in 2ml of LB liquid containing ampicillin and incubated overnight at 37°C and 250 rpm centrifugation.

Bacterial pellets were poured to an microfuge tubes then collected by centrifugation at 13000rpm for 1 min, resuspended in 350 µl of STET (Annex) and 15 µl lysozyme (20 mg/ml), vortexed and then incubated in boiling water for 40 sec and immediately chilled in ice for 2 min. After a centrifugation at 14,000 rpm for 20 min, and elimination of the pellet, soluble

plasmid DNA were extracted with addition of 175 µl of phenol and 175 µl of isomilic alcool (24:1) followed by vortexing and then centrifugation at 13,000 rpm for 10 min, 300 µl of surfactant were transferred then in fresh tubes containing 200 µl of Ammonium acetate (NH4OAC) and 1ml of cold absolute ethanol and centrifuged at 13,000 rpm for 10 min.

The pellet was washed with 500 µl of 70% cold ethanol, centrifuged at 13,000 rpm for 10 min and, after surfactant elimination and drying into Vacuum pump for 1h, was resuspended in 50µl TE buffer (Annex) containing 1 µl of RNase A (10 µg/µl), vortexed then conserved in ice.

Enzymatic digestion of plasmid

To verify if extracted plasmids contained the appropriate DNA insert, a digestion with the restriction endonuclease EcoRI (Amersham) was carried out; 10-15 ng of plasmid DNA were incubated with 1 µl 10X buffer H (Roche), 2 U EcoRI, (10 µl, final volume of digestion) at 37°C for 1h30.

Size of restricted DNAs was determined by electrophoresis in 1% TAE 1x agarose gel. The gel was then stained by ethidium bromide (10 mg/ml). DNA bands were observed under an UV transilluminator.

The tubes that contains plasmids carrying the CTV coat protein insert, confirmed by enzymatic digestion, were transferred from ice to 37°C for 30min, then 30 µl of PEG-NaCl (Annex) was added, put on ice for 1h, centrifuged at 14,000 rpm for 5 min, washed by 100 µl of 70% cold ethanol after liquid phase elimination, centrifuged 14,000 rpm for 5 min, dried for 1h into vacuum pump after liquid phase elimination and finally the pellet was dissolved in 30 µl of sterile water.

PCR Amplification of recombinant DNA and CP gene Sequence analysis

White colonies are picked and added to PCR mixture, for recombinant DNA amplification using the same primers as in the RT-PCR before SSCP analysis, in order to check on the genetic properties of selected CTV isolates and to study the conformation complexity of the SSCP patterns obtained from each isolate.

Clones that present different SSCP pattern originated from each isolate were chosen and cDNA sequences of the selected recombinant plasmid were obtained by automatic sequencing (Primm Company, Italy).

The sequences analysis and alignment were done using the software packages BioEdit (Hall, 1999). The homology with other known CTV sequences was determined with the BLAST program (Altschul *et al.,* 1990).

The sequences were compared to previously published sequences overlapping the CP gene retrieved from the GenBank entries: T30(AF26065), M2 (AY190048), SY568 (AF01623), ArgC269 (AY750771), Cal81 (AY995567), Isr VT (U56902), Jordan (AY550252), Arg C257 (AY750757), Por 28C (AF184118), Qaha (AY340974), T36 (AY170468), 19-121(AF184114).

Calculation of pair wise nucleotide distances between sequences and clustering were done using the Molecular Evolutionary Genetic Analysis software MEGA version 2.1 (Kumar *et al.,* 2001) after excising the 20 terminal bases on both sides of the amplified products corresponding to the primers used.

CE-SSCP

DNA amplification: the p20, p23, p25 and p18 gene were amplified using internal fluorescent primers (Table 3).

Polymer synthesis: The POP conformation analysis polymer (CAP) was prepared following the formulation for 5g of polymer (5% CAP, 10% glycerol); 2.78 g of 9% Conformational Analysis Polymer, 0.5g of 10X Genetic Analyzer Buffer (running buffer), 0.5g 100% Glycerol, H2O up to 5.0g. .

Sample Preparation: 1µl of PCR products diluted serially were added to the SSCP analysis master mixture (10µl of Hi-Di™ deionized formamide, 0.25µl of GeneScan-500 ROX size standard [Applied Biosystems]) in each well of the sample plate. The samples were subsequently denatured by heating at 95°C for 5 minutes at 95°C and were immediately cooled on ice for 5 minutes to avoid re-annealing of the complementary strands prior to loading onto the Genetic Analyzer. Run the sample plates using the newly created run modules, and then analyze the results.

SSCP electrophoresis. SSCP electrophoresis was performed with the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) (fig.9). The anode-cathode buffer consisted of a TBE solution. Capillary of 35cm length were used, and the electrophoresis conditions were set at a 10-s injection time, a 7-kV injection

voltage, a 13-kV electrophoresis voltage, a 120-s syringe pump time, a constant temperature of 30°C, and a 24-min collection time. The retention times of the phosphoramidite (HEX)- or 6-carboxyfluorescein (FAM)-labeled fragments were determined by the electrophoretic mobility value relative to the electrophoretic mobility for the ROX-labeled internal standard, shown as red peaks, obtained with the ABI Prism 310 GeneScan Analysis Software (Applied Biosystems).

Fig.9. performed with the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems)

Virus viroid interaction

Immunocapture RT-PCR (IC/RT-PCR)

Indexed plants that were found to be infected simultaneously by CTV and citrus viroids were subjected to an ELISA test, as reported in the serological test above, for capturing CTV particles in an ELISA plate. Then a PCR mixture was prepared, as for RT-PCR detection for viroids. Each extracted sample was loaded three times in the ELISA plate for the 3 different mixture with the appropriates primers for each viroid. These was done for the three studied viroids CEVd, CVd-II and CDVd. After washing, the wells were loaded with the different mixture and then put on an incubator at 50°C for 1h. These mixture were then taken from the wells and put on PCR tubes to complete the PCR in the thermocycler, with the conditions reported above for viroid detection.

PCR products were then checked on 1% agarose gel, for the presence of viroid amplicons indicationg that the viroids particles have been encapsidated by the CTV coat protein, and some excised bands were cloned then sequenced.

CHAPTER 4. RESULTS AND DISCUSSION

Biological indexing:

Biological properties of the Mediterranean CTV isolates were investigated by indexing onto a standard of indicator plants. Biological characterization remains the most reliable procedure to accurately document the severity of CTV isolates (Garnsey *et al.*, 1995; 2005).

Twenty seven CTV isolates were compared for symptom expression on five citrus indicator plants; Mexican lime, Duncan grapefruit, Madam Vinous sweet orange grafted on sour orange, sour orange seedlings, and Madam Vinous sweet orange seedlings. Reactions were relatively consistent among the three replicate plants per host inoculated with each isolate, and some differences in symptom severity between isolates were readily apparent.

Mexican lime reactions.

Inoculated plants were observed for vein clearing (Fig.10), leaf cupping (Fig.11), stunting and stem pitting. Symptoms in Mexican lime showed a broad range of severity (Table 5).

Some isolates expressed a pronounced vein clearing that could be seen without having to put the leaf in backlighting (Fig.10), these include mostly isolates from the Middle East region including Lebanon, Syria and Palestine. These last were also strongly stunted (Fig.12) with pronounced stem pits in Mexican lime (Fig.13), whereas the milder CTV isolates had minor stem pitting.

Sweet/sour reactions.

Sweet/sour plants were evaluated for stunting and leaf chlorosis, especially chlorosis along the major veins, which indicated a girdling effect expected from CTV induced phloem necrosis at the bud union (Table 5). Precocious flowering and fruit set were also regarded as indicators of phloem dysfunction. Some CTV isolates caused stunting of the scions relative to controls

Fig. 10: Pronounced vein clearing

Fig. 11: Leaf cupping

Fig. 12: Stunting on Mexican lime

Fig. 13: SP on Mexican lime

Grapefruit reaction.

Inoculated Duncan seedlings were monitored for foliar symptoms including, vein clearing and corking and leaf chlorosis. Stunted Duncan plants often had an abnormally thickened bark (Fig. 14) that indicated an abnormal

differentiation of phloem and xylem tissues. Stem pitting was determined by peeling the main stem above the point of inoculation

Fig.14: SP on Duncan Grapefruit

Fig. 15 : Discrete SP on Madam vinous SwO

Fig. 16: CPsV symptoms mature leaf of Madam vinous SwO induced by the isolate Q110

Sweet orange reactions.

CTV isolates inducing stem pitting on sweet orange are considered as the most dangerous as they occur in decline-tolerant scion–rootstock combinations. Few mediterranean isolates induced discreat stem pitting (Fig. 15) on Madam vinous (Table 5), these include mostly isolates from the middle East area.

Three CTV isolates were found to be coinfected with CPsV with ELISA test. The coinfection with all CTV group did not preclude ring spot–type young leaf symptom expression of CPsV in sweet orange (Fig.16), in accordance with the finding of Vidalakis *et al.,* (2004), in their investigation on the efficacy of the bioindexing and the symptom expression on the primary indexing hosts for citrus graft-transmissible pathogens in the presence of additional pathogens.

Similarly, symptom expression of CTV in the primary indexing host ML was essentially unaffected by the presence of other tested pathogens as reported also by Vidalakis *et al.,* (2004) including citrus viroids.

