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Genetic variability of the coat protein gene of isolates of *Citrus variegation virus* from Campania (southern Italy)

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Summary. Six new sequences of the coat protein (CP) gene of *Citrus variegation virus* (CVV) isolates, some producing crinkly leaf symptoms and some asymptomatic, and collected in Campania (southern Italy), are presented. Comparison with previously reported CP gene sequences of isolates from various locations worldwide confirmed a high degree of coat protein gene conservation in Campania (lowest similarity among all CVV sequences 92.4%). No relationship was found between amino-acid substitutions and host species or symptoms. Phylogenetic analysis proved that CP sequences from Campania isolates clustered in a new group when compared to those reported in the data bank.

Key words: CVV, *Ilarvirus*, molecular variability, RNA 3, phylogenetic analysis.

Introduction

Citrus variegation virus (CVV), a member of subgroup 2 of *Ilarviruses* in the family Bromoviridae, is the causal agent of infectious variegation, a disease occurring all over the world and affecting a wide range of citrus species and cultivars. Two strains of the virus have been described on the basis of the symptoms they induce on citrus trees in the field: infectious-variegation strain, and crinkly leaf strain. The infectious-variegation strain induces more or less severe chlorotic mottle on the leaves, with or without crinkle. The crinkly leaf strain causes distorted, puffed or puckered leaf segments but without variegation. Affected leaves may increase in size. No specific CVV vector has been identified, and the only known way of infection or spread is by graft-inoculation, graft-propagation or mechanical transmission. The CVV genome consists of three plus-sense RNA molecules of 3–4 kb (RNA 1), 2.9 kb (RNA 2) and 2.2 kb (RNA 3). RNA 1 and RNA 2 are monocistronic and encode non-structural proteins involved in replication. RNA 3 is bicistronic with ORF 1 at the 5'proximal end, coding for a putative movement protein, and ORF 2 at the 3'distal end, coding for the viral coat protein (CP). The CP is translated from a subgenomic mRNA (RNA 4) of 0.9 kb, which is also encapsidated (Symons, 1985).

An important contribution to understanding the molecular variability of CVV was made by Bennani *et al*. (2002), who analyzed the capsid protein sequence of isolates from different geographic origins and displaying symptoms of infectious variegation or crinkly leaf. They found a relationship between the CP gene and symptom expression, but no correlation between amino-acid substitutions and host species or geographic origin.

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Results of the molecular characterization of 6 CVV isolates collected from different citrus species and cultivars growing in areas of Campania (southern Italy) are presented in this study.

Materials and methods

Infected plant material

The 6 CVV sources used in the study were collected in a survey during the citrus-growing period (March–June) from naturally infected leaves of citrus trees testing positive for the virus by the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using CVV polyclonal antiserum provided by S. Garnsey and M. Hilf (USDA-ARS-USHRL, Fort Pierce, FL, USA), and by RT-PCR with the specific primers CVVa and CVV4 (Bennani *et al*., 2002). The survey was carried out in 2004 in the major fruit growing areas of Campania (Damiano *et al*., 2005). The trees examined included three lemon (*Citrus limon* [L.]) trees, 'Ovale di Sorrento' and 'Zagara', two sweet orange trees (*C. sinensis* [L.] Osb) 'Valencia' and 'Moro', and one sour orange tree (*C. aurantium* [L]). The lemon trees 'Ovale di Sorrento' showed severe warping, pocketing and crinkling on the leaves, and protuberances and bumps on the fruits (Figure 1). The remaining trees were symptomless.

PCR amplification and sequencing of the CP gene

Total RNA was extracted from 100 mg of healthy and CVV-infected citrus leaf tissues using Trizol LS reagent (Invitrogen Corp., Carlsbad, CA, USA), as described by Vives *et al*. (2002).

To amplify the entire CP gene of all six isolates by RT-PCR, the primer CVV-UTR (5'-GGGATGACACTTT-GACTCGG-3'), complementary to nt 2001 to 2020 of the CVV reference sequence (Scott and Ge, 1995), was designed and used for the reverse transcription (RT) step. The same primer was also used together with primer CVVa (Bennani *et al*., 2002) for RT-PCR amplification of a 755 nt fragment between positions 1266 and 2020 of RNA 3 of CVV (Scott and Ge, 1995). This fragment comprised 63 nt of the intergenic region upstream of the CP gene, the entire CP gene, and 38 nt of 3'UTR.

