Oral Session VI

Infectious uncloned full-lenght cDNAs as a tool for the study of the etiology of fruit tree viral diseases

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Frequent mixed viral infections make it difficult to understand the respective contribution to the observed symptomatology of individual viruses present in fruit trees. As a tool for the study of the etiology of fruit tree viral diseases, we have developed a simple and generic method to separate viral complexes through the use of uncloned infectious full-length cDNAs, thus bypassing several tedious or limiting steps (need for an herbaceous host, problems of viral purification or for cloning of infectious cDNAs...). As a model system we are using *Apricot pseudo-chlorotic leaf spot virus* (APCLSV) and the closely related *Apple chlorotic leaf spot virus* (ACLSV). In the first strategy tested, the 7.6 kbp full-length viral cDNA is amplified from crude total nucleic acid extracts using a forward primer containing the bacteriophage T7 RNA polymerase promoter. Viral RNAs are then produced by in vitro transcription and directly inoculated to host plants. In the second strategy (Fakhfakh et al., 1996), megaprimers linking signals for transcription in planta (CaMV 35S promoter and Nopaline synthase terminator) to the ends of the viral genome are used for the synthesis of a long PCR product integrating the promoter, the full-length viral cDNA and the terminator, which is biolistically inoculated to host plants.

The preliminary results obtained with the first strategy show infection rates of the inoculated herbaceous hosts of between 10 and 50%. Results of inoculation of GF 305 peach seedlings with the same transcripts and of herbaceous and woody hosts with the PCR products developed using the second strategy will also be presented. These techniques should find wide application for the definite association of specific symptoms to a given agent, even when it is only available as mixed infections. (Fakhfakh et al. 1996. Journal of General Virology, 77, 519-523.).

Expression of the coat protein genes of PNRSV and PDV in the synergistic disease peach stunt.

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Simultaneous infections of peach (*Prunus persica* Batsch L.) with the two ilarviruses, Prunus necrotic ringspot virus (PNRSV) and Prune dwarf virus (PDV), produce a synergistic disease referred to as “peach stunt”. Previous work showed significant differences in the expression of the coat protein (CP) genes. In the presence of PNRSV, an up to 17-fold reduction in the amount of (+) strand RNA 3 of PDV, as compared to similar trees infected with PDV alone, was observed. However, the presence of PDV had no effect on the concentration of (+) strand RNA 3 of PNRSV (Scott et.al., 2001. Acta Hort. 550:229-236). This work re-examines and extends these observations using multiplex real-time PCR. Probes to both the plus and minus strands of the RNA 3 of each virus were designed and synthesized. The comparative CT method (ΔΔCT) was used for relative quantitation of gene expression. A reduction in the amount of (+) strand RNA 3 of PDV observed in the earlier work was not seen using real-time PCR. However, in a time course experiment with samples collected at 14-day intervals for 6 weeks, there was a substantial increase in the concentration of the (+) strand of RNA 3 of PDV after 14 days irrespective of whether the virus was present as a sole infecting agent or as a co-infection with PNRSV. At this same point in time there was a decrease in concentration of (+) of the RNA 3 of PNRSV. By the next sample date the concentrations of the (+) strand of RNA 3 of both viruses had returned to “normal”. The results are discussed in relation to the most extensively studied plant viral synergism synergism (PVX and PVY) and to known changes in concentration of PNRSV and PDV based on earlier observations made using ELISA.

Investigations on virus occurrence in different tissues throughout the year and sequence variability of Apple stem pitting virus

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Apple stem pitting virus (ASPV) is a latent virus of apple and belongs to the genus Foveavirus in the family Flexiviridae. Mixed infections with *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) can lead up to 60% yield loss.

Virus RNA extractions were made from buds or leaves, phloem and roots from four different trees at least once a month throughout the year. A Real- Time PCR assay was evaluated and the virus titer was determined.

The complete sequence of the ASPV isolate PB 66 was obtained. It is 9363 bp in size and consists of 5 open reading frames. Comparison with the isolate PA 66 of the coat protein sequence showed identity of 82% in the sequence and 81% amino acid similarity. The whole sequence showed 80% sequence identity to isolate PA 66. Selected portions of the newly analyzed genotype were compared to database entries to analyze the variability of ASPV.

