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Citrus variegation virus: molecular variability of a portion of the RNA 3 containing the coat protein gene and design of primers for RT-PCR detection

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Abstract

The coat protein gene and part of the intergenic region of the RNA 3 of several isolates of *Citrus variegation virus* (CVV) producing either infectious variegation or crinkly leaf symptoms were amplified by RT-PCR, cloned and sequenced. Some isolates were composed of a mixture of sequence variants. The coat protein gene appeared to be highly conserved (lowest similarity among all CVV sequences 93%), especially at the N-terminal, indicating at the molecular level that both types of symptoms are indeed produced by the same virus species. No relationship could be found with the geographic origin. Sequences obtained from isolates producing infectious variegation clustered in a different branch of a dendrogram than those originating from crinkly leaf symptoms. Both clusters could further be distinguished by two parsimonious sites in the coat protein gene. In the short stretch of the intergenic RNA 3 region analysed, a stable hairpin exists in addition to the previously reported hairpin that constitutes the core promoter for the RNA 4 transcription. This second hairpin could also be recognised in the other subgroup 2 Ilarviruses. Surprisingly, at the nucleotide and amino acid levels and in the secondary features of the intergenic region, CVV appeared closer to the other subgroup 2 Ilarviruses than to *Citrus leaf rugose virus* to which it is serologically related and has been considered to be evolutionary related. Using primers designed for the conserved regions, the virus was detected with a prevalence of 25% and 13% in Portuguese and Moroccan citrus collections. A group of RT-PCR positives was further confirmed by ELISA and biological indexing.

Introduction

Citrus variegation virus (CVV, genus *Ilarvirus* family *Bromoviridae*), is a member of the subgroup 2 of Ilarviruses. Citrus trees infected with CVV exhibit a range of symptoms that are usually mild on oranges and mandarins but may be severe on citron and lemons with an associated reduction in yield and fruit malformation. Two strains of the virus have been distinguished based on symptomatology on Citrus: infectious variegation and crinkly leaf. Usually, infectious variegation is a more severe disease than crinkly leaf. Symptoms typical of this include crinkling of leaves associated with areas displaying various degrees of chlorosis. Leaves may be narrower and have an irregular outline. The crinkly leaf symptoms includes warping, pocketing and crinkling without variegation or reduction in leaf size (Desjardins and Bové, 1980).

The virus is serologically related to other members of the same genus: *Elm mottle virus* (EMoV), *Tulare apple mosaic* (TAMV), *Citrus leaf rugose* (CiLRV), *Spinach latent virus* (SpLV) and *Asparagus virus 2* (AV-2). There exists some contradictory evidence that needs further elucidation: *Hydrangea mosaic virus* (HdMV), which according to serology, is grouped in subgroup I (Bol, 1999), has an almost identical genome to EMoV, according to sequences published on the Genebank (accession numbers AF172965–AF172969) and

should be considered the same virus as EMoV (Scott, personal communication). In addition, the sequences deposited on the Genebank as belonging to SpLV (accession numbers U93192–U93194) are also from EMoV (Scott, personal communication). In this work the sequences corresponding to the accession numbers AF172965 and U93194 will be referred as belonging to EMoV (HdMV) and EMoV (SpLV) respectively. As an Ilarvirus, CVV has a tripartite, message-sense, single-stranded RNA genome encapsulated by a 24 kDa protein. The two larger genomic RNAs, RNA 1 and RNA 2, are monocistronic and encode non-structural proteins involved in replication. In contrast, RNA 3 is bicistronic with ORF 1 coding for a putative movement protein and ORF 2 coding for the putative viral coat protein (CP). The coat protein is expressed from a fourth, sub-genomic RNA (RNA 4) of 0.9 kb which is encapsulated. It has been shown that CP is required for infection (van Vloten-Doting, 1975; Gonsalves and Fulton, 1977). For years, only one genomic sequence of CVV RNA 3 (Scott and Ge, 1995) and parts of the replicase have been available at Genebank.