Reaction patterns:

Multiple isolates shared similar patterns of symptom expression. These are summarized in Table 5. Some isolates showed mild vein-clearing symptoms on Mexican lime but did not cause stem pitting, these are considered to be mild CTV isolates.

The isolates selected for comparison were from 13 different countries, mostly from the Mediterranean area, and belong to different biogroups, based on their symptom expression pattern (Table 6).

Correlation of symptom profile to MCA13 reactivity

The biologically characterized isolates were also tested for reactivity to the selective MAb MCA13 (Daden, 2006), known to differentiate between most of the severe and mild isolate (Permar *et al.,* 1990; Nikolaeva *et al.,* 1998) and which constitute a good indication of potential aggressiveness of an isolate, based on observations that it reacted with most isolates that cause decline, SY, GSP and OSP alone or in combination, and did not react to isolates that failed to cause those symptoms.

A comparison of symptom patterns and MCA13 reactivity is summarized in Table 6.

Interestingly, 17 CTV isolates out of 27 studied isolates that expressed severe symptoms reacted to MCA13. This included all isolates that induce GSP and SY and belongs to the following countries China, Croatia, Cyprus, Egypt, Lebanon, Montenegro, Morocco, Palestine and Syria gave a clear cut positive reaction with this MAb, even if the number of the tested CTV sources was not always large to be significant.

While MCA 13 MAb failed to react only with the Algerian and all the Apulian (Italian) CTV isolates which reacted only in Mexican lime (pattern 1).

Correlation of biological reaction to isolate genotype:

The CTV isolates belonging to the Middle East region including Lebanon, Syria and Palestine presented a severe SP on Pompelmo grapefruit and a light SP on Madam vinous Sweet orange on Volkameriana rootstock, the SP on Sweet orange being an indicator of the extreme severity of a CTV isolate.

As shown in Table 6 symptom profile 1 were predominantly associated with the T30 genotype while symptom profiles 4, 5, 6 and 8 were most frequently associated with the T3 and VT genotype.

Table.5: Symptoms induced on universal indicator plants and MCA13 MAbs reactivity.

Indicators / Isolates	Mexican lime	Sour orange	Madam vinous/ Sour orange	Madam vinous/ volkameriana	Pompelmo duncan seedlings	MCA13 reactivity
Q S 6	**LC, VC, ST, SP**	**ST**	**ST**	**dSP**	**mSP**	**+**
Q S7	**LC, VC, ST, SP**	**ST**	**ST**	**dSP**	**mSP**	**+**
Q 57	**VC, ST**	**ST**	**ST**	**dSP**	**NS**	**+**
Q 7	**LC, VC, ST, SP**	**ST**	**ST**	**dSP**	**mSP**	**+**
Q 12	**LC, VC, LD, ST, SP**	**ST**	**ST**	**dSP**	**mSP**	**+**
Q 40	**LC, VC, ST, SP**	**ST, LD**	**ST**		**sSP**	**+**
Q 52	**LD, ST, sVC, SP**	**ST, LD**	**ST**	**dSP**	**sSP**	**+**
Q 75	**LC, VC, ST, LD, K**	**ST**	**ST**	**dSP**	**NS**	**+**
Q 73	**LC, sVC, SP, ST**	**ST**	**ST**	**NS**	**mSP**	**+**
Q 97	**sVC, LC, ST, SP**	**ST, K**	**ST**	**NS**	**sST, dSP, K**	**+**
Q 108	**sLC, VC, ST**	ST	**ST**	**dSP**	**NS**	**+**
Q 76	**LC, sVC, LD**	**NS**	**NS**	**NS**	**NS**	**--**
Q 21	**sLC, LD, SL, sVC, SP**	**sST, LD**		**NS**	**sST**	**+**
Q 32	**LC, VC, ST, SP**	**ST, LD**	**ST**	**ST**	**sST**	**+**
Q 102	**LC, VC, ST**	**NS**	**ST**	**NS**	**NS**	**--**
Q 103	**LC, ST, LD**	**NS**	**ST**	**NS**	**NS**	**--**
Q 110	**VC, ST, K**	**NS**	**ST, LD**	**NS**	**NS**	**--**
Q 112	**VC**	**NS**	**ST**	**NS**	**NS**	**--**
Q113	VC	**NS**	ST	**NS**	**NS**	**--**
Q 118	VC	**NS**	ST	**NS**	**NS**	**--**
Q 120	VC	**NS**	ST	**NS**	**NS**	**--**
Q 124	VC, ST	**NS**	ST	**NS**	**NS**	**--**
Q 134	VC	**NS**	ST	**NS**	**NS**	**--**
Q 5	sVC, LC, K	ST	ST,K	ST	NS	+
Q 30		ST	ST	dSP	NS	+
Q 25	SVC, sST, SP	ST, LD	ST	ST, SP	dSP	+
Q 26	SVC, sST, SP	ST, LD	ST	ST, SP	dSP	+

M.L : Mexican lime, **S.O :** Sour orange, **Sw.O/S.O :** Sweet orange/Sour orange, **P.D :** Pompelmo duncan, **LC**: leaf cupping; **sLC:** severe leaf cupping;**VC:** vein clearing; **sVC:** severe vein clearing; **ST:** stunting; **LD: leaf deformation; SP: stem pitting, mSP: moderate stem pitting; dSP: discrete stem pitting ; K: chlorosis, NS: no symptom**

Table6. CTV isolates biogroups and MCA13 reactivity.

		Symptoms induced on various indicator plants						
Isolates	Origin	M. lime (VC, SP)	SwO/SO (D)	SO (SY)	D.Gft (SP)	SwO (SP)	Biogroup	MCA13 Reactivity
Q 5	Albania	+	+	+	--	--	3	+
Q76	Algeria	+	--	--	--	--	1	--
Q25	China	+	+	+	+	+	5	+
Q26	China	+	+	+	+	+	5	+
Q73	Cyprus	+	+	+	+	--	4	+
Q57	Egypt	+	+	+	+	--	4	+
Q7	Lebanon	+	+	+	+	+	5	+
Q12	Lebanon	+	+	+	+	+	5	+
Q97	Iran	+	+	+	+	--	4	+
Q21	Italy	+	+	+	+	--	4	+
Q32	Italy	+	+	+	+	--	4	+
Q 102	Italy	+	+/--	--	--	--	1; 2	--
Q 103	Italy	+	+/--	--	--	--	1; 2	--
Q 110	Italy	+	+/--	--	--	--	1; 2	--
Q 112	Italy	+	+/--	--	--	--	1; 2	--
Q113	Italy	+	+/--	--	--	--	1; 2	--
Q 118	Italy	+	+/--	--	--	--	1; 2	--
Q 120	Italy	+	+/--	--	--	--	1; 2	--
Q 124	Italy	+	+/--	--	--	--	1; 2	--
Q134	Italy	+	+/--	--	--	--	1; 2	--
Q108	Montenegro	+	+	+	+	--	4	+
Q75	Morocco	+	+	+	+	--	4	+
Q40	Palestine	+	+	+	+	+	5	+
Q52	Palestine	+	+	+	+	+	5	+
QS6	Syria	+	+	+	+	+	5	+
QS7	Syria	+	+	+	+	+	5	+
Q30	Trinidad	+	+	+	+	--	4	+

The presence (+) or absence (–) of vein clearing (VC), stem pitting (SP) and decline (D) symptoms were observed in seedlings of Mexican lime (M. lime), Duncan grapefruit (D. Gft.), sweet orange (SwO), or in plants of sweet orange grafted on sour orange (SwO/SO). Seedling yellows (SY) was observed on sour orange.

Assessment of the sanitary status of the CTV collection:

Presence of other important virus:

The whole CTV collection was thus tested by ELISA for the presence of Citrus Psorosis Virus (CPsV) and Citrus Infectious Variegation Virus (CVV).

Six CTV isolates out of 90 were find to be superinfected with CPsV, those were Q110, Q103, Q118, Q112, Q120 and Q145, all Italian isolates, while no CVV infection was detected.

This was done to know more about the geographical distribution of viral diseases in the Mediterranean basin, this collection was subjected then also to an viroid indexing.

Viroid indexing:

Three months after inoculation Etrog plantlets demonstrated characteristic symptoms of citrus viroids (Fig 17), including short internodes, leaf epinasty, mid-vein necrosis, differing in intensity according to the isolates. Interestingly, isolates belonging to mid-east countries were found to be the most severe one, probably because of the hot climate in the region.

a **b** **c**

Fig 17. Symptoms induced by citrus viroids on Etrog (C. medica) citron; a. Short internodes, b. Leaf epinasty, c. Mid-vein necrosis.

PCR results:

The mediterranean CTV isolates resulted infected by at least one viroid (table7). Interestingly, all isolates that were found to be infected with CDVd were found to be doubly infected by CVd-II.

