Investigation on Occurrence of *Tricho-, Fovea-* and *Capilloviruses* in Ancient Fruit Tree Cultivars in Campania

M. Barone, D. Alioto and A. Ragozzino
Facoltà di Agraria
Università di Napoli "Federico II"
Dip. AR.BO.PA.VE
Via Università 100, 80055 Portici (NA)
Italy

T. Candresse
Virology group, UMR GD2P
IBVM, INRA
BP81
33883 Villenave d'Ornon Cedex
France

Keywords: ACLSV, APCLSV, ALV, CGRMV, ASPV, ASGV, CVA

Abstract

During a survey of ancient local fruit tree cultivars, the occurrence of *Apricot pseudo chlorotic leaf spot virus* (APCLSV), a recently described *Trichovirus* closely related to *Apple chlorotic leaf spot virus* (ACLSV) (Liberti et al., 2005a), was investigated. Samples were collected from 361 symptomatic and asymptomatic trees and analyzed by nested RT-PCR. APCLSV was detected in 7 of 112 apricot, 1 of 29 peach and 2 of 26 plum trees. All apple, pear and cherry trees tested negative for APCLSV. Compared with those in databanks, the sequences of APCLSV PCR products showed an average pairwise divergence of 11.6% (nt) and 4.5% (aa). The majority of isolates clustered with those characterized by Liberti et al. (2005a), but three additional divergent clusters could be identified. These results significantly extend our knowledge of the variability of APCLSV and suggest that contrary to ACLSV, APCLSV could be restricted to stone fruit hosts. Forty-eight samples were also screened for the presence of viruses belonging to the *Trichovirus, Foveavirus* and *Capillovirus* genera by PDO Nested RT-PCR (Foissac et al., 2005). The results revealed a very high prevalence of ACLSV in all species tested. Additional viruses detected include *Cherry green ring mottle virus* (CGRMV, 2 of 4 cherry trees), *Apricot latent virus* (ALV, 1 of 14 apricot trees), *Cherry virus A* (CVA, 6 of 14 apricot, 1 of 6 plum and 1 of 4 cherry trees), *Apple stem pitting virus* (ASPV, 4 of 9 apple and 1 of 8 pear trees) and *Apple stem grooving virus* (ASGV, 1 of 9 apple and 1 of 8 pear trees). This is the first record for APCLSV on peach in Campania and of CVA in Italy. These results provide information on the prevalence of *Fovea-*, *Tricho-* and *Capilloviruses* in old local cultivars and extend our knowledge on the diversity of these agents.

INTRODUCTION

The *Trichovirus, Foveavirus* and *Capillovirus* genera belong to the family *Flexiviridae* and are characterized by flexuous, elongated viral particles and phylogenetically related, monopartite, plus sense, single stranded RNA genomes (Adams et al., 2004). Most viruses belonging to these genera infect temperate fruit trees of the family Prunoidae (peach, apricot, plum, cherry, and Japanese plum and apricot) and Maloidae (apple, pear, quince and Japanese pear). At the present, 4 different viruses are recognized by the ICTV as being members of the genus *Trichovirus*, 3 of the genus *Foveavirus* and 3 of the genus *Capillovirus*; however, new viruses belonging to these genera are frequently discovered on fruit trees affected by diseases of unclear etiology. Recently, during a survey of *Prunus* material from Campania (Southern Italy), a novel *Trichovirus* named *Apricot pseudo chlorotic leaf spot virus* (APCLSV) was discovered in mixed infection with ACLSV on Japanese plum with severe stem pitting/grooving symptoms and on a local variety of apricot with ring-pox symptoms (Liberti et al., 2005a). Further studies demonstrated the presence of APCLSV, always in association with ACLSV, in *Prunus* samples (apricot and Japanese plum) from Spain, France and Australia (Liberti et al., 2005a). The spread and presence of APCLSV in Pomoidae and in other Prunoidae has not been studied till now.

The aim of the present study is to try to define the natural host range of APCLSV by analyzing a wide range of old Italian local cultivars belonging to varietal collections of Prunoidae and Maloidae in Campania (Southern Italy). We also hoped through this study to verify the existence of a constant association between APCLSV and ACLSV and to extend our knowledge on the variability of APCLSV. The same germplasm collections were analyzed in parallel for the presence of others *Tricho*-, *Fovea*- and *Capilloviruses*.

MATERIALS AND METHODS

Surveys and Sample Collection

Field inspections and sample collection were carried out in germplasm collections of the Campania region during the spring-summer periods of 2004 and 2005. Samples (petals, leaves or fruits) were collected from 238 stone fruit trees (112 apricots, 26 plums, 29 peaches, 67 cherries and 4 plumcots) and 123 pome fruit trees (93 apples and 30 pears) of different cultivars.