The virus does not seem to be naturally transmissible by pollen (Lovisolo, 1993), but is highly transmissible by mechanical means from citrus to citrus and to herbaceous hosts (Roistacher, 1991). The chief measure to control CVV should be its detection at an early stage of infection and the use of virus-free propagation material. Antibodies for ELISA are available (Davino and Garnsey, 1984) and this assay is considered a reliable technique for the detection of the virus in young tissue of field trees until late spring or early summer. However, ELISA performed poorly in winter and fall (Davino et al., 1988). No molecular technique has yet been developed to detect the virus and there is no data regarding the molecular variability of the virus.

In order to gain a clearer view of the position of the two CVV strains in the Ilarvirus subgroup 2 and to study the variability of the *CP* gene we isolated and characterized a short stretch of the intergenic region of the RNA 3 and the *CP* gene of isolates that produce different symptoms and originate from different geographic locations.

Material and methods

Virus isolates

Seedlings of *Citrus sinensis*, Cv. Madam Vinous, were graft-inoculated with CVV infected tissue from different geographical areas. These included the previously characterized infectious variegation isolates from Florida: severe isolate CVV-1 (= PV196) (Garnsey, 1968), mild isolate CVV-2 (Garnsey et al., 1984), obtained from USDA, ARS, Orlando FL, USA, CVV-E1234 characterized by Dr. Grant (Desjardins and Bové, 1980), obtained from the Station de San Giulliano, Corsica and CVV-IV400 from IVIA, Spain (Duran-Vila et al., 1988). Previously characterized Crinkly leaf isolates were obtained from Corsica CVV-81A65 (Desjardins and Bové, 1980) and from Spain, CVV-CL903. Uncharacterised isolate CVV-3 was obtained in the field near Marrakech and CVV-ES86 obtained in a citrus repository in Portugal. Virus-free citrus was included as healthy control. All plants were grown in an insect-proof greenhouse. Bark samples were collected from young branches and used fresh, frozen at −20 °C or lyophilised.

RNA extraction

Total RNA was extracted from plant tissue by means of a lithium chloride based protocol according to Hughes and Galau (1988), with minor modifications. Bark samples (200 mg), were powdered in liquid nitrogen and extracted 1/10 (v/w) in 200 mM Tris–HCl (pH 8.5), 300 mM LiCl, 10 mM EDTA, containing 1.5% SDS, 1% sodium deoxycholate, 1% NP-40 and 0.5% 2-mercaptoethanol. The extract was collected in a 1.5 ml microfuge tube and centrifuged for 5 min at 12,000*g* at 4 °C. One volume of 5 M potassium acetate (pH 6.5) was added to the supernatant, the mixture was incubated for 10 min at −20 °C and centrifuged for 15 min at 13,000*g* at 4 °C. The supernatant was recovered and the nucleic acids were precipitated with an equal volume of isopropanol. After drying, the pellet was re-suspended in 30 µl of sterile deionised water.

RT-PCR amplification

At the time this work started only the U17389 RNA 3 sequence of CVV was available in the Genebank. To amplify the *CP* gene of most CVV isolates, the available Genebank sequences of the RNA 3 of the subgroup 2 Ilarvirus (EMoV – accession numbers U85399 and U57048, AV-2 – X86352, CiLRV – U17390, and EMoV (HdMV) – AF172965) were compared. The primers CVVa: 5′-GGAGGAGATTTGTCTTGAAG-3′ and CVVb: 5′-GTCATTCYTCAACAACCA-3′ were designed in

such way that they had a perfect homology to CVV, EMoV, EMoV (HdMV) and AV-2 and low homology to CiLRV, respectively 55% and 61%. These primers were chosen to amplify a fragment between positions 1266 and 1983 of RNA 3 of CVV which comprises 63 nt of the intergenic region upstream to the *CP* gene and the entire *CP* gene. The RT-PCR reactions were performed by a one tube single-step protocol in a total volume of 25 µl using 2 µl of RNA template. The reaction mixture consisted in 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 400 nM of each of the primers, 0.4 mM of each dNTP and contained 0.08% NP-40, 0.5 U *Taq* DNA polymerase (MBI-Fermentas), 5 U RNAguard (Amersham Pharmacia), and 6 U M-MLV reverse transcriptase (Perkin Elmer). The thermocycling programme was as follows: 30 min at 39 °C, 2 min at 94 °C, then 35 cycles of 92 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min and finally 5 min at 72 °C. Assessment of the amplified products was by conventional agarose gel electrophoresis.