Table.7: PCR results for viroid infection

CTV sources	CDVd	CVd-II	CEVd
Q109	--	--	--
Q102	+	+	+
Q113	+	+	--
Q7	+	+	+
Q97	--	--	+
Q30	+	+	+
Q12	+	+	+
Q75	--	--	+
Q73	+	+	+
Q40	+	+	+
Qs7	+	+	+
Q76	+	+	+
Q52	+	+	+
Q3	--	--	+
Q110	+	+	+
Qs6	+	+	--
Q57	+	+	+
Q134	+	+	--
Q118	+	+	+
Q120	+	+	--
Q103	+	+	+
Q112	+	+	+
Q25	+	+	+
Q124	+	+	+
Q21	+	+	+
Q32	+	+	+

All the positive CTV isolates displayed at least symptoms on the primary CTV indicator M. lime even if they were found to be infected also by at least one of the most economically important citrus viroids, in accordance with the study conducted by Vidalakis et al., (2004), reporting that the coinfection of M. lime with CTV and the mixture of the viroids Citrus viroid-Ia (CVd-Ia), CVd-IIb, CVd-IIIb, and Citrus exocortis viroid (CEVd) did not preclude symptom expression of either the T30 or the VT CTV groups in the primary indicator M. lime.

Cloning and sequencing:

Differences in the nucleotide sequence are reported in Fig 18.

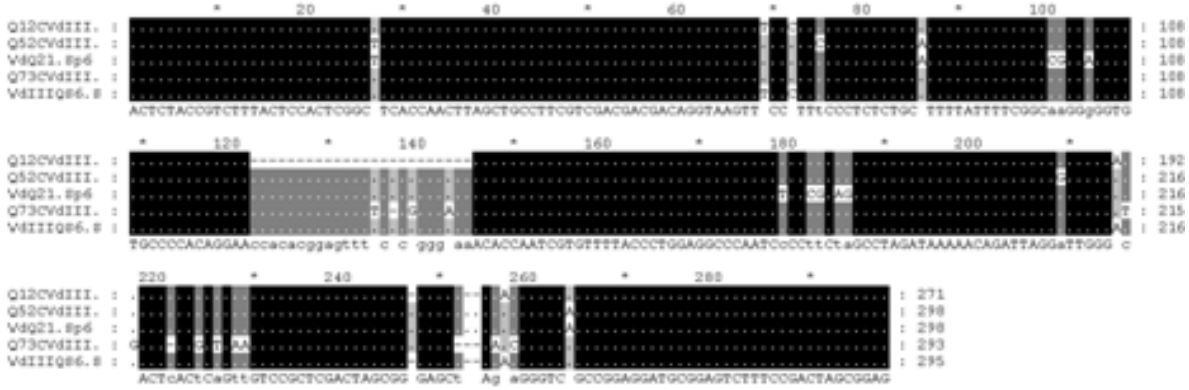


Fig 18. Differences in CDVd DNA sequences

Conventional SSCP:

Four CTV genomic parts (p18, p20, p23 and CP) were amplified by the respective primers then analyzed by the conventional SSCP for a preliminary discrimination between CTV isolates.

Tissue samples yielding different SSCP patterns in the same gel under any of the electrophoretic conditions were considered to contain a different population of sequence variants, hereafter called a haplotype.

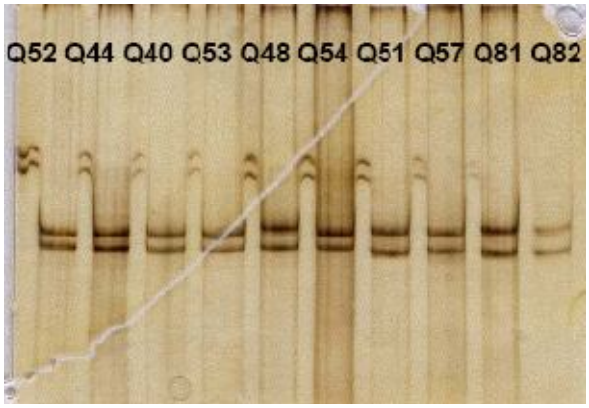
P20 profiles:

Previously, it was observed that the SSCP profile of cDNA of the p20 gene faithfully reflected the composition of the RNA population, and that sequence variants accounting for at least 10% of the RNA population could be detected in the SSCP profile (Rubio *et al*., 2000). Therefore, SSCP analysis of cDNA is an easy procedure to compare the population composition of two CTV isolates for the gene analysed and to see if the major component of these populations is the same in both isolates.

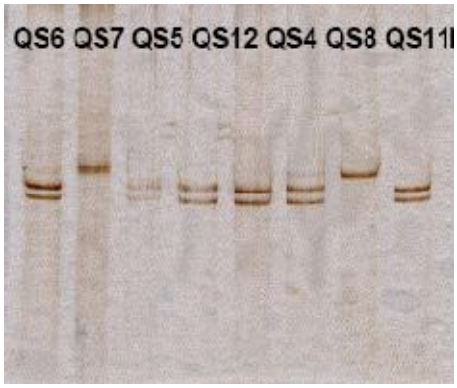
Great variability was observed in p20 haplotypes within the 44 CTV isolates belonging to different Mediterranean citrus growing countries that yielded 21 different haplotypes.

The number of different haplotypes found in each region varied from one to five for p20. Overall, the highest diversity of haplotypes was found in

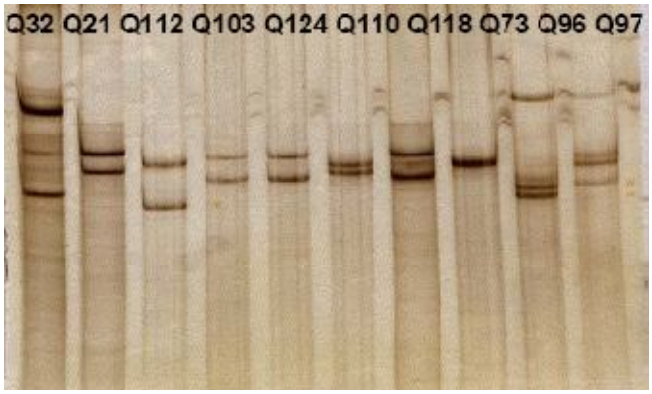
Italy, where for 7 isolates analyzed, 5 haplotypes were displayed (Fig c), accounting for 71% of variability within Italian isolate for the p20 gene, and the lowest diversity in Albania, where for 5 isolates analyzed, only 1 haplotype was displayed.



a. a.

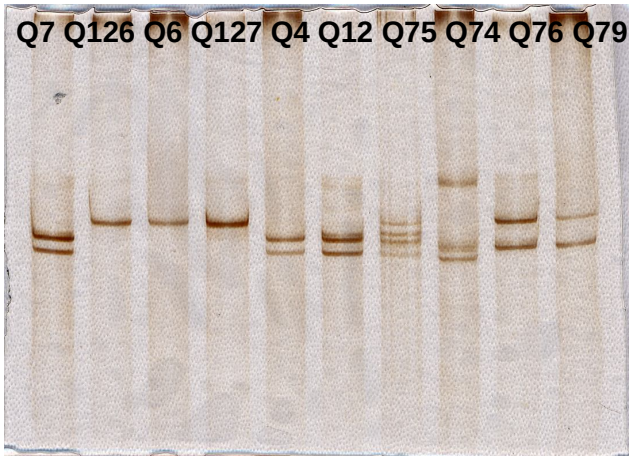


b.

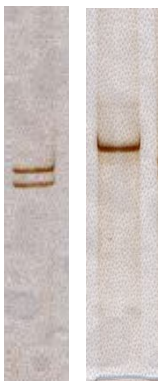


c.

d.



e.



f.

Fig 20. SSCP profiles (a, b, c, d, e) and haplotype (f) detected for the *p20* gene of the CTV genome using single-strand conformation polymorphism (SSCP) analysis of the cDNA obtained by RT-PCR using specific primers.

The isolates belonging to the Middle East region (Lebanon, Palestine, Syria) and Egypt seems to display two haplotypes “1 and 2” for p20 gene (Fig 20. f). Interestingly, when some of these isolates their CP gene were cloned and sequenced (Daden, 2006) they differed by less than 1% at nucleotide level from the well characterized VT (Fig. 19) from Israel (Mawassi *et al.,* 1993).

These haplotypes “1 and 2” (Fig 20. f) represented 43% and 14% of all samples analysed, respectively, making from the haplotype “1” the predominant detected one for the analyzed isolates.

Some of the SSCP profiles obtained consisted of just two DNA bands, as in the case of haplotype1 and 2, indicating that the viral population in those samples contained a predominant sequence variant (Kong *et al*., 2000), whereas others had more than two bands suggesting the presence of two or more sequence variants at various ratios, as it is for the two Moroccan isolates Q74 and Q75. Rubio *et al.* (2000) observed that sequence variants accounting for at least 10% of the total population in the infected tissue could be detected in the SSCP pattern.

An association was observed between specific haplotypes and symptom expression,

P18 profiles:

In contrast with p20 gene, SSCP of p18 gene displayed more conserved profiles (Fig. 21). In fact , some isolates that resulted to have different SSCP profiles using P20 gene displayed the same SSCP profile for the p18 gene, this is the case of the Lebanese isolates Q7, Q126, Q6, Q127, Q4, Q12, that displayed one haplotype, while with p20 gene they displayed two haplotypes “1 and 2”.

Similar observations have been made by other workers when analyzing the p18 gene or the 5′ untranslated region of various CTV isolates (Ayllón *et al.*, 2001). In such studies, it was found that mildly pathogenic CTV isolates contained one sequence type whereas severely pathogenic isolates contained two or three sequence types.

