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# Variability of a portion of RNA 3 containing the coat protein gene of *Citrus variegation virus* (CVV) using single-strand conformation polymorphism (SSCP)

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**Summary.** A portion of RNA 3 of *Citrus variegation virus* (CVV), comprising part of the intergenic region and the coat protein (CP) gene from eight viral isolates, was amplified by RT-PCR and cloned. The clones were compared for intra and inter-isolate variations by single-strand conformation polymorphism analysis. Some of the results were compared with sequence data previously obtained. The test discriminated between clones differing in as little as 3.2% of the nucleotides. Most isolates included several variants, in some cases with a predominant pattern, which, however, could no longer be recognised in new RT-PCR products obtained 13 months later. This procedure can therefore be used to identify and detect variations between CVV isolates. It is rapid, inexpensive and may reduce the amount of sequencing needed for comparing viral isolates.

**Key words:** Citrus viruses, infectious variegation-crinkly leaf, molecular variability, RT-PCR, SSCP.

## Introduction

Citrus variegation virus (CVV) is a widespread virus (Bennani *et al.*, 2002) that has not been much studied. It is a member of the ilarvirus group of the *Bromoviridae* and has a tripartite single-stranded RNA genome. Infected citrus trees exhibit a range of symptoms, which are usually mild on oranges and mandarins but may be severe on citron and lemon trees; on these yield reduction and fruit malformation may also occur. Two types of symptoms, attributed to different strains of the virus are commonly distinguished: infectious variegation, characterised by crinkling of the leaves associated with chlorosis, and crinkly leaf, which includes warping, pocketing and crinkling but without variegation or reduction in leaf size (Desjardin and Bové, 1980). Detection and characterisation of CVV in the field is by ELISA and more recently, at the molecular level, by RT-PCR and sequencing (Bennani *et al.*, 2002). In this study, the coat protein gene and part of the intergenic region were cloned and sequenced to compare several CVV isolates with different geographic origin and biological properties. Nucleotide sequencing is the most accurate procedure for strain identification, but it is too expensive and time consuming for routine

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purposes. Single-strand conformational polymorphism (SSCP) analysis (Orita *et al.*, 1989) appears an attractive alternative due to its suitability and has been used to assess the variability of several viruses. In SSCP analysis, double-stranded DNA is heat-denatured in the presence of formamide and both DNA strands are separated by electrophoresis in non-denaturing polyacrylamide gel. Under these conditions, part of the single-stranded DNA acquires a globular conformation that depends on the existence of self-complementary regions. The occurrence of mutations in these regions strongly affects the single-strand conformation, resulting in electrophoretic mobility shifts.

SSCP has been used for different purposes, such as: evaluating sequence heterogeneity among isolates of hepatitis B (Yap *et al.*, 1992) and C (Enomoto *et al.*, 1994) viruses; assigning beet necrotic yellow vein virus isolates to a known strain group (Koenig *et al.*, 1995); differentiating *CTV* isolates on the basis of their genetic variability (Rubio *et al.*, 1996; Ayllon *et al.*, 1999); studying the population structure of *CTV* (Kong *et al.*, 2000); and characterising different viroids (Palacio and Duran-Vila, 1999). Recently, fluorescent SSCP has also been used to detect variants of avocado sunblotch viroid (Schnell *et al.*, 2001).

In this paper SSCP was used to study intra and inter-isolate variability of CVV and results were compared with data recently obtained.

## Materials and methods

### Virus isolates

Virus isolates from different geographical areas were those used in previous studies (Bennani *et al.*, 2002) and were maintained in citrus seedlings grown in insect-proof greenhouses. They included: isolates CVV-1 (Garnsey *et al.*, 1968) and CVV-2 (Garnsey *et al.*, 1984), obtained from USDA, ARS, Orlando FL, USA; CVV-E1234, characterised by Grant (Desjardins and Bové, 1980), obtained from the Station de San Giuliano, Corsica, France; CVV-IV400 from IVIA, Spain (Duran-Vila *et al.*, 1988); CVV-81A65 from Corsica; CVV-CL903 from Spain (Desjardins and Bové, 1980); CVV-3 from a field near Marrakech, Marocco; and CVV-ES86 obtained from a citrus repository in Portugal (Bennani *et al.*, 2002). Virus-free citrus was included as a healthy control.