Close similarities between Cherry chlorotic rusty spot disease from Italy and Cherry leaf scorch from Spain

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Cherry chlorotic rusty spot (CCRS), a disease affecting sweet and sour cherry in southern Italy was regularly found associated with an unidentified fungus and with a complex pattern of viral-like double-stranded RNAs as well as with two small circular RNAs (cherry small circular RNAs, cscRNAs). Further studies revealed that i) the ds-RNAs correspond to the genome of different micoviruses belonging to the genera *Chrysovirus*, *Partitivirus* and *Totivirus* and ii) the two viroid-like RNAs consist of two groups of variants with similar sequence but differing in size (394–415 and 372–377 nt for cscRNA1 and cscRNA2, respectively). Here we report that the dsRNAs of *Chrysovirus* and Parti*t*ivirus have been detected by RT-PCR analysis with CCRS specific primers in nucleic acid preparations from cherry leaves affected by cherry leaf scorch (CLS) in Spain, a disease whose etiological agent is the ascomycetes *Apiognomonia erythrostoma*, order *Diaporthales*. Moreover, Northern-blot hybridization assays showed that a viroid-like RNA co-migrating and sharing high sequence similarity with the cscRNA1 previously reported in Italy, accumulate in leaves from CLS affected trees in Spain. These data, together with other evidences showing similar symptoms, disease cycle and fungal fructifications in CCRS and CLS affected trees, suggest a close relationship between the two cherry disorders.

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Vertical transmission of Prunus necrotic ringspot virus: hitch-hiking from gametes to seedling.

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The aim of this work was to follow infection by Prunnus necrotic rigspot virus (PNRSV) in apricot reproductive tissues and transmission of the virus to the next generation. PNRSV was detected in repoductive tissues during gametogenesis. The virus distribution was studied at diferent developmental stages such as at the gloular, torpedo, bent cotyledon and mature stages. The results obtained shed light on the PNRSV vertical trasnmission from gametes to seedlings.

Molecular characterization of a new Prunus-infecting Flexiviridae member

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During a survey in Iran, the occurrence of Trichoviruses, Foveaviruses, and Capilloviruses was investigated in symptomatic stone fruit trees, by the PDO nested-RT-PCR technique, which allows the detection of viruses belonging to these three genera of the family *Flexiviridae* (Foissac et al., 2005). The PDOamplified cDNAs obtained from a *Prunus persicae tree* displaying mottle symptoms was cloned and sequenced, revealing two different sequences, one belonging to an isolate of Apricot pseudo-chlorotic leaf spot trichovirus, and the other showing its highest identity (only 73%) with Citrus leaf blotch virus, a member of the tentative Citrivirus genus in the family *Flexiviridae*. The nucleotide sequence of the 3' end of the genome (3055 bp) of this second agent was determined. The molecular organization is similar to that of some trichovirus members such as *Peach mosaic virus* (PcMV), with 4 open reading frames coding respectively for a replication-associated protein, a movement protein, a capsid protein, and a nucleic acid-binding protein. However, the identity levels between the PcMV proteins and those of the new agent are very low (at the most, 51.5% for the partial replicase). Phylogenetic reconstructions for the three complete ORFs and the partial replicase suggest that this agent should be considered as a new virus defining a new genus in the *Flexiviridae* family. The biological characterization of this virus is underway. Interestingly, a second isolate of this new agent was recovered from a Japanese plum collected in France but of unknown origin and test-inoculated on GF305 Prunus persicae seedlings in greenhouse. After two or three months the peach seedlings displayed red marbling symptoms on leaves (co-infected with Prunus necrotic rusty mottle virus, PNRSV). This second isolate has a similar genomic organization and over 90% nucleotide identity in the sequenced region. (Foissac et al., 2005. Phytopathology, 95,617-625).

Widespread occurrence of Tomato ring spot virus in deciduous fruit trees in Iran

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Despite long precedence of fruit tree growing in all provinces in Iran, the information on tree viruses in this country is scant. In the present study, presence of tomato ring spot virus (ToRSV) was surveyed in various woody plants in this country by mechanical transmission to herbaceous hosts, ELISA using a commercial antiserum, and PCR with specific primers. ToRSV was identified in the following plant-symptom combinations: Walnut with mottling, deformation, necrosis, and yellowing of main veins from Tehran Province; plum with yellowing of main veins, peach with yellowing of main veins and marginal necrosis, and hazelnut with interveinal chlorosis and marginal necrosis from Ardabil Province; apple with yellowing of main veins, mosaic and necrotic lesions, quince with large necrotic spots, and almond with leaf deformation and rossetting from Khorasan Province; and raspberry with marginal necrosis of leaf and necrotic lesions from Mazandaran Province. Mechanical inoculation from walnut,

plum, peach, hazelnut, apple, quince, almond, and raspberry to *Nicotiana tobaccum* cv. Samsun resulted in systemic infection. The virus isolates induced local lesions, leaf deformation, and necrosis in N. rustica, chlorotic local lesions on *Chenopodium quinoa*, and large local lesions on Gomphrena globosa. All samples were ELISA positive. PCR with specific primers resulted in the amplification of expected fragment (490 bp). This study shows extensive occurrence of ToRSV in Iran.

Virus diseases of stone fruit trees in Belarus

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The purposes of the work were studying of plum and cherry orchards viral contamination, selection of virus free trees of varieties and vegetative propagated rootstocks of *Prunus L.*, monitoring of the nuclear rootstocks collection. Serological test (DAS-ELISA) was used for virus disease detection. For this time investigation of viruses distribution of stone fruit trees have been carried out and the first collection of industrial cultivars nuclear has been created. Sanitary selection didn't allowed to find virus free plants of rootstocks for sour cherry and sweet cherry. Virus elimination *in vitro* (meristem culture and chemotherapy) was successful for 5 forms of rootstock.

ApMV, ACLSV and PDV were often detected in plum orchard (20,63 %, 30% and 41,79 % accordingly). As in many European countries, the presence of Sharka is the major threat for the stone fruit orchard. In Belarus Sharka is not included in the list of quarantine objects. In 2000 year contamination by PPV was revealed for the first time (1 tree from Grodno region), in 2005 PPV was registered in 3 trees, in 2007 – in 30.

High contamination of all sour and sweet cherry plantings by PNRSV (86,23 %) was established. Other viruses were detected in the industrial orchards of sour and sweet cherry considerably below (ACLSV, ArMV, PDV, ApMV, RRV – no more then 13 %), than in propagation plantation. All tests for CLRV were negative and only one positive result was established at testing PPV. From the most widespread viruses of fruit cultures in the adjacent countries, in our researches only CLRV was not revealed, all other viruses in different quantity were presented in Belarussian orchards and nurseries.

Detection, monitoring and characterization of LChV-1 isolates from southern italy

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Little cherry virus 1 (LChV-1), one of the causal agents of the Little Cherry Disease in cherries, has unknown impact in other stone fruit hosts. LChV-1 was diagnosed by different molecular techniques (one-step RT-PCR, dot-blot hybridization, tissue-printing hybridization), serological technique (ELISA), and serological-molecular techniques (IC RT-PCR and IC one-step RT-PCR). Different LChV-1 Prunus isolates from Southern Italy were assayed from leaf samples collected randomly during late summer and early autumn 2007. The molecular techniques described here showed to be more reliable in virus detection than the serological and serological-molecular assays. LChV-1 was monitored also during different seasons over the year by the use of tissue-printing hybridization from different tissues (leaf petioles, branches, dormant cuttings and buds). LChV-1 was detectable throughout the year in all types of tissues tested, with the most reliable detection during the summer and autumn. Virus-specific primers were used to amplify fragments of ORF1a, ORF4, CP and CPm of selected LChV-1 isolates. Italian LChV-1 isolates showed high sequence identity among them (90% or more) in all sequenced genomic regions, even though they came from different stone fruit species and orchards.

Keywords: stone fruits, diagnosis, monitoring, sequence analyses