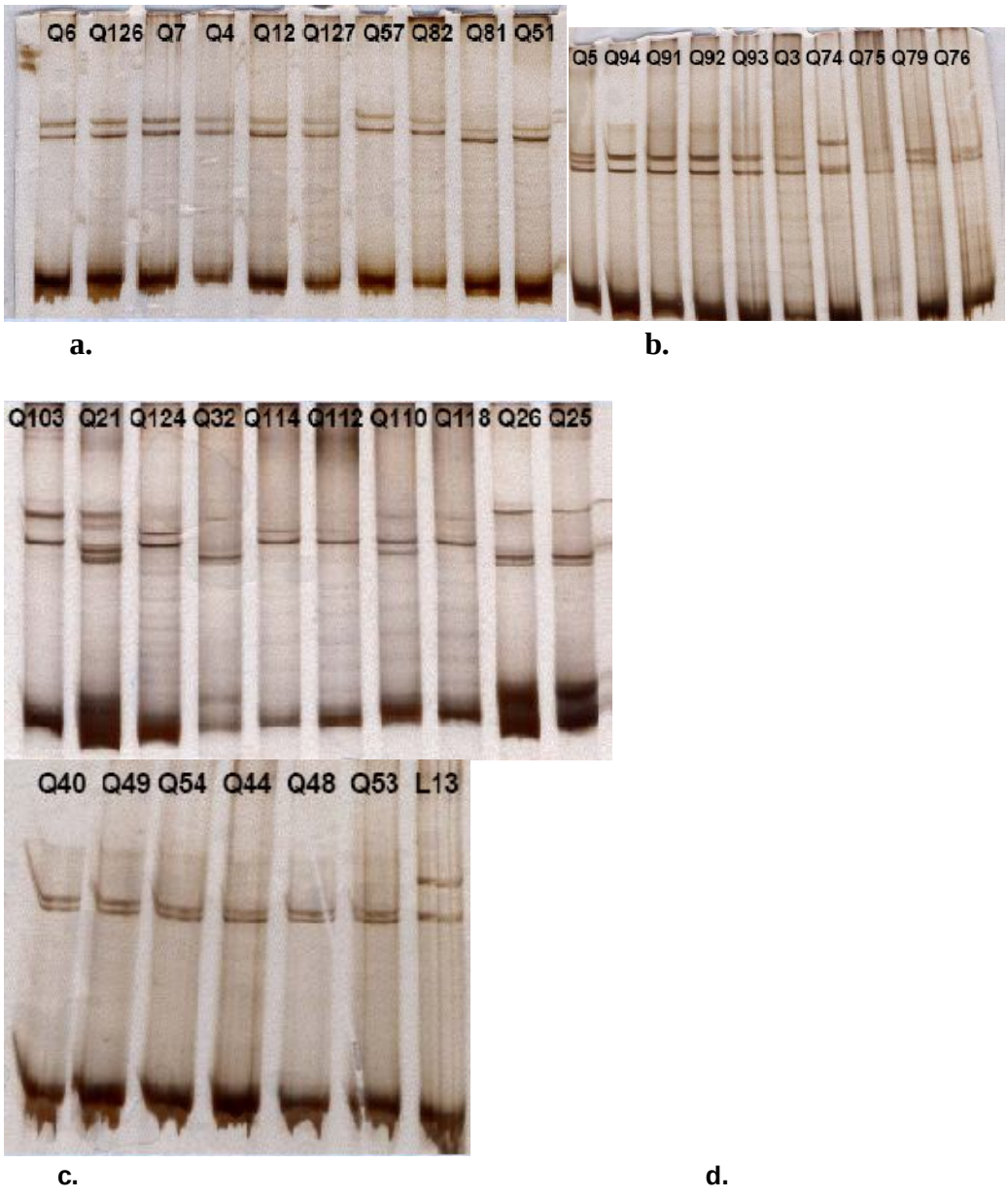


Fig 21. SSCP profiles (a, b, c, d) detected for the *p18* cDNA of the CTV genome obtained by RT-PCR using specific primers.

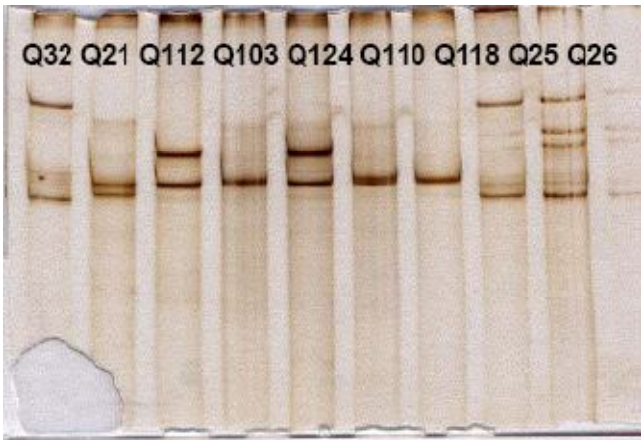
The electrophoretic profile of cloned DNA fragments sometimes shows three instead of two bands due to the presence of two stable conformations for one of the strands (Rubio *et al*., 1996); however, the presence of multiple DNA bands in a SSCP profile of a RT-PCR product usually indicates that the cDNA analyzed contains more than one sequence variant.

P23 profiles:

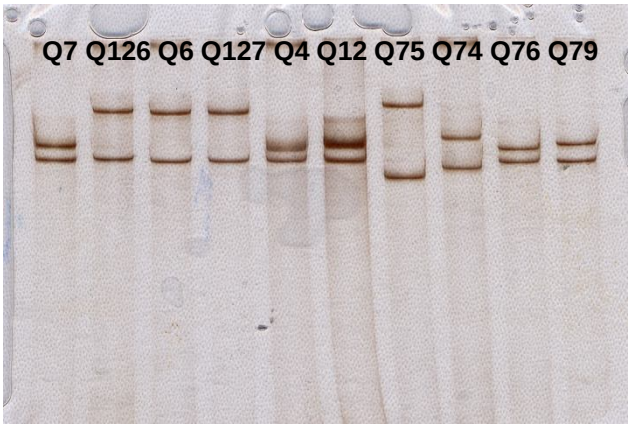
The CTV p23 is an RNA-binding protein involved in regulating the asymmetrical accumulation of viral RNA strands, when over-expressed in transgenic Mexican limes it displayed typical CTV symptoms, showing thus its involvement and importance in symptoms expression.

In a study on CTV p23 polymorphism, Sambade *et al*., (2003) found that the nucleotide sequence in a region of this gene enables discrimination between mild and severe CTV isolates, that there was low nucleotide diversity (from 0 to 0.0035±0.0014) in groups of clones with the same SSCP pattern, and that SSCP analysis discriminated clones with a 0.0030 nucleotide distance confirming the reliability of this approach to differentiate sequence variants of gene p23.

Considering this potential involvement of gene p23 in symptom expression, here we compared the predominant sequence variants of this gene from 27 CTV isolates of different geographic origin and biological characteristics with the conventional SSCP as primary molecular screening of CTV strains.



a.



b.

c.

d.

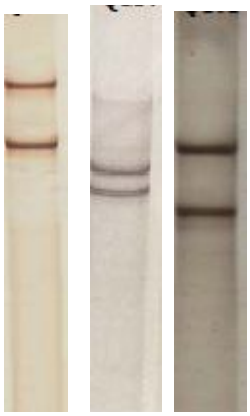
Fig 22. SSCP profiles (a, b, c, d) and haplotype (e) detected for the *p23* gene of the CTV genome using single-strand conformation polymorphism (SSCP) analysis of the cDNA obtained by RT-PCR using specific primers.

Interestingly, some isolates that displayed multiple band SSCP profiles with p20 gene showed just two clear bands when analyzed with p23 gene, this is the case of isolates Q74, Q75

Some of the banding patterns were indistinguishable. For instance, H9, H10, H11, and H12 all separated products at similar distances to each other, although the citrus-indexing data suggest different biology of the CTV source. And the same associations between geographical origin of the CTV sources and SSCP profile were observed as for the p20 and p18 profiles for the samples of Middle East origin.

P25 haplotypes: (Daden, 2006)

A B C D



Developing SSCP markers by F-SSCP:

Conventional SSCP analysis is mostly used in primary CTV strain differentiation because it combines simplicity, low cost and the possibility of being used with many samples. Also, it has enough sensitivity to detect a single nucleotide difference in relatively large DNA fragments up to 700 nucleotides (Rubio *et al.*, 1996) and differences between SSCP profiles are consistent if experimental conditions are carefully maintained. The main limitation of conventional SSCP analysis is that profile differences cannot be quantified in terms of the genetic distance between the corresponding DNA fragments (Rubio *et al*., 1996; Garcìa-Arenal *et al.,* 2001), and therefore, it provides only a qualitative picture of the variation.