Total Nucleic Acids Extractions

Total RNAs were extracted using a silica-capture method previously described by Foissac et al. (2001).

Detection of APCLSV by Nested RT-PCR

The amplification of short region of the polymerase gene was carried out using the primers NT1 and NT3 and the procedure described by Liberti et al. (2005a). The PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide, purified with a commercial kit according to the instruction of the manufacturer (NucleoSpin Extract II kit, Macherey-Nagel) and directly sequenced without any prior cloning step.

ACLSV Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

The APCLSV-infected samples were also analysed by ELISA to verify the presence of ACLSV, which had previously been reported to occur in mixed infection with APCLSV (Liberti et al., 2005a). Double antibody sandwich (DAS) ELISA tests were performed using immunoglobulins from a polyclonal antiserum raised against the P863 ACLSV isolate (INRA Bordeaux) or using a commercial kit (Loewe Biochemica, GmbH Sauerlach, Germany). Triple antibody sandwich (TAS) ELISA was carried out using the 29.13 and 8.20 monoclonal antibodies (Mabs), developed by INRA (Bordeaux, France) against the P863 ACLSV isolate. All ELISA procedures were according to Alioto et al. (1999). The optical densities (ODs) were measured at 405 nm with a Biorad 3550 Microplate Reader (Richmond, CA, USA) at intervals from 20 min up to 2 h after substrate addition. Values three times above the average obtained for healthy samples were assumed to indicate infected samples. Each test was repeated at least twice. Further tests to confirm the presence/absence of ACLSV were performed using the ACLSV-specific A52-A53 primer pair and the RT-PCR amplification scheme of Candresse et al. (1995).

Detection of *Tricho*-, *Fovea*- and *Capilloviruses* by PDO Nested RT-PCR

Total RNAs extracted from 48 samples (14 apricot, 6 plum, 4 plumcot, 4 cherry, 3 peach, 9 apple and 8 pear trees) were submitted to the PDO Nested RT-PCR as described by Foissac et al. (2005) and PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. All PDO products were purified using a NucleoSpin Extract II kit (Macherey-Nagel) and cloned in the pGEM-T Easy vector (Promega). Five clones for each isolate were further sequenced (B.M.R., Padova, Italy), and analysed.

Sequence Analysis

The sequences were compared to databanks using BLAST programs (Altshul et al., 1997) on the NBCI server (www.ncbi.nlm.nih.gov). Multiple sequence alignments and phylogenetic analysis were performed using MEGA 3.1 (Kumar et al., 2004).

RESULTS

Detection of APCLSV and Analysis of Its Sequence Variability

Analysis by Nested RT-PCR of total nucleic acid extracts of 238 stone and 123 pome fruit trees revealed the presence of APCLSV in 7 of 112 apricots (6.2%), 2 of 26 plums (7.7%) and 1 of 29 peaches (3.4%). All apple, pear and cherry trees tested negative. Symptoms observed on APCLSV infected apricot and plum trees mainly consisted of stem pitting, stem grooving and graft-incompatibility problems. Ring-pox symptoms on fruits were detected only in one infected apricot tree. No symptoms were observed on the peach tree which tested positive. Sequence comparisons between the PCR fragments and with databanks showed an average pairwise divergence between isolates of 11.6% (nucleotide) and 4.5% (amino acid), respectively. Phylogenetic analysis of the nucleotide sequences showed that the majority of APCLSV isolates cluster in a major group (Fig. 1A), containing 6 isolates from this study (5 from apricot and 1 from plum) and all previously characterized isolates from Italy, France, Spain and Australia with the exception of the Susino2 (Sus2) isolate (Liberti et al., 2005). In addition, this analysis provided evidence for the existence of three additional divergent groups consisting of the only peach isolate detected (Fig. 1B), of two plum isolates including the previously characterized Sus2 one (Fig. 1C) and of two "new" apricot isolates (Fig. 1D).

Analysis of the Possible Presence of ACLSV in Co-Infection with APCLSV

Results obtained by DAS and TAS ELISA revealed the presence of ACLSV in 8 of the 10 APCLSV samples tested (5 apricots, 2 plums and 1 peach). On the other hand, two apricot samples yielded negative ELISA results, using the ACLSV-specific monoclonal antibodies. Further evidence for the absence of ACLSV in these samples was obtained through RT-PCR analysis using the ACLSV-specific A52-A53 primer pair (Candresse et al., 1995).