Design of primers for diagnosis and screening of citrus germplasm repositories for CVV

To assure that a large spectrum of CVV strains could be detected apart from other Ilarviruses, an additional primer CVV4 (reverse): 5′-ATGCACGGCACCAGTTG-3′, was designed in such a way as to have a perfect homology with all determined CVV sequences and low homology with CiLRV and other Ilarviruses of the subgroup 2. Primers CVVa and CVV4 amplify a fragment of 624 bp. These primers were used in a one tube single-step RT-PCR as described above to screen a collection of Portuguese traditional varieties from diverse regions maintained at the Centro de Citricultura, Portugal. An extensive screening was also carried out in Morocco on the Souihla Citrus Germplasm Repository at Marrakech. A group of 10 random positives from Souihla was tested by ELISA for further confirmation and indexed on Eureka lemon to evaluate the severity of the isolates.

Cloning of RT-PCR product and sequencing

The RT-PCR products with the expected size were ligated into pGEM T-Easy vector system I according to the manufacturer's instructions. The recombinant plasmids were used to transform competent INVαF' *E. coli* cells (Invitrogen). The selection of the transformed colonies was done by α-complementation followed by direct PCR amplification from the white colonies using primers CVVa/CVVb. The plasmid DNA was purified using alkaline lysis methods or Wizard plus SV miniprep Kit (Promega) according to the manufacturer's instructions. Confirmation of the presence of the insert was accomplished by digestion with *Eco*RI. Minipreps from selected clones were ethanol precipitated and sent to be sequenced in both senses by a specialized firm. The sequence data obtained in this work was submitted to Genebank with successive accession numbers from AF434911 to AF434922.

Genomics

Multiple sequence alignments and amino acid sequence deduction were done using the BioEdit sequence alignment editor program package (Hall, 1999). Dendrograms were generated by the ClustalX program using the NJ method with the default parameters.

Results

RT-PCR amplification and cloning of the CP *gene*

When RNA extracts from CVV infected tissues were used for RT-PCR amplification, the expected 718 bp product was the sole product conspicuous over a smeared background (Figure 1). No amplification occurred when RNA extracts from non-infected tissue were used. Apparently, the smeared background did not interfere with the cloning as most of the recombinant *E. coli* colonies had the full length product as verified by PCR.

Molecular variability of the CP *gene*

The *CP* gene region was retrieved from the sequence data, aligned jointly with the previous existing Genebank sequences of CVV, CiLRV, EMoV, EMoV (SpLV), EMoV (HdMV) and AV-2 and the similarity between the sequences computed. The similarity between the CVV *CP* gene and that of CiLRV was as low as 62–63%. Surprisingly, the CVV *CP* gene appeared to be closer to EMoV (SpLV), AV-2, EMoV (HdMV) or EMoV with similarities around 70%, 76%, 83% or 83%, respectively. Within the CVV sequences, the similarity ranged from 93% to almost 100% in the case of CVV-1 and U17389, showing a highly

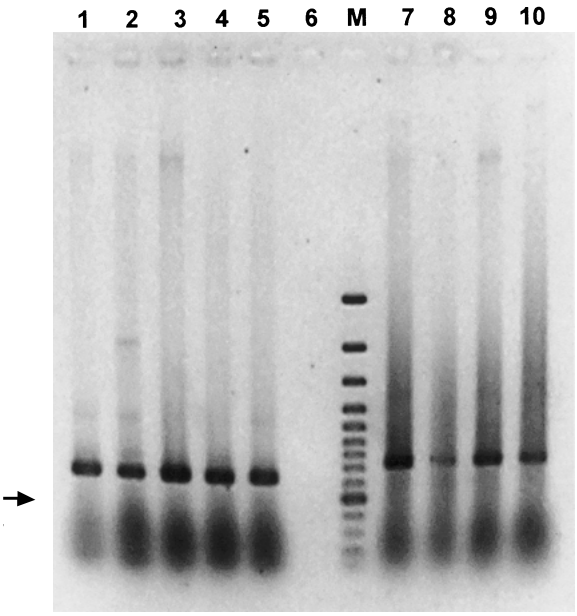

Figure 1. Comparison of RT-PCR products obtained with primers CVVa – CVV4 (lanes 1–6) and CVVa – CVVb (lanes 7–10). The samples analysed on each lane are respectively 85A65, CVV-ES86, CVV-CL903, CVV-1, CVV-2, Healthy control, 85A65, CVV-ES86, CVV-CL903 and CVV-1. Lane M, 100 bp ladder (arrow pointing at 500 bp). Agarose gel at 1% stained with ethidium bromide. Picture printed as a negative.