To screen the genetic diversity of CTV isolates and be able to quickly classify them in an appropriate group that reflect their real severity, there is the need of developping new molecular markers. Fluorescent fragments amplified by PCR were added…

The tecnique was able to detect a set of already known mutations by using a collection of 13 isolates previously sequenced

The repeatability of the results within and between different runs was also evaluated

Visual analysis of the electropherograms suggests that the

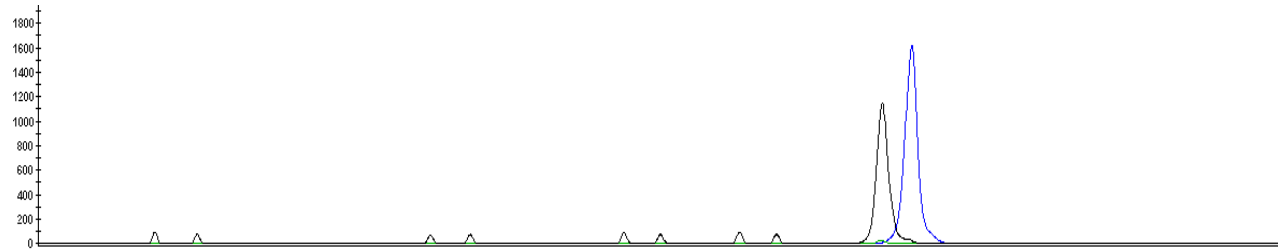
SSCP Data Analysis. The peaks of the SSCP profiles were detected with the GeneMapper^{TM} Software version 3.7 (Applied Biosystems), using Genescan-500HD

ROX as a size standard, defining an apparent size for each peak. Thus, the location of peaks is arbitrarily expressed in base pairs (bp). For statistical comparison of SSCP profiles, the list of peaks detected by GeneMapper^{TM} and their surfaces were analyzed. Because the data files did not follow a log-normal distribution, we used Spearman_s correlation coefficient (S) to compare the SSCP profiles.

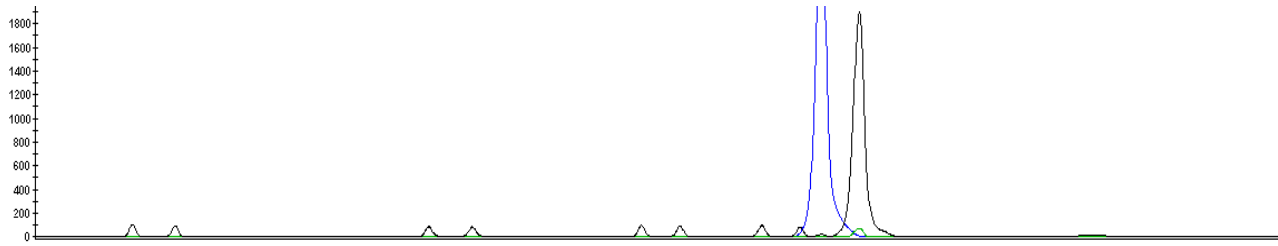
P20 F-SSCP profiles:

So far 27 isolates have been analysed and 7 different haplotypes detected these **(Fig.)** are reported in details in the Annex

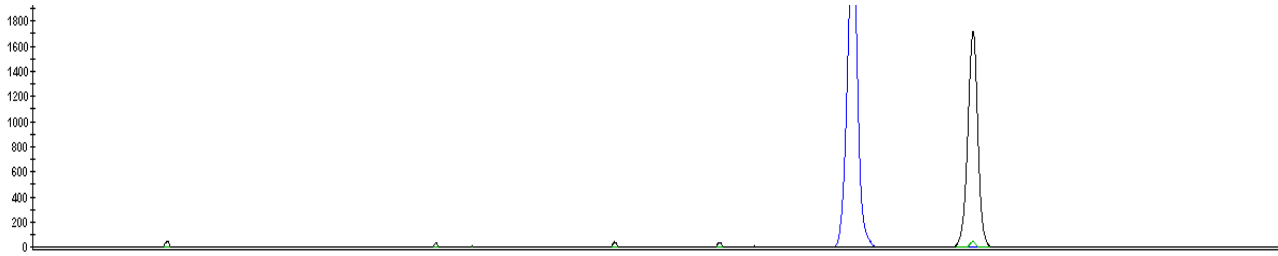
Q52:


1800
1600
1400
1200
1000
800
600
400
200
0

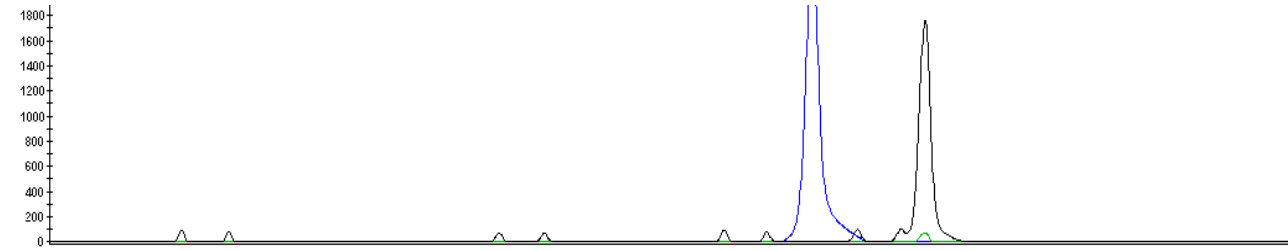
Q112


1800
1600
1400
1200
1000
800
600
400
200
0

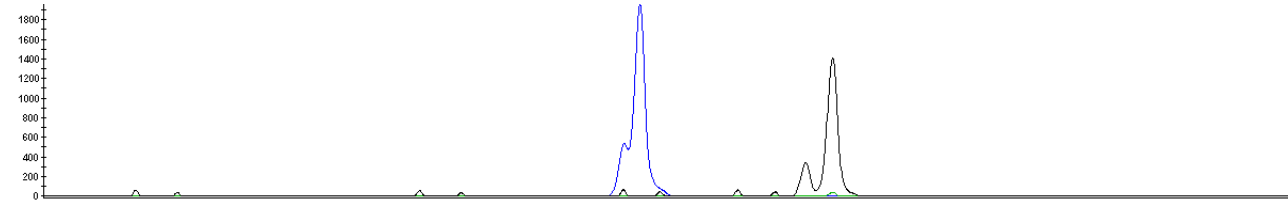
Q73


1800
1600
1400
1200
1000
800
600
400
200
0

Q96



Q93



Q3

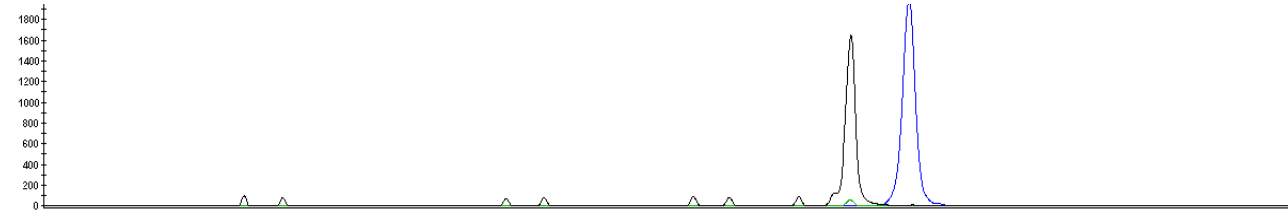


Fig.23 P20 CTV haplotypes detected by F-SSCP

Mixed infection were also well detected by F-SSCP, this was the case of two CTV isolates Q21 and Q75 (Fig.24)

Q21

Q75

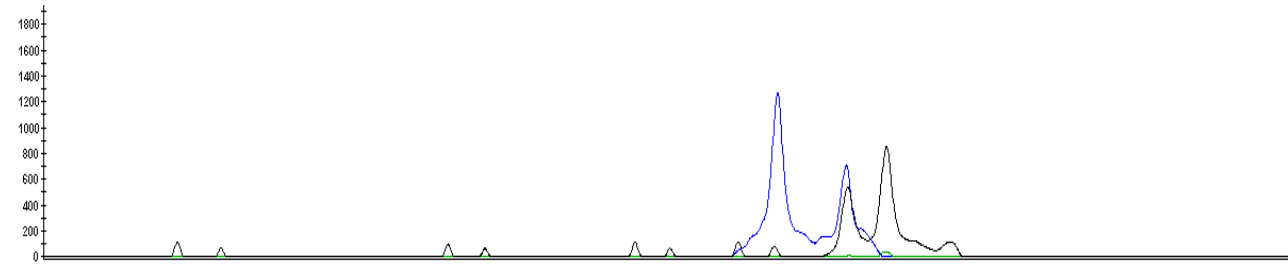


Fig.24 P20 CTV mixed infection haplotypes detected by F-SSCP

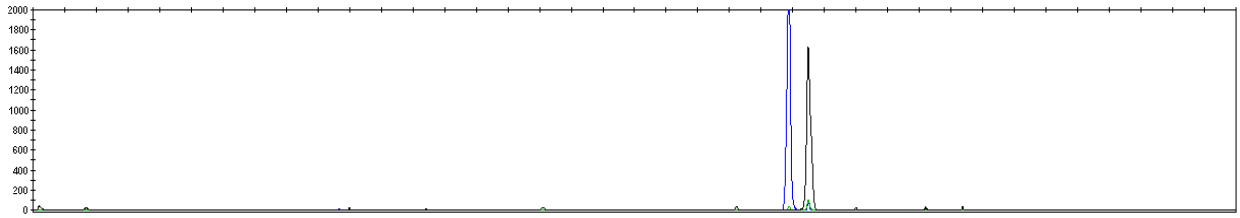
Interestigly, the same was noticed for the VT group that conserved the same haplotype (profile 1, Fig.) for p20 gene even in F-SSCP profiles, this was the case of the CTV isolates belonging to the Middle East region (Lebanon, Palestine, Syria) and Egypt. The haplotype 1 for p20 gene (Fig.) account for