### cDNA synthesis, RNA amplification and cloning

Total nucleic acid of each isolate was extracted from 0.2 g of fresh bark tissue by a lithium-chloride-based protocol as described in an earlier study (Bennani *et al.*, 2002). CVVa and CVVb primers (Bennani *et al.*, 2002), designed from conserved regions of ilarvirus subgroup 2 (except Citrus leaf rugose virus (CiLRV)) were used to amplify the full-length coat protein (CP) gene and 63 nt of the intergenic region upstream of this gene. Amplification was carried out with 2 µl of RNA and RT-PCR mixes (25 µl final volume) containing 10 mM Tris (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 400 nM each of the primers, 0.4 mM of each dNTP, 0.08% NP-40, 0.5U Taq DNA polymerase (MBI-Fermentas, Vilnius, Lithuania), 5U RNAguard (Amersham Pharmacia, Buckingamshire, UK) and 6U MuLV reverse transcriptase (Perkin Elmer, Wellesley, MA, USA). The amplification conditions were: 30 min at 39°C, 2 min at 94°C, followed by 35 cycles at 92°C for 30s, 56°C for 45s and 72°C for 1 min and finally 5 min at 72°C. The amplified products were separated by 1% agarose gel electrophoresis. RT-PCR products of the expected size were cloned into the EcoRV-cut PGEMT-Easy vector system I (Promega, Madison, WI, USA) and transformed into *E. coli* INVα F' cells (Invitrogen, S. Giuliano Milanese, Italy). For all isolates, positive clones were identified by direct PCR amplification from the white colonies using primers CVVa/CVVb (Bennani *et* al., 2002). The plasmid DNA was purified using a Wizard plus SV miniprep Kit (Promega) according to manufacturer's instructions. The identity of the insert was confirmed by digestion with *EcoRI*.

### SSCP analysis

Fifty-one clones of 63 nt intergenic region upstream of the CP gene, and the entire CP gene obtained from eight isolates were compared by SSCP.

SSCP analysis was done on PCR products amplified from recombinant *E. coli* colonies or, in some experiments, directly from RT-PCR products. For SSCP analysis, 1 to 3 µl of the amplified products was mixed with 10 µl of denaturing buffer (19.6 mM EDTA, pH 8, in formamide containing a minimal amount of bromophenol blue), denatured for 5 min at 90°C, and loaded on a non-denaturing 8% polyacrylamyde gel. After running for 3 h at 200 V, 4°C, the gels were stained with silver nitrate (Orita *et al.*, 1989).

**Sequence comparison**

The sequences compared were those of clones CVV-1-U, CVV-2-L, CVV-3-O, CVV-E1234-11, CVV-E1234-13, CVV-IV400-12, CVV-IV400-13, CVV-81A65-12, CVV-81A65-27, CVV-CL903-15, CVV-CL903-21 and CVV-ES86, with accession numbers AF434918, AF434917, AF434916, AF434915, AF434914, AF434922, AF434921, AF434911, AF434912, AF434920, AF434919 and AF434913 respectively.

## Results

**SSCP analysis of the amplicon**

Amplification from the plasmids and from RNA isolates in all cases yielded a single fragment of 718 bp, encompassing the CP gene and 63 nt of the intergenic region upstream of this gene. Electrophoresis used to compare the RT-PCR products of different isolates and the 51 clones by SSCP, as described in Materials and methods, yielded very reproducible patterns when repeated analysis was done (data not shown). All SSCP results are illustrated in Fig. 1.