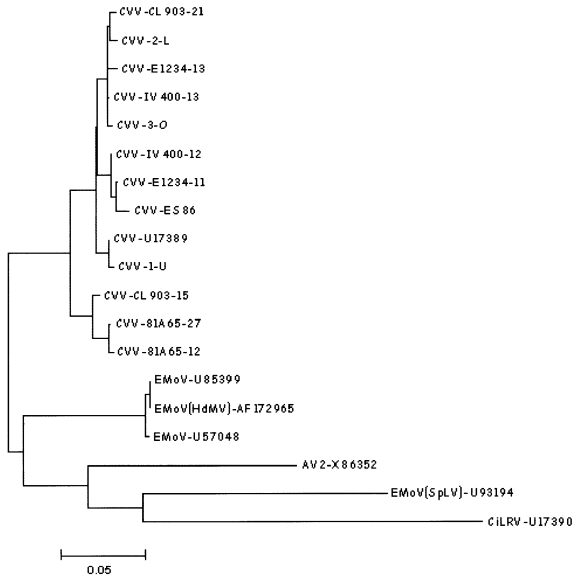

Figure 2. Dendrogram constructed from the alignment of the *CP* gene sequences of the CVV clones sequenced in this work and other subgroup 2 Ilarviruses obtained from the Genebank (accession number following virus acronym).

conserved *CP* gene in which most of the differences were due to synonymous substitutions.

The geographic origin of the CVV isolates did not correlate with the pattern of clustering of the *CP* gene sequences (Figure 2): isolates from Florida and Corsica are dispersed through the dendrogram. In cases in which more than one clone was sequenced, the sequences obtained were frequently positioned in different branches. Conversely, there seems to exist a good agreement with the biological properties. Thus, both sequenced clones of isolate CVV-81A65 and one clone of isolate CVV-CL903, which originated from the crinkly leaf symptoms, clustered together in a separate branch.

The similarity between the deduced CVV amino acid sequences ranged from 94% to 99%. Excluding three conserved stretches at positions 20–40, 81–116 and 184–200, the differences occurring in 21 sites were evenly distributed averaging 1.4 variable sites every 10 amino acids. However only 9 parsimony informative sites were present. Six of these sites could differentiate isolate CVV-81A65 from the others. The amino acid sequences of the other subgroup 2 viruses were also included in the comparison (Figure 3). A high homology could be noticed in the first 30 amino acid residues of the CVV isolates. The relative molecular mass of the deduced CPs of the CVV isolates was computed and shown to be in agreement with the value of 24,040 Da previously published (Scott and Ge, 1995). The highest difference being as low as 311 Da.

Intergenic region

Only a short stretch of the intergenic region immediately upstream of the *CP* gene was analysed. A very conserved region extended from the end of CVVa primer (pos 20) to position 46, and then a variable region started just before the beginning of the *CP* gene.

Design of primers for diagnosis and screening of citrus germplasm repositories for CVV

The RT-PCR amplification of RNA extracted from leaf samples of infected citrus trees, using CVVa/CVV4 primers, yielded a specific DNA band of about 624 pb, with higher intensity over a clear background than the CVVa/CVVb couple of primers (Figure 1). When used for diagnosis purposes the pair of primers CVVa/CVVb