Most of the mild Italian isolates displayed the hapltotype 2, accounting for

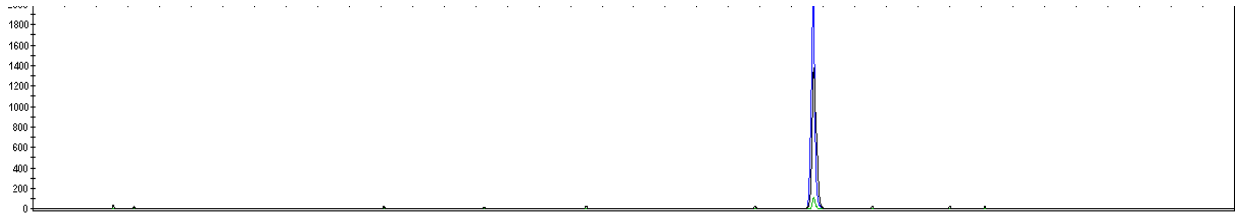
These last reacted negatively to MCA13 MAbs

P18 profiles:

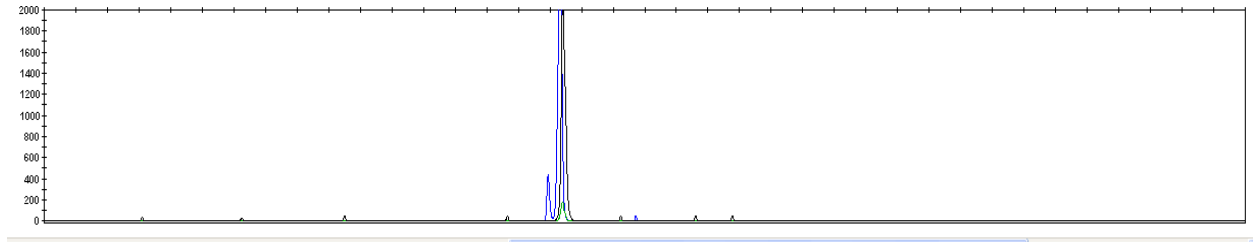
Q93:



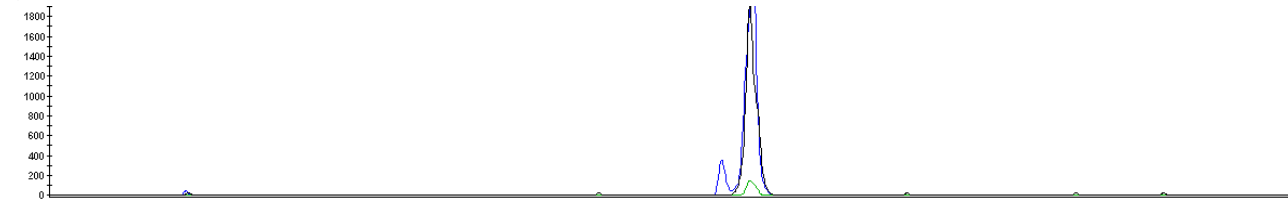
Q73 :



Q6:

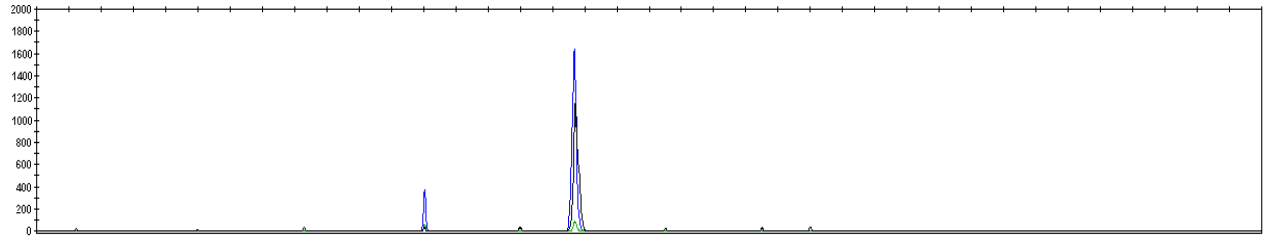


Q52

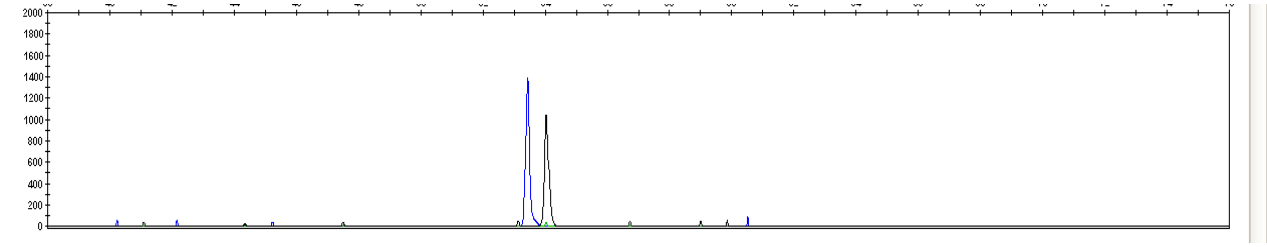


The severe CTV isolates Q52, Q12 displayed the same F-SSCP profiles also for the p18 gene.

Q112:

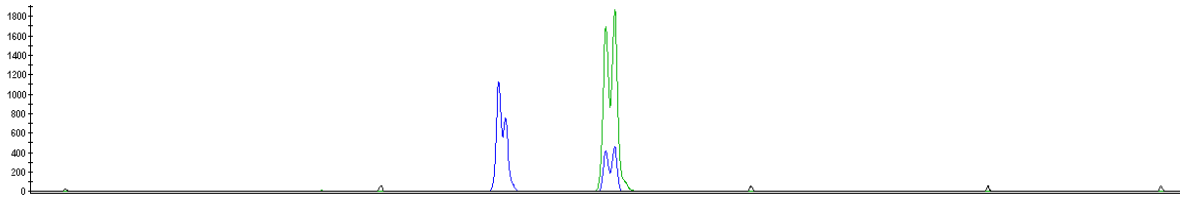


Q118:

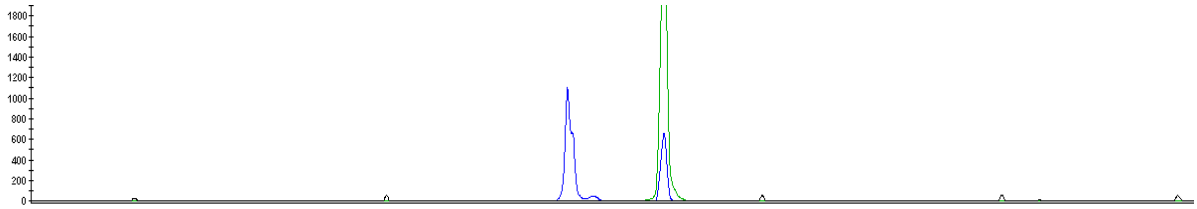


P23 F-SSCP profiles

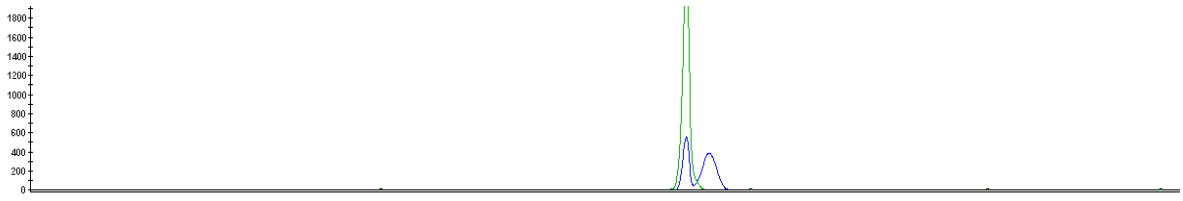
Q110



Q12



Q52



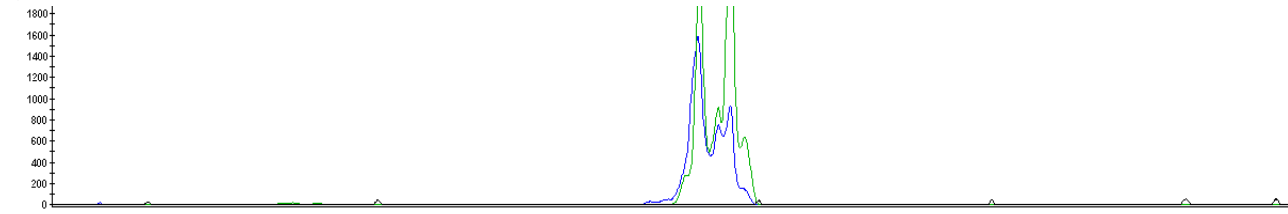
Q5

The mild Italian CTV isolates Q110 (Fig.), Q124, Q112, Q102, Q118 and the Algerian mild isolates Q76 and Q79 (Annex), displayed the same F-SSCP profile.

Similarly, the severe Lebanese CTV isolates Q12 (Fig.), Q6 and the Syrian QS6 isolate(Annex) displayed the same F-SSCP profile.

The Palestinian isolates Q52, Q44, Q40 conserved the same F-SSCP profile.