For cDNA synthesis, 2 µL of total RNA extract and the 0.5 µM primers were denatured for 5 min at 95°C and chilled on ice. RT was performed in a total volume of 20 µL containing 0.5 mM of each of the four dNTPs, 300 U Superscript™ II reverse transcriptase (Invitrogen), 40 U Rnase OUT™ recombinant ribonuclease inhibitor (Invitrogen), 5 mM DTT and 4 µL first strand buffer (250 mM Tris-HCl pH 8.3; 375 mM KCl; 15 mM $MgCl_2$). RT was carried out in an Eppendorf Mastercycler Gradient. Cycling parameters were: 42°C for 1 h, 94°C for 5 min. The PCR was performed in a total volume of 50 µL containing 2 µL RT product, 2 mM $MgCl_2$, 0.2 mM of each of the dNTPs, 0.2 µM CVVa, 0.2 µM CVV-UTR, 5 U Taq polymerase recombinant (Invitrogen) and 5 µL PCR buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl). PCR conditions were: pre-incubation 94°C for 2 min, 35 cycles at 94°C for 40 s, 58°C for 40 s, 72°C for 1 min, and a final elongation step at 72°C for 10 min.

PCR products of the six isolates were cloned into plasmid pGEM-T Easy Vector System I (Promega Corp., Madison, WI, USA), following manufacturer's instructions, and five distinct cDNA clones per PCR product were used for sequencing.

PCR products obtained using the primers CVVa and CVV4 were directly sequenced.

Sequencing was performed by the BMR Sequencing Service (Padova, Italy). All mutations were individually confirmed using the original electrophoregrams.

Sequence analysis

The sequences were compared with the sequences in the databanks using BLAST programs (Altshul *et al*., 1997) on the NBCI server (www.ncbi.nlm.nih.gov). Multiple alignment of the coat protein gene was performed with CLUSTAL W (Thompson, 1994).

Mean diversities, genetic distances (p-distances calculated on amino-acid or nucleotide identity), and Nei-Gojobory synonymous/non-synonymous substitution rates (Nei and Gojobory, 1986) were calculated using MEGA 3.1 (Kumar *et al*., 2004). Phylogenetic trees of the deduced amino-acid sequences were constructed by the neighbour-joining method (Saitou and Nei, 1987) and node values were estimated by bootstrap analysis using 1000 replicates.

Results and discussion

PCR amplification with the specific primers (CVVa and CVV-UTR) yielded a single DNA fragment of the expected size (755 bp) in all isolates analysed (Figure 2). Nucleotide sequence alignments of the 5 clones per CVV isolate showed very low diversity values ranging from 0 to 0.3%. In view of these results, the major sequence variant of each isolate was used in subsequent analyses (GenBank accession numbers: EU650673-EU650678). A comparison of these sequences with those obtained using primers CVVa/CVV4 showed that the sequences of the overlapping segments were substantially identical for each isolate.