Analysis of the Presence of Other Members of the *Trichovirus*, *Foveavirus* and *Capillovirus* Infections in Old Italian Cultivars Using PDO Nested RT-PCR Polyvalent Amplification

A subset of 48 stone and pome fruit sources was selected for analysis using the polyvalent PDO nested RT-PCR amplification procedure (Foissac et al., 2005). Product(s) of the expected size (362 bp) was (were) obtained from each of the samples used. Following gel purification, the PCR products were cloned and 5 individual cDNA clones were sequenced for each plant. Sequence comparisons revealed the presence of ACLSV in all species tested and in a total of 44 of the 48 tested samples. Additional viruses detected included *Cherry green ring mottle virus* (CGRMV, 2 of 4 cherry trees), *Apricot latent virus* (ALV, 1 of 14 apricot trees), *Cherry virus A* (CVA, 6 of 14 apricot, 1 of 6 plum and 1 of 4 cherry trees), *Apple stem pitting virus* (ASPV, 4 of 9 apple and 1 of 8 pear trees) and *Apple stem grooving virus* (ASGV, 1 of 9 apple and 1 of 8 pear trees) (Table 1).

DISCUSSION

The present survey provides broad information on the presence of *Fovea-*, *Tricho-* and *Capilloviruses* in old pome and stone fruit cultivars from the Campania region of Southern Italy. ACLSV appears to be by far the most widely spread virus among those analysed, since it was detected in all pome and stone fruits species tested (Table 1) and in an overall 91% of all tested samples. The analysis of the sequences corresponding to these isolates showed a remarkable degree of variability, with an average pairwise nucleotide

sequence divergence between isolates of 18.9% (±1.3%); ASPV and ASGV were both found on both susceptible species tested (apple and pear). CGRMV and ALV, already reported from Campania (Gentit et al., 2001; Liberti et al., 2005b), were observed on cherry and apricot, respectively.

APCLSV was not detected among the 93 apple, 30 pear, 67 cherry and 4 plumcot trees analysed. On the other hand, it was recovered on plum (2 of 26; 7.6%) and apricot (7 of 112; 6.2%) and for the first time in Campania on peach (1 of 29; 3.4%). Thus, its prevalence appears to be approximately 15 times lower than that of ACLSV, despite the fact that these viruses are two closely related members of the *Trichovirus* genus. Contrary to the situation reported by Liberti et al. (2005a), two of the 10 APCLSV-positive samples were found free of ACLSV infection, indicating that APCLSV is not dependent on ACLSV for the successful establishment of infections in its *Prunus* hosts. Furthermore, despite the fact that it was initially discovered in apricots showing ring-pox-like symptoms, APCLSV does not seem to be specifically associated to ring-pox symptoms on fruits since only 1 out of the 7 APCLSV-infected apricots showed this symptomatology.

The sequence comparison of the PCR products derived from the APCLSV isolates discovered in this study and of those in databanks showed an average pairwise divergence between isolates of 11.6 (nt) and 4.5% (aa), respectively. This level of variability is substantially higher that that initially reported by Liberti et al. (2005a), in part because of the identification of some divergent isolates, which form three additional divergent clusters. As compared to the diversity found for the closely related ACLSV (18.9%), the diversity of APCLSV still appears to be substantially smaller. Similarly, the host range of ACLSV includes both the Prunoidae and the Maloidae whereas this study found only evidence of APCLSV infection in Prunoidae (excluding cherry), which suggests that APCLSV natural host range could be restricted to stone fruits. Given the very different situations observed for these two closely related viruses, it is tempting to speculate as the nature and origin of the factor(s) that have ensured the higher evolutionary success and 15 times higher prevalence of ACLSV.

During this study, CVA was detected for the first time in Italy in apricot, plum and cherry trees, often in mixed infection with ACLSV, APCLV and/or CGRMV. This virus was not recovered from plumcot or peach, the latter being described as a potential natural host of CVA (James and Jelkmann, 1998). The case of the plumcot deserves more wide-ranging detection efforts (only 4 trees tested) because it is a hybrid of european plum and apricot, both of which are potential hosts of CVA or APCLSV. The discovery of these two viruses never or only recently described in Italy in old local cultivars indicate that they may have been introduced a long time ago probably by infected propagation material.

ACKNOWLEDGEMENTS

Thanks to Antonio Peluso for technical assistance.

Literature Cited

Adams, M.J., Antoniw, J.F., Bar-Joseph, M., Brunt, A.A., Candresse, T., Foster, G.D., Martelli, G.P., Milne, R.G. and Fauquet, C.M. 2004. The new plant virus family *Flexiviridae* and assessment of molecular criteria for species demarcation. Arch. Virol. 149:1045–1060.

Alioto, D., Gangemi, M., Deaglio, S., Sposato, P., Noris, E., Luisoni, E. and Milne, R.G. 1999. Improved detection of Citrus psorosis virus using polyclonal and monoclonal antibodies. Plant Pathol. 48:735–741.