Q75

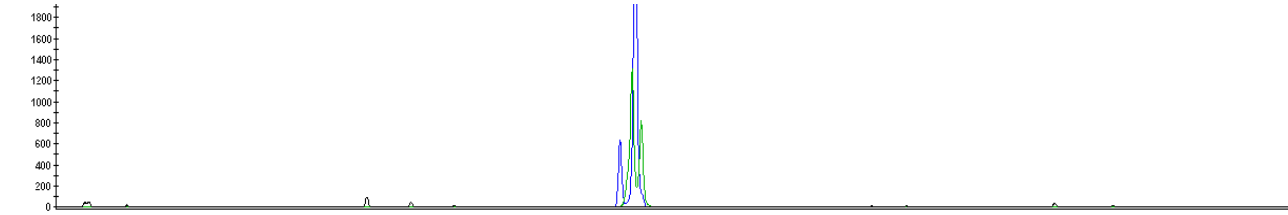


Q74

The Moroccan CTV isolates Q75 and Q74 displayed a complex profile indicating the presence of a mixed infection.

P25 F-SSCP profiles:

QS7



The Syrian CTV isolates QS7 (Fig.) and QS6, the Palestinian isolate Q52, Q40, Q44, and the Lebanese Q12, Q4 displayed the same F-SSCP profile for the p25 gene.

Cloning and sequencing:

Fifteen CTV isolates were chosen for multiple sequence alignment and genetic diversity analysis using the CP, p20 and p23 genes. Their PCR

products were cloned in the linearized and thymidylated pGem-T vector (Promega) using standard protocols.

Of these, analysis with both conventional and CE-SSCP were performed of several clones, in order to confirm the occurrence of infections with a mixture of genomic variants.

An haplotype is defined as an isolate showing a specific electrophoretic mobility of one or of the two strands of the PCR fragment. Both SSCP methods detected the different haplotype existing, with the

The nucleotide sequence of selected haplotypes were determined in both directions using vector specific primers.

by direct sequencing of the PCR products amplified with the same primers of the respective genomic region (table).

of co-infections, suggested by the high intra-isolate variability,

showed a variety of different patterns, suggesting the occurrence (Fig.).

random selected minority SSCP haplotypes were sequenced.

Phylogenetic trees were obtained using these sequences. Minority haplotypes of some isolates did not cluster with the corresponding predominant haplotype, indicating the presence of diverged sequence variants within individual isolates.

The predominant sequence variant present in each CTV isolate was selected by SSCP analysis [33, 40]. For this purpose, p23 clones were PCR-amplified with the same primers and conditions described above, and the resulting DNA was SSCP analyzed in parallel with the RT-PCR product from which the clones were obtained. Those clones whose DNA strands comigrated with the most intense DNA bands of the starting RT-PCR product were sequenced.

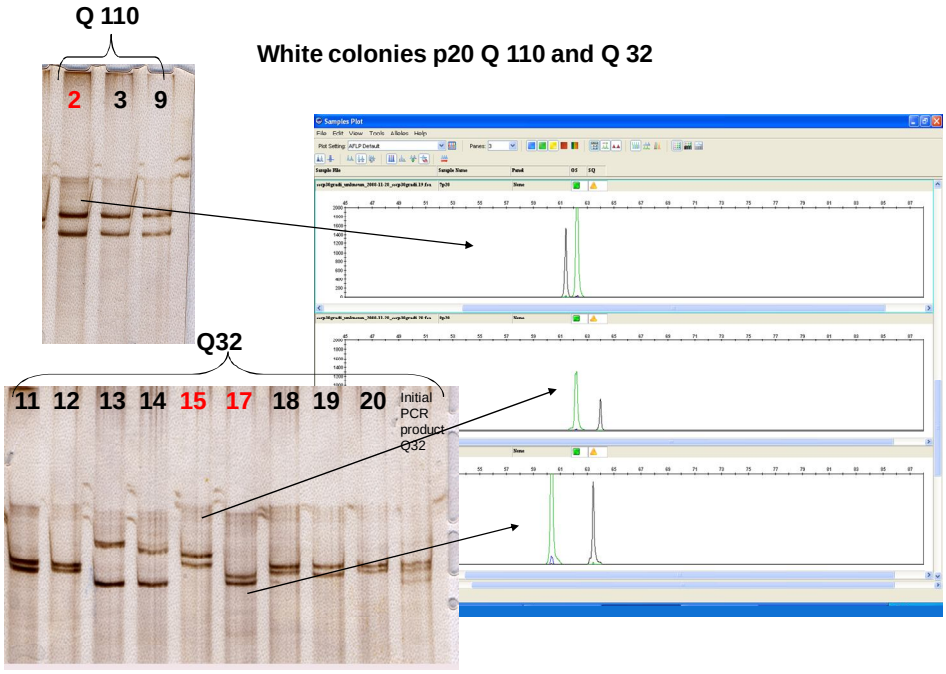
In total, six isolates contained mixed infections of 2 to 3 of the known CTV genotypes.

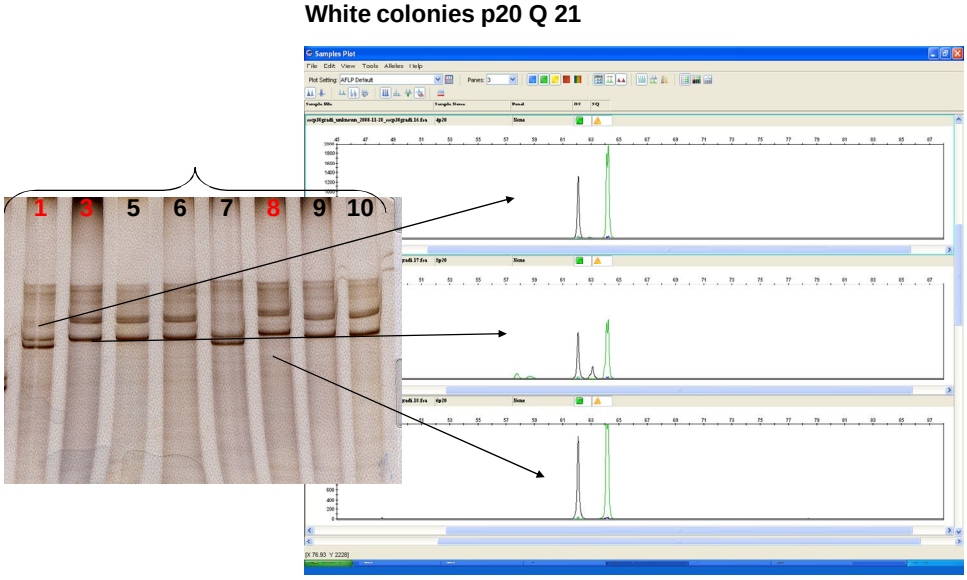
Sequence analysis of different variants showed a *CP* gene with 669 nucleotides having greater than 90% nucleotide identity to most CTV *CP* gene sequences available in GenBank. A genomic variant (GenBank Accession No. AY764154) was closely related (98.5% nucleotide identity) to the T30 mild strain from Florida (GenBank Accession No. AF260651). However, other sequences obtained

showed only 93% nucleotide identity with this variant and were closely related to other *CP* gene sequences obtained from Croatian isolates.

two isolates contained the T36 genotypes, six isolates contained the VT genotypes, five isolates contained T30 genotypes, ….T3 genotypes and…. B165 genotypes isolates.

White colonies p20 Q 32


Q 110
2 3 9
White colonies p20 Q 110 and Q 32
Q32
11 12 13 14 15 17 18 19 20
Initial PCR product Q32