The alignment of the CP sequences of the Campania

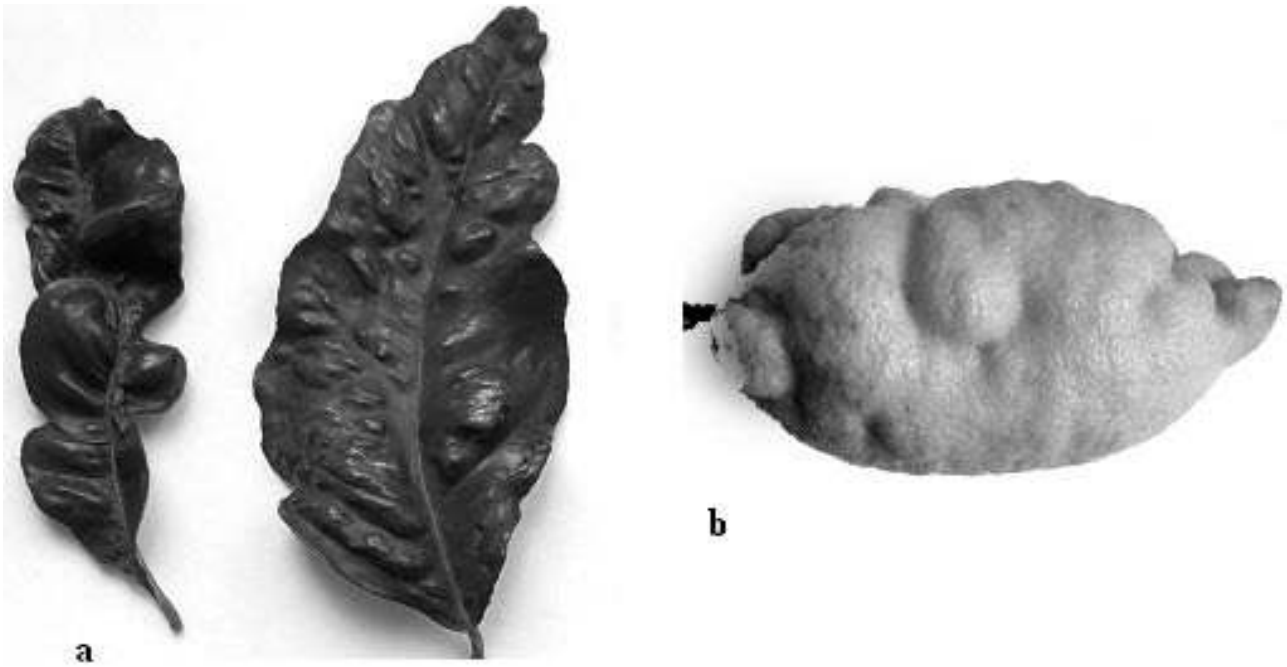

Figure 1. Severe symptoms of crinkling on the leaves, and bumps on the fruits of lemon 'Ovale di Sorrento'.

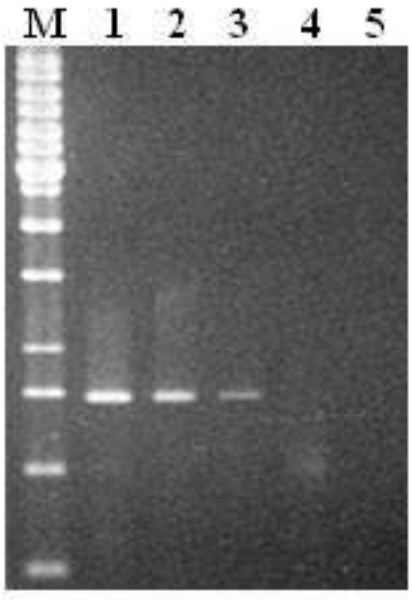

Figure 2. Agarose gel electrophoresis of RT-PCR of the coat protein from 2 CVV isolates. Lane 1, positive sample; lane 2, Campania Zagara; lane 3, Campania Ovale; lane 4, healthy sample; lane 5, no DNA; M, 1kb ladder DNA marker.

isolates together with those available at GenBank showed a nucleotide and amino-acid average pairwise divergence between isolates of 3.7±0.4% and 3.5±0.6% respectively, confirming the low genetic variability of CVV isolates, as already reported by Bennani *et al*. (2002).

Phylogenetic analysis of the nucleotide sequences showed that CVV isolates clustered in three groups (Figure 3), two of which, already described by Bennani *et al*. (2002), including isolates of different geographic origin with symptoms of infectious variegation (group A) and crinkly leaf (group B). The isolates from Campania clustered in a new divergent group (group C) (Figure 3).

The short stretch of the intergenic region and the 3' UTR analysed appeared to be very conserved in all isolates from Campania and was similar to those reported by Scott and Ge (1995) (data not shown).

The alignment of the amino-acid sequences showed 36 amino-acid (aa) positions, 19 of them parsimony-informative, which varied within the 218 aa long protein of the CP gene. Most of the differences were due to synonymous substitutions in the C-terminal half of the protein. The N-terminal part of the CP, and in particular, the amino-acid residues surrounding an arginine, crucial for RNA-binding activity in all members of the family Bromoviridae (Yusibov and