The SSCP patterns obtained directly from RT-PCR products amplified with primers CVVa–CVVb (Fig. 1-I) differed between most isolates, except those for ES86, E1234 and CVV3. Usually the patterns consisted of only two conspicuous bands, suggesting that the isolates were closely related variants. However, when SSCP analysis was carried out with the CVVa–CVVb PCR product amplified directly from transformed *E. coli* colonies, some isolates consisted of more than one variant. Isolates 81A65 (Fig. 1F), CVV3 (Fig. 1C), CVV1(Fig. 1A) and CVV2 (Fig. 1B) each produced a predominant pattern that was common to the majority of that isolate's clones. On the other hand, only about 50% of the clones from isolates IV400 (Fig. 1D) and CL903 (Fig. 1G) yielded similar patterns. The patterns of clones IV400-13, CVV1-U and ES86 were very similar among each other (Fig. 1H).

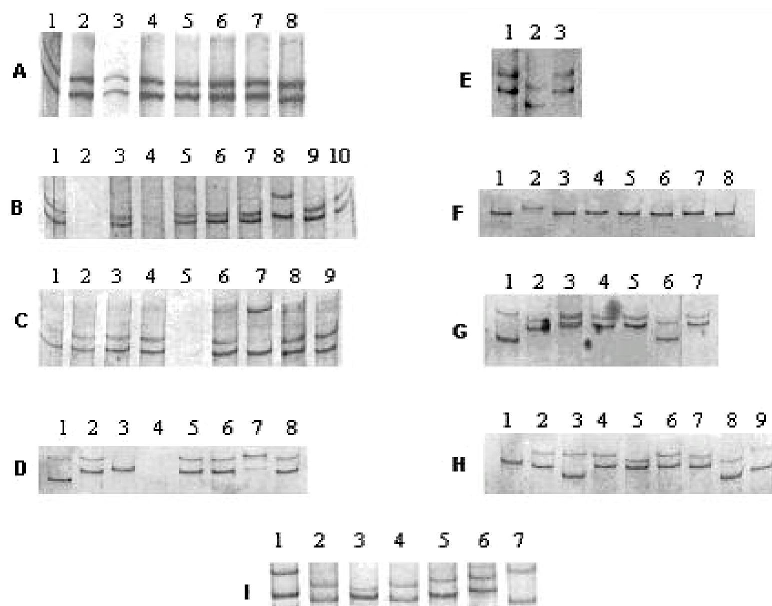


Fig. 1. SSCP analysis of PCR and RT-PCR products obtained with primers CVVa and CVVb. Panels A to G, direct PCR on isolates CVV-1, CVV-2, CVV-3, CVV-IV400, CVV-E1234, CVV-81A65 and CVV-CL903 respectively, carried out with *E.coli* colonies harbouring the RT-PCR product. Sequenced clones are presented in lanes A6 (CVV-1-U), B7 (CVV-2-L), C4 (CVV-3-O), D1 (IV400-12), D2 (IV400-13), E1 (E1234-11), E2 (E1234-13), F1 (81A65-12), F8 (81A65-27), G5 (CL903-15) and G6 (CL903-21). Panel H, side-by-side comparison of SSCP patterns from some sequenced clones: CVV-81A65-12, CVV-IV400-13, CVV-IV400-12, CVV-ES86, CVV-CL903-15, CVV1-U, CVV2-L, CVV-E1234-13 and CVV-E1234-11 (lanes 1 to 9 respectively). Panel I, SSCP patterns of RT-PCR products obtained from plants infected with isolates CVV-IV400, CVV-E1234, CVV-81A65, CVV-ES86, CVV-2, CVV-1, and CVV-CL903 (lanes 1 to 7 respectively).