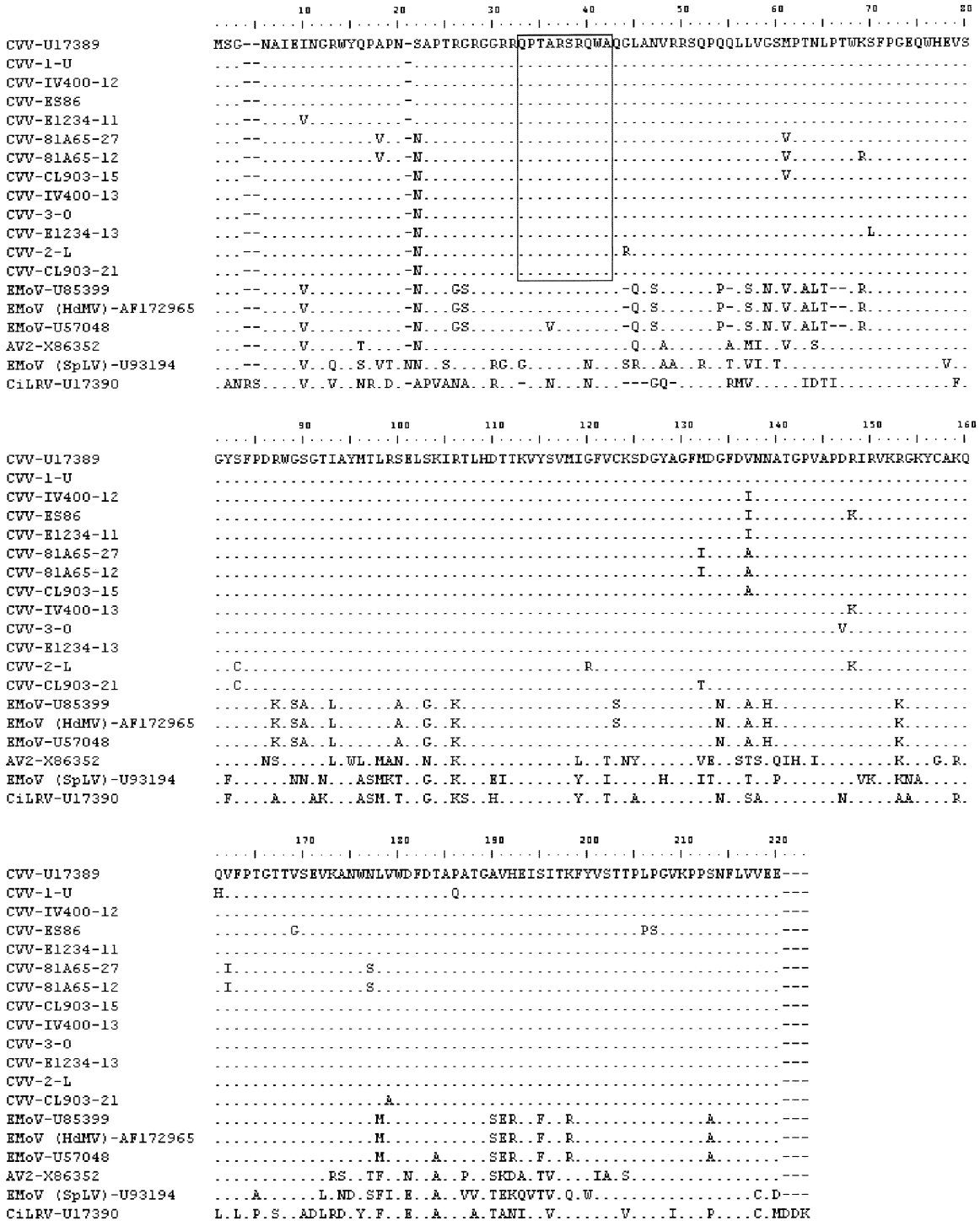

Figure 3. Alignment of the deduced amino acid sequences of the CP of CVV clones sequenced in this work and other subgroup 2 Ilarviruses whose sequences were obtained from the Genebank (accession number following virus acronym). Identities to the CVV-U17389 Genebank sequence are marked as a dot. Boxed residues correspond to the conserved RNA binding sequence (see text).

often failed to detect samples that were positive by CVVa/CVV4 and thus were abandoned for diagnosis. Primers CVVa/CVV4 were used to test 44 samples of Portuguese traditional varieties and showed that about 25% of the samples were infected. In Morocco the prevalence of the virus in the citrus repository at Souihla was about 13% (193 samples tested). All the samples of the group of 10 random positives were confirmed by ELISA. Their biological indexing on Eureka lemon revealed a diversity of symptoms ranging from very mild for Sunrise orange and Thompson grapefruit to very severe for Mandarinette and Homossassa orange.