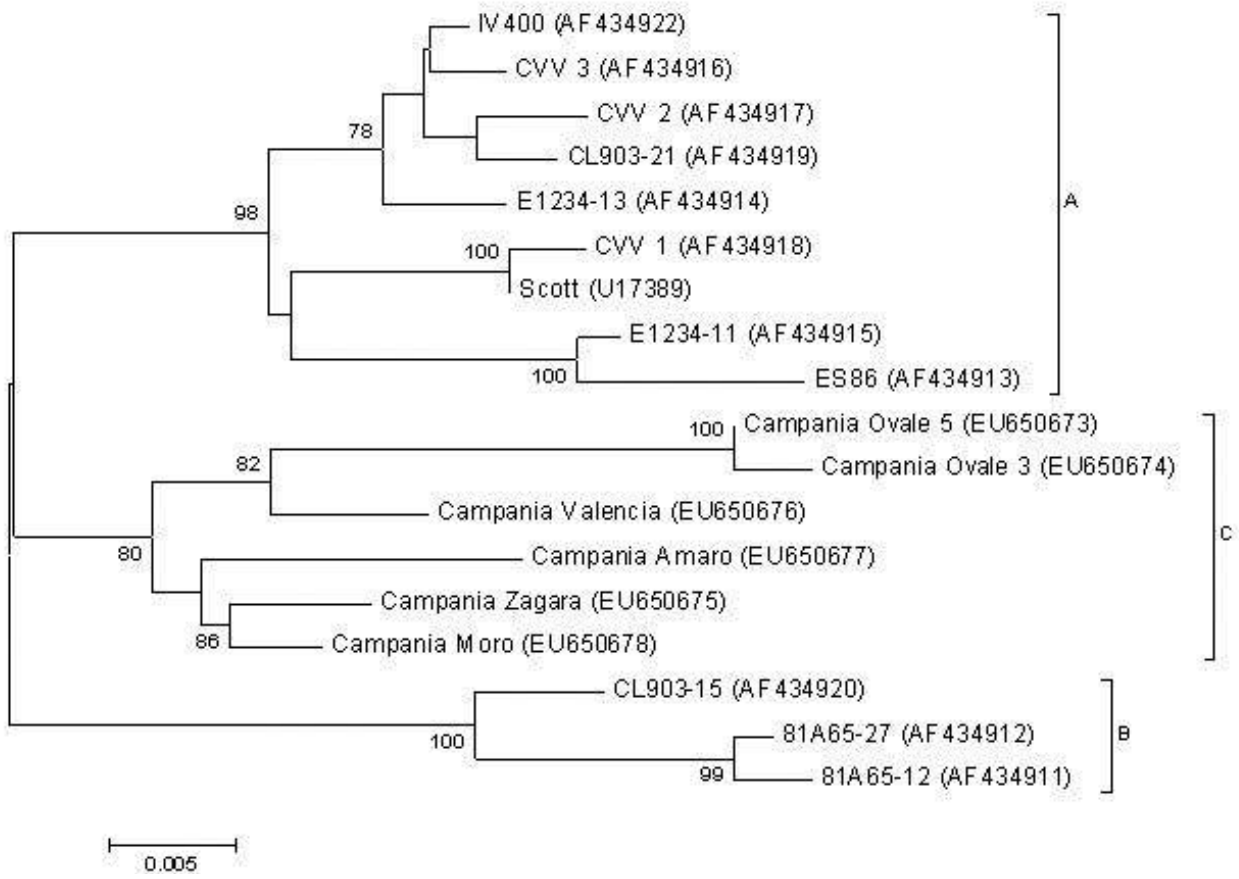

Figure 3. Neighbour-joining tree of CP nucleotide sequences of CVV isolates. Numbers near nodes indicate bootstrap values. GenBank accession numbers are in brackets.

Loesch-Fries, 1998; Swanson *et al*., 1998), turned out to be very well conserved. In CVV, the crucial arginine is located in the QPTA**R**SRQWA at position 37 of the amino-acid sequence (Scott and Ge, 1995). Our results confirmed, as already reported earlier (Bennani *et al*., 2002), the very low variability of this region since, of all the clones and isolates analysed, we found just one clone (Zagara isolate) containing a G_{37} instead of the prevailing R_{37} (data not shown).

Analyses of the amino-acid sequences of the Campania isolates did not show any correlation between the amino-acid substitutions and symptom expression. In a previous report, Bennani *et al*. (2002) hypothesised that limited changes at the amino-acid level in the CP correlated well with symptom types (pathotypes) and identified at positions 61 and 137, two amino-acid substitutions, of which M_{61} and V_{137} were associated with variegation symptoms, and V_{61} and A_{137}, with crinkly leaf symptoms.