Table 1. Homology percentage and number of nucleotide differences (in bold type) among 12 clones in the region considered. U17389 was obtained from GenBank.

| Seq | U17389 | IV400-12 | IV400-13 | CVV1-U | CVV2-L | CVV3-O | E1234-11 | E1234-13 | CL903-15 | CL903-21 | ES86 | 81A65-27 | 81A65-12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| U17389 | 100 | 98.3 | 98.3 | 99.7 | 97.4 | 98.1 | 97.9 | 97.7 | 95.6 | 97.9 | 97.2 | 94.7 | 94.5 |
| IV400-12 | **11** | 100 | 98.3 | 98 | 97.4 | 98.1 | 99.5 | 97.7 | 95.1 | 97.9 | 98.6 | 94.1 | 94.0 |
| IV400-13 | **12** | **12** | 100 | 98 | 99.1 | 99.5 | 97.9 | 99.1 | 95.4 | 99.3 | 97.4 | 94.4 | 94.2 |
| CVV1-U | **2** | **14** | **14** | 100 | 97.2 | 97.9 | 97.6 | 97.4 | 95.4 | 97.6 | 96.9 | 94.4 | 94.2 |
| CVV2-L | **18** | **17** | **7** | **19** | 100 | 98.7 | 97 | 98.6 | 95.1 | 99 | 96.6 | 94 | 93.8 |
| CVV3-O | **13** | **12** | **3** | **15** | **10** | 100 | 97.7 | 99 | 95.2 | 99.1 | 97 | 94.2 | 94.1 |
| E1234-11 | **14** | **3** | **15** | **16** | **21** | **16** | 100 | 97.3 | 94.7 | 97.4 | 98.7 | 94 | 93.8 |
| E1234-13 | **16** | **16** | **4** | **18** | **10** | **7** | **19** | 100 | 95.9 | 99 | 96.6 | 94.9 | 94.8 |
| CL903-15 | **31** | **33** | **31** | **33** | **35** | **32** | **38** | **29** | 100 | 95.2 | 94.2 | 98 | 97.9 |
| CL903-21 | **15** | **15** | **5** | **17** | **7** | **6** | **18** | **7** | **33** | 100 | 96.7 | 94.2 | 94.1 |
| ES86 | **22** | **10** | **18** | **22** | **24** | **21** | **9** | **24** | **41** | **23** | 100 | 93.5 | 93.4 |
| 81A65-27 | **38** | **42** | **32** | **40** | **43** | **40** | **43** | **36** | **14** | **41** | **46** | 100 | 99.5 |
| 81A65-12 | **39** | **43** | **41** | **42** | **44** | **42** | **43** | **36** | **14** | **41** | **47** | **3** | 100 |

**Nucleotide differences between clones**

Nucleotide sequence homology of clones previously sequenced (Bennani *et al.*, 2002) and obtained with the program Clustal (Thompson *et al.,* 1994) is summarised in Table 1. Differences in the nucleotide sequence were not always correlated with differences in electrophoretic mobility. When sequences of the very similar clones IV400-13, CVV1-U and ES86 were compared, they differed from each other by 14 to 22 bp. All clones differing by more than 23 bp yielded different patterns. Conversely, in the case of clones that differed only for 3 nt different patterns were produced by IV400-12 and E1234-11, and by CVV3 and IV400-13, and identical patterns by 81A65-12 and 81A65-27. In summary, any two amplicons producing different patterns differed by at least 3 bp (0.4%), while amplicons with similar patterns always differed by less than 23 bp (3.2%).

## Discussion

SSCP analysis of several clones of each CVV isolate showed that some of the isolates (e.g. CVV1, CVV2, CVV3, 81A65) consisted of one predominant variant. In these cases the pattern of the predominant variant was recognised in the SSCP pattern of the RT-PCR products from which the clones were obtained. To have an idea of the accuracy of this indication, sequences of two clones from 81A65 having the same pattern were compared and a total of 3 nucleotide differences were found. This suggests that SSCP gives a fair approximation of intra-isolate variability. On the other hand, patterns from clones obtained from isolates CL903, E1234 and IV400 produced different patterns, suggesting that each of these isolates consisted of several variants, typical of a viral quasi-species. However, in these cases there was no straightforward correspondence between the SSCP patterns of the clones and the sequence of the RT-PCR products which yielded them. It was expected that the SSCP pattern of the RT-PCR product would be similar to the most frequent pattern of the clones or would be the same as the individual patterns, but this was not the case. SSCP analysis also did not reveal any particular feature of the RT-PCR patterns that could be associated with either infectious variegation or crinkly leaf symptoms. Although conditions were far from those usually considered ideal for SSCP, in that short PCR products (Sheffield *et al.*, 1993) were used, it was possible to discriminate among clones differing by at least 3.2% of their nucleotide sequence. Other Authors (Rubio *et al.*, 1996) using products of approximately the same length, were able to distinguish clones differing by 1.4%. Field isolates of CVV consist of a large number of CP gene variants. Under the conditions used, SSCP of RT-PCR products appeared not to be adequate to reveal the sequence variants that compose a particular isolate. The SSCP of individual clones should be used for that purpose.