Discussion

Based on the limited amount of information available at Genebank, the pair of primers CVVA/CVVB was designed. These proved to have a sufficiently broad spectrum to amplify CVV isolates from diverse origins and with diverse biological properties.

Sequencing the amplified product showed that the *CP* gene of CVV is a conserved gene in which most of the differences were due to synonymous substitutions. Additional nucleotide substitutions resulted in a small number of non-synonymous non-parsimonious amino acid substitutions (10) which were found scattered among the CP sequences. The possibility of these being the result of misincorporations due to the high error rates of *Taq* polymerase (Bracho et al., 1998) or M-MLV reverse transcriptase (Malboeuf et al., 2001) cannot be excluded. However, the random nature and small amount of these putative artefacts should not significantly change the global pattern of clustering in view of the fact that the CP appears to be conserved and most substitutions occur in parsimonious sites.

Similarity of the CVV sequences with CiLRV was found to be approximately of same value as computed by Scott and Ge (1995). However, it was surprising to find that CVV appeared closer to other non-infecting citrus viruses than to CiLRV, which may have implications on the evolutionary picture we have of these viruses (see below). When a dendogram was constructed, no relationship with the geographic origin could be found but two clusters were conspicuous. One in which were mapped all the clones originated from isolates with infectious variegation symptoms (IV cluster), differing among themselves at the nucleotide level less than 5%. A second cluster gathered the clones from isolates with crinkly leaf symptoms (CL cluster); these differed less than 2%. However, one of the two clones from the crinkly leaf isolate CVV-CL903 was grouped into the IV cluster, close to a clone from the mild IV isolate CVV-2. These results confirm at the molecular level that crinkly leaf and infectious variegation should be regarded as two strains of the same virus and suggest a relationship between symptoms and the CP sequence. It is interesting that if both types of strains may coexist in the same tree (isolate CVV-CL903), these strains do not cross-protect to one another (in the sense of inhibiting replication of the other strain). At the amino acid level, the three clones belonging to the CL cluster could be distinguished from those of the IV cluster by two parsimonious sites at positions 61 and 137, which were common to EMoV (HdMV) and EMoV. Additional parsimonious sites were common to the CVV-81A65 isolate and EMoV (SpLV) at positions 18, 132 and 177. Within the *Ilarvirus* and the closely related *Alfamovirus* genera a relationship between the *CP* gene and symptom determination has been shown for *Alfalfa mosaic virus* (Neeleman et al., 1991) and for PNRSV (Hammond and Crosslin, 1998), in which limited amino acid substitution correlated with symptoms. On the other hand, studies of the molecular variability of PDV (Vaskova et al., 2000) and PNRSV (Scott et al., 1998; Aparicio et al., 1999) did not report any relationship with symptoms, host species or geographic origin. The N-terminal part of the CP of *Alfamovirus* and *Ilarvirus* genera has been shown to be involved in the replication cycle of these viruses and an RNA binding consensus sequence was proposed by Ansel-McKiney et al., (1996). In this work, the N-terminal part of the CP was highly conserved, including the CVV specific consensus sequence (boxed region in Figure 3) and the crucial arginine at position 37.

Lovisolo (1993) proposed a common origin for CiLRV and CVV in the Mediterranean and North American areas, involving a rare event of transmission from wild plants to cultivated citrus. Additionally, Scott and Ge (1995) reasoned that if such event is rare it would be unique, with the citrus Ilarviruses (CVV and CiLRV) resulting from divergence from a common ancestor that moved to *Citrus*. However, our data do not support this hypothesis because the *CP* gene of all the CVV so far sequenced have a closer homology to the subgroup 2 Ilarviruses that are not described as infecting citrus (EMoV, EMoV (HdMV), EMoV (SpLV), AV-2), than with CiLRV. Additionally CiLRV has a noticeable divergence at the N-terminal part of the CP. Considering the CP data, the closest candidates for