In these two positions, the Campania isolates exhibited not only the amino-acids associated with variegation symptoms, but also those inducing crinkly leaf symptoms or no symptoms at all. These findings indicated that there is a no strict correlation between the CP gene and symptom determination, as previously reported for other Ilarviruses (Aparicio *et al*., 1999; Vaskova *et al*., 2000). At the amino-acid level, isolates from Campania were distinguished from the other isolates by four parsimonious sites, two of them represented by changes that potentially alter amino-acid polarity: H_{128} instead of $Y_{128,}$ and A_{184} instead of T_{184}.

It is concluded that, as evidenced by phylogenetic analysis, all Campania isolates so far identified belong to a new population of isolates. This could be explained by the introduction into Campania, in the past, of an initial CVV isolate in infected propagation material, and by the subsequent geographical isolation of citrus within the region.

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Literature cited

Altschul S.F., T.L. Madden, A.A Schäffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Aparicio F., A. Myrta, B. Di Terlizzi and V. Pallas, 1999. Molecular variability among isolates of prunus necrotic ringspot virus from different *Prunus* spp. *Phytopathology* 89, 991–999.

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, 155–162.

Chare E.R. and E.C. Holmes, 2004. Selection pressure in the capsid genes of plant RNA viruses reflect mode of transmission. *Journal of General Virology* 85, 3149–3157.

Damiano A., M. Malfitano, N. Duran-Vila and D. Alioto, 2005. Occurence of *Citrus variegation virus* (CVV) in Campania (Southern Italy). *Journal of Plant Pathology* 87, (4, Special issue), 292.

Davino M. and S.M. Garnsey, 1984. Purification, characterization and serology of a mild strain of citrus infectious variegation virus from Florida. In: *Proceedings of the International Organisation of Citrus Virologists* 9, 196–203.

Garcıa-Arenal F., A. Fraile and J.M. Malpica, 2001. Variability and genetic structure of plant virus populations. *Annual Review of Phytopathology* 39, 157–186.

Kumar S., K. Tamura and M. Nei, 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150–163.

Nei M. and T. Gojobori, 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Molecular Biology and Evolution* 3, 418–423.

Roistacher C.N., 1991. Graft-transmissible diseases of citrus. *Handbook for Detection and Diagnosis*. FAO, Rome, Italy, 286 pp.

Roistacher C.N., 1993. Psorosis-a review. In: *Proceedings of the 12th Conference of the International Organization of Citrus Virologists* (P. Moreno, J.V. da Graça, L.W. Timmer, ed.), IOCV, Riverside, CA, USA, 139–154.

Saitou N. and M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.

Scott S.W. and X. Ge, 1995. The complete nucleotide sequence of RNA3 of citrus leaf rugose and citrus variegation Ilarviruses. *Journal of General Virology* 76, 957–963.

Swanson M.M., P. Ansel-McKinney, F. Houser-Scott, V. Yusibov, L.S. Loesch-Fries and L. Gehrke, 1998. Viral coat protein peptides with limited sequence homology bind similar domains of alfalfa mosaic virus and tobacco streak virus RNAs. *Journal of Virology* 72, 3227–3234.

Symons R.H., 1985. Viral genome structure. In: *The Plant Viruses* Vol. 1, *Polyhedral Virions with Tripartite Genomes* (R.I.B. Franchi ed.), Plenum Press, NY, USA and London, UK, 57–81.

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, 4673–4680.

Vives M.C., L. Rubio, L. Galipienso, L. Navarro, P. Moreno and P. Guerri, 2002. Low genetic variation between isolates of *Citrus leaf blotch virus* from different host species and of different geographical origins. *Journal of General Virology* 83, 2587–2591.

Vaskova D., K. Petrzic and J. Spak, 2000. Molecular variability of the capsid protein of the prune dwarf virus. *European Journal of Plant Pathology* 106, 573–580.

Yusibov V. and L.S. Loesch-Fries, 1998. Functional significance of three basic N-terminal amino-acids of alfalfa mosaic virus coat protein. *Virology* 208, 405–407.

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