ABSTRACTS

Variations in the RNA Population of *Citrus tristeza virus* (CTV) Isolates after Graft-Inoculation to a New Host

M. A. Ayllón, J. Guerri, and P. Moreno

ABSTRACT. *Citrus tristeza virus* (CTV) shows a wide biological variability, which affects the disease incidence and the damage caused in different citrus areas. As with other RNA viruses, CTV isolates contain a population of RNA sequence variants derived from the error-prone nature of the RNA polymerase, whose composition likely affects their biological characteristics. A quick picture of the RNA population can be obtained by single-strand conformation polymorphism (SSCP) analysis of the cDNA obtained by reverse transcription (RT), and PCR amplification of selected RNA regions. We examined the effect of host change on the composition of the RNA population by graft-inoculating four CTV isolates (T385, T317, T318 and T388) on four plants each of Mexican lime, Pineapple sweet orange, Etrog citron and sour orange, and periodically analyzing the SSCP profile of gene p18. All isolates studied showed variations between the SSCP profiles obtained from plants of different citrus species, and sometimes also between profiles obtained from individual plants of the same species. Overall, the host inducing less population changes was Mexican lime and the most stable population was that of isolate T317. This isolate induced only two types of SSCP profile, whereas isolate T318 induced up to 11 different profiles in the 16 plants inoculated. The SSCP profile of some individual plants varied in successive analyses at different times. These findings indicate that, after CTV inoculation to a new host, the RNA population suffers constraints that may alter its final composition. Shifting to the new population probably occurs after several months of unstable composition.

Transgenic Expression of *Citrus tristeza virus* p23 Protein Induces Viral-like Symptoms in Mexican Lime Plants

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ABSTRACT. The 23-kDa protein (p23) coded by the 3'terminal gene of *Citrus tristeza virus* (CTV) is an RNA-binding protein that contains a motif rich in cysteine and histidine residues in the core of a putative zinc-finger domain. Furthermore, p23 sgRNA is the second most abundant viral mRNA in infected tissues and protoplasts, and it accumulates earlier than the other sgRNAs in infected protoplasts. On this basis, a regulatory role for p23 in CTV replication or gene expression has been suggested. To explore whether constitutive expression of this protein could affect the normal CTV infectious process, we generated transgenic Mexican lime plants carrying the p23 transgene, or a truncated version thereof, under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Expression of the p23 protein induced phenotypic aberrations resembling symptoms produced by CTV in non-transgenic lime plants, whereas transgenic plants expressing the p23 truncated version were normal. Symptoms shown by the p23-transgenic plants included severe vein clearing, leaf cupping and stem pitting, as well as leaf epinasty, apical necrosis and growth interruption or stunting in the most severe cases. CTV-like symptoms displayed by the transgenic plants expressing p23 were generally more intense than those induced by most CTV isolates in non-transgenic limes. Interestingly, the amount of p23 protein in transgenic plants was higher than that of CTV-infected plants. The difference in the accumulation of p23 most likely results from the action of the strong constitutive CaMV 35S promoter. In addition, a direct correlation was found between the intensity of CTV-like symptoms and the p23 accumulation in the transgenic plants. This demonstrates that p23 is involved in symptom development and that it likely plays a key role in CTV pathogenesis. Our finding also delimits a small region of the large CTV genome for future mapping of specific pathogenic determinants.

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Separation of *Citrus tristeza virus* Subisolates Using Aphid Transmission and their Molecular Analyses

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ABSTRACT. *Citrus tristeza virus* (CTV) exists in field isolates as a complex of virus. This complex may contain both mild and severe strains. Using single and multiple aphid transmissions, subisolates of various field isolates were separated. CTV isolates that tested negative with the monoclonal antibody MCA13 were found to contain MCA13 positive subisolates. Using primers to specific and variable regions of the CTV genome, reaction profiles of the isolates were generated. The profiles of the subisolates sometimes were very different from the parent field isolates from which they were transmitted.

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A New Entity in Citrus Associated with *Citrus tristeza virus* and with Similarities to *Oat blue dwarf virus* and Grapevine fleck virus

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ABSTRACT. A cDNA clone with high similarity to part of the replicase region *of Oat blue dwarf virus* (OBDV), type species of the genus *Marafivirus*, was obtained from