White colonies p20 Q 21
1 3 5 6 7 8 9 10

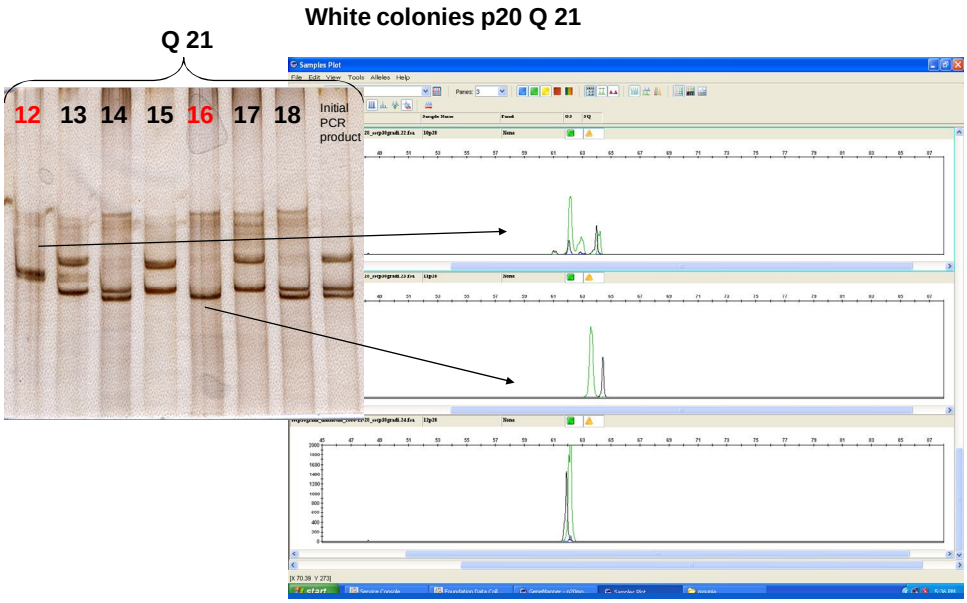


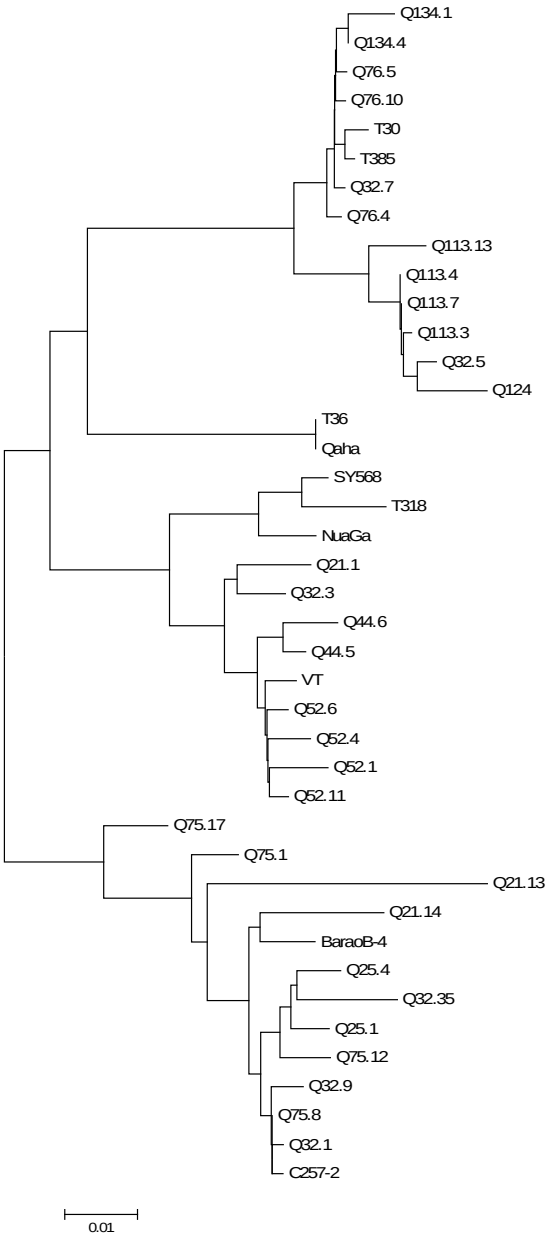
Fig. SSCP analysis of CTV isolate Q44 CP clones

To assess reliability of SSCP analysis to differentiate between sequence variant and select the predominant sequence variant of each CTV isolate, SSCP patterns of individual cDNA clones were compared with the pattern of the RT-PCR product from which they had been obtained.

The SSCP pattern of some isolates had only two DNA bands, while others yielded a more complex pattern [34]. In the first case, most of the clones showed the same SSCP pattern as the corresponding RT-PCR product

and one of them was selected for sequence analysis. In the second case, a higher variability was observed between clones, but only those whose SSCP pattern coincided with the most intense bands in the pattern of the starting RT-PCR product were sequenced [33]. Alignments showed that the complete sequence of gene p23 was preserved in all cases, without insertions, gaps or unexpected stop codons, indicating that each selected clone corresponded to a translatable sequence.

The Palestinian isolate Q44 showed the lowest number of SSCP profiles, whereas Q21 and Q32 exhibited the highest number of them. The percentage of predominant pattern was calculated for all isolate. The Palestinian Q44 isolate exhibited the highest value (93%), being the most homogeneous isolate for CP gene, followed by the Italian isolate Q110 with 86%. On the contrary, Q32 showed the lowest value (31%) for the major clone, being the most heterogeneous for the CP gene region among the analyzed isolates, (Daden, 2006) and also for p20 gene, followed by the Italian isolate Q21 with 40%.


Q134.1
Q134.4
Q76.5
Q76.10
T30
T385
Q32.7
Q76.4
Q113.13
Q113.4
Q113.7
Q113.3
Q32.5
Q124
T36
Qaha
SY568
T318
NuaGa
Q21.1
Q32.3
Q44.6
Q44.5
VT
Q52.6
Q52.4
Q52.1
Q52.11
Q75.17
Q75.1
Q21.13
Q21.14
BaraoB-4
Q25.4
Q32.35
Q25.1
Q75.12
Q32.9
Q75.8
Q32.1
C257-2
0.01

Citrus Viroids-CTV interaction:

CTV virions are covered with two capsid proteins of 25 and 27 kDa, coating about 95 and 5% of the virion length, respectively (Febres *et al*., 1996; Satyanarayana *et al*., 2004), and are single-stranded, positive sense genomic RNA (gRNA) approximately 20 kb in size, readily transmitted by citrus aphids species. Viroids are, in the contrary, small naked RNA particles (359 nt) that completes their infection cycle without generating either a capsid or other viroid-specific proteins.

An essential role of the viral capsid protein in virus transmission by aphids has been proved (Syller, 2000). The specific recognition of viral genomic RNA by capsid protein CP is a critical event in the infection cycle of all single-stranded RNA viruses. This recognition event plays a crucial role in specific encapsidation of the viral genome as well as other steps in the virus life cycle.

Heterologous encapsidation between viruses in mixed infections may allow transmission by aphids of normally non-aphid-transmissible viruses or change virus-vector interactions, it is known also by transcapsidation, meaning the coating of the nucleic acid of one virus or virus strain with the protein of another in a double infections.

This phenomenon has been demonstrated even for the Nigerian cowpea virus (CV) and the tobacco mosaic virus (TMV), which are distantly related serologically, in conditions in which both viruses multiply readily (Kassanis and Bastow, I971).

Samples were analyzed using ELISA with a CTV polyclonal antibodies to ascertain for CTV infection. After plate development, wells of positive samples were used for IC-RT-PCR analysis targeting the three citrus most economically

important citrus viroids; CVd-II, CEVd and CDVd, that have been already found to infect the CTV isolate collection (table), using their respective pairs of primers (table).

When analyzed using agarose gel electrophoresis, PCR products from the positive samples consisted of a single amplicon of viroids expected size compared with a positive control. That the CDVd RNA was contained in the coat protein of CTV was indicated by the amplification of the RNA viroid with its specific primers after the immunocapture of CTV particles in the wells of an ELISA plate with an polyclonal antisera. This phenomenon occurs even at 25°C, the temperature at which the M. lime plantlets inoculated with CTV isolates were kept in the greenhouse.

When sap was extracted from *C. medica* used for biological indexing of citrus viroids, plantlets that were kept in the greenhouse at 35°C, the phenomenon was not verified, this can be explained by the low CTV title, found in ELISA test value, and it's reduced ability to multiply due to the high temperature. While when other *C. medica* plantlets inoculated with CTV isolate were kept at 25°C, the CDVd was amplified from ELISA plate, indicating that both CTV and CDVd could multiply in *C. medica* and in ambient temperature.

In contrast, no amplified products were obtained when the sample was taken from citrus plants with only one infection CTV or citrus viroids.

The expected bands were excised from agarose gel, eluted and then cloned in (pGEM-T Easy Vector; Promega, Madison, WI) and sequenced.

This fact can allow to viroid particles to be transmitted by citrus aphids as it was demonstrated for the Potato Tuber Spindle Viroids (PTSVd) that was found to be vectored by aphids only when potato was doubly infected by Potato Leafroll Luteovirus (PLRV) whose coat protein encapsidates the viroids.

The transmission of citrus viroids encapsidated in the viral capsid protein by aphids is even more likely to happen in the open field if the most efficient CTV aphid vector *Toxoptera citricidus* continue its spreading and get more diffused in the area.

This phenomenon was more likely to happen when the conditions were favorable for CTV multiplication, and was confirmed for the available CTV strains in the collection and for the three most economically important citrus viroids.

This will change the idea about the non transmissibility of the citrus viroids by insects (Laird *et al.,* 1963), as the encapsidation of citrus viroids can lead to a dependent transmission.

This finding strongly suggests that the dependent transmission is a phenomenon that can occur easily in nature, and that apart from the risks of mutations that have to be taken in consideration when inoculating a mild CTV isolate for cross protection, as a strategy to contain damages in an area where the CTV is established, there is also the risk that the encapsidation by CTV coat protein, of a normally non-aphid-transmissible virus particle, occurs in a mixed infection allowing thus its transmission by citrus aphids in the commercial orchards.

Chapter 5: Conclusion

The objective of this work was to find a potential molecular marker that could be used for quick identification of virulent CTV isolates through the study of a mediterranean CTV collection. For this purpose 27 CTV isolates were compared for symptom expression and by conventional and CE-SSCP analysis of four 3′ terminal genes (P18, P20, P27 and P23). A great diversity of SSCP profiles was observed among isolates for the genes studied.

Bioindexing coupled with laboratory-based diagnostic techniques offers the best available detection for viral citrus pathogens of uneven tree distribution and/or low titer.

Although whole genome DNA sequence data and molecular markers are now available for many CTV isolates, the ability to identify genetical variation associated with pathogenicity and symptoms expressed is still limited.

Regardless their geographical origin, F-SSCP profiles of the p23, p20 and p25 appears to be a useful marker for quick distinction between CTV isolates that do not cause seedling yellows or stem pitting (mild group) and those that do so (severe). This could be a useful tool for containment of potentially damaging CTV isolates in citrus areas, as the Mediterranean basin, where mild isolates are predominant but where reservoirs of damaging CTV isolates exist.

This could be a useful tool for containment of potentially damaging CTV isolates in citrus areas, as the Mediterranean basin, where mild isolates are predominant, as well as for predicting the outcome of cross-protection assays.

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