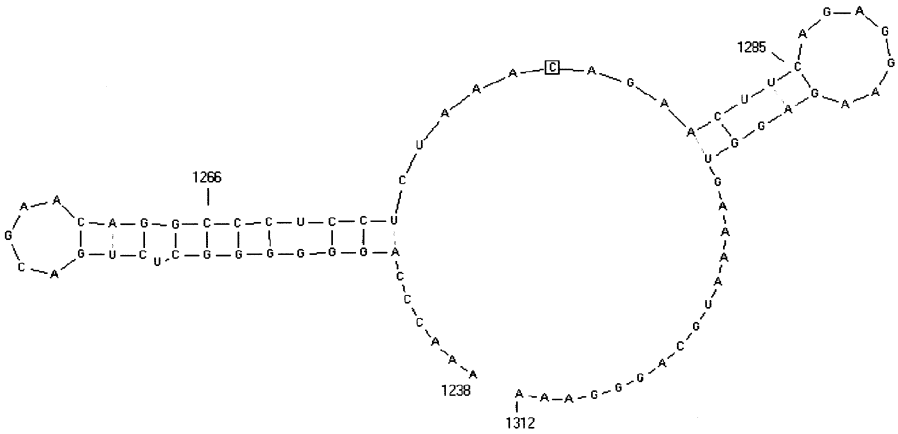

Figure 4. Predicted stem-loop structures adjacent to the putative transcriptional start of the RNA 4. The sequence is presented as the complement to the RNA 3 plus strand, being numbered from the 3′ to 5′ end. The hairpin on the left is the putative core promoter; the hairpin on the right corresponds to the conserved structure reported on this work. The boxed C is the transcriptional start of RNA 4. The CVVa primer extends from nt 1266 to 1285. The region extending from 1285 to 1312 is conserved in all the CVV sequences so far analysed. The same structure (here ranging from position 1281 to 1297) could be found in EMoV, EMoV (HdMV) and AV-2 with the G at position 1287 substituted for an A and in CiLRV in which the A–U bonding at the base of the stem was reversed and three additional substitutions were present in the bulge (G for A at 1286 and A for G at 1289 and 1290).

an ancestral relationship are EMoV (including EMoV (HdMV) as a strain) and AV-2. Both have a wide distribution with areas of superimposition to citrus cultivation (Buchen-Osmond, 2001). It is also interesting to note that these viruses are closer to the CL than to the IV cluster.

It has been shown that in the *Bromoviridae* a hairpin exists immediately upstream of the transcriptional start of the RNA 4 in the RNA 3 minus strand, which has a particular stability for those viruses that require genome activation by the CP as in the subgroup 2 Ilarviruses (Jaspars, 1998). In the sequences we studied, the upstream primer CVVa encompasses the 6 terminal nucleotides of the stem loop of the hairpin and the putative transcriptional start of the RNA 4, thus preventing the direct analysis of the variability of that region. However, the 26 nucleotides that follow the primer are totally conserved among the 13 sequences studied. When the RNA 3 minus strand sequence was analysed for its secondary structure, this region together with the 5 terminal bases of the CVVa primer appeared involved in another stem-loop structure (Figure 4) whose free-energy was 4.4 kcal/mol. The same structure was also present in EMoV, EMoV (HdMV), CiLRV and AV-2 with minor variations in the bulge part. Once again, CVV appeared closer to the other viruses than to CiLRV. In the case of EMoV (SpLV) a stem-loop structure is also present but with a shorter stem. This suggests the existence of a second stable hairpin downstream of the transcriptional start that is conserved in the subgroup 2 Ilarviruses.

There are no data concerning the variability of the region where the CVVb primer lies. On the opposite, primer CVV4 lies in a region that is conserved among the CVV isolates studied. These primers proved their efficacy in detecting isolates with diverse biological properties as indicated by our biological indexing assay, suggesting that they are able to react with a broad spectrum of CVV isolates. The prevalence of CVV in both citrus repositories tested was surprisingly high, considering that no conspicuous symptoms were visible and that the virus had not been previously reported in either country. The occurrence of mild strains and the fact that the virus is readily transmitted by mechanical means could account for that. Quoting Garnsey et al. (1984), 'Although CVV has not been a major citrus production problem, repeated discovery of the virus in unexpected and unrelated sites indicates that continued surveillance is necessary, especially in certification programs'.

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