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## Literature cited

Ayllon M.A., L. Rubio, A. Moya, J. Guerri and P. Moreno, 1999. The haplotype distribution of two genes of citrus tristeza virus is altered after host change or aphid transmission. *Virology* 255(1), 32–39.

Bennani B., C. Mendes, M. Zemzami, H. Azeddoug and G. Nolasco, 2002. *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. *European Journal of Plant Pathology* 108(2), 155–162**.**

Desjardins P.R. and J.M. Bové, 1980. Infectious variegation and crinkly leaf. In*: Description and Illustration of Virus and Virus-like Diseases of Citrus*. A collection of colour slides. Proceedings of the International Organisation of Citrus Virologists (J.M. Bové, R. Vogel, ed.), Vol. 2, IRFA, 1–9.

Duran-Vila N., M. Cambra, J.A. Pina, J.F. Ballester and L. Navarro, 1988. Virus content and growth patterns of callus cultured *in vitro* from healthy and virus infected Citrus species. *Proceedings of the International Organisation of Citrus Virologists* 9, 310–313.

Enomoto N., M. Kurosaki, Y. Tanaka, F. Marumo and C. Sato, 1994. Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. *Journal of General Virology* 75, 1361–1369.

Garnsey S.M., 1968. Additional non-citrus hosts for the Florida isolate for citrus variegation virus. *Phytopathology* 58, 1433–1434.

Garnsey S.M., N. Baksh, M. Davio and J.P. Agostini, 1984. A mild isolate of Citrus Variegation Virus found in Florida Citrus. *Proceedings of the International Organisation of Citrus Virologists* 9, 188–195.

Hall T.C., 1979. Transfer RNA-like structures in viral genomes. *International Review of Cytology* 60, 1–26.

Koenig R., P. Lüddecke and A.M. Haeberle, 1995. Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *Journal of General Virology* 76, 2051–2055.

Kong P., L. Rubio, M. Polek and B.W. Falk, 2000. Population structure and genetic diversity within California Citrus tristeza virus (CTV) isolates. *Virus Genes* 21(3), 139–145.

Orita M., H. Iwahana, K. Kanazawa and T. Sekia, 1989. Detection of polymorphism of human DNA by gel electrophoresis as single strand conformation polymorphism, *Proceedings of the National Academy of Sciences of the USA* 86, 2766–2770.

Palacio A. and N. Duran-Vila, 1999. Single-strand conformation polymorphism (SSCP) analysis as a tool for viroid characterization. *Journal of Virological Methods* 77(1), 27–36.

Rubio L., M.A. Ayllon, J. Guerri, H. Pappu, C. Niblett and P. Moreno, 1996. Differentiation of citrus tristeza closterovirus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *Annals of Applied Biology* 129, 479–489.

Schnell R.J., C.T. Olano and D.N. Kuhn, 2001. Detection of avocado sunblotch viroid variants using fluorescent single-strand conformation polymorphism analysis. *Electrophoresis* 22(3), 427–432.

Sheffield V.C., J.S. Beck, A.E. Kwitek, D.W Sandstrom and E.M. Stone, 1993. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 16, 325–332.

Thompson J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22(22), 4673–4680.

Yap E.P.H., Y-M.D. Lo, K. Cooper, K.A. Fleming and J.O.D. McGee, 1992. Exclusion of false-positive PCR viral diagnosis by single-strand conformation polymorphism. *The Lancet* 340, 736.

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