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

Candresse, T., Lanneau, M., Revers, F., Grasseau, N., Macquaire, G., German, S., Malinovsky, T. and Dunez, J. 1995. An immunocapture PCR assay adapted to the

detection and the analysis of the molecular variability of the *Apple chlorotic leafspot virus*. Acta Hort. 386:136–147.
Foissac, X., Svanella-Dumas, L., Dulucq, M.J. and Candresse, T. 2001. Polyvalent detection of fruit tree *Tricho*, *Capillo* and *Foveaviruses* by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). Acta Hort. 550:37–43.
Foissac, X., Svanella-Dumas, L., Gentit, P., Dulucq, M.J., Marais, A. and Candresse, T. 2005. Polyvalent degenerate oligonucleotides reverse transcription-polymerase chain reaction: a polyvalent detection and characterization tool for *Trichoviruses*, *Capilloviruses* and *Foveaviruses*. Phytopathology 95:617–625.
Gentit, P., Foissac, X., Svanella-Dumas, L., Peypelut, M. and Candresse, T. 2001. Characterization of two different apricot latent virus variants associated with peach asteroid spot and peach sooty ringspot diseases. Arch. Virol. 146(8):1453–1464.
James, D. and Jelkmann, W. 1998. Detection of *Cherry virus A* in Canada and Germany. Acta Hort. 472:299–303.
Kumar, S., Tamura, K. and Nei, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. in Bioinf. 5:150–163.
Liberti, D., Marais, A., Svanella-Dumas, L., Dulucq, M.J., Alioto, D., Ragozzino, A., Rodoni, B. and Candresse, T. 2005a. Characterization of *Apricot pseudo-chlorotic leaf spot virus*, a novel *Trichovirus* isolated from stone fruit trees. Phytopathology 95:420–426.
Liberti, D., Marais, A., Svanella-Dumas, L., Ragozzino, A. and Candresse, T. 2005b. Partial genome sequence of an apricot isolate of *Cherry green ring mottle virus* (CGRMV). Arch. Virol. 150(1):185–188.

Tables

Table 1. Viruses detected by PDO RT-PCR analysis in pome and stone fruit germplasm collections of old Italian cultivars.

Host	Virus detected		
	Trichoviruses	*Foveaviruses*	*Capilloviruses*
Apricot	ACLSV, APCLSV	ALV	CVA
Plum	ACLSV, APCLSV		CVA
Plumcot	ACLSV		
Peach	ACLSV, APCLSV		
Cherry	ACLSV	CGRMV	CVA
Apple	ACLSV	ASPV	ASGV
Pear	ACLSV	ASPV	ASGV

Figures

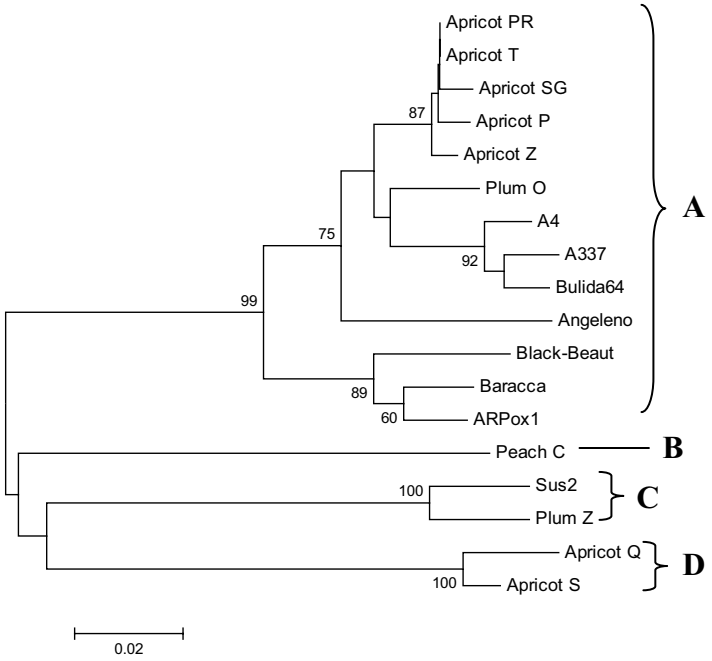

Fig. 1. Phylogenetic tree of APCLSV isolates reconstructed from the nucleotide sequence of the APCLSV-specific nested PCR assay. Tree was constructed using the neighbour-joining method with 1000 bootstrap replicates. Only bootstrap values above 60 are shown. The bar represents 0.02 substitutions per site. The following sequences were used: Sus 2 (AY713379), Angeleno (AY713381), Black-Beaut (AY713385), Baracca (AY713384), Bulida 64 (AY713383), ARPox 1 (AY713380), A4 (AF413932), A337 (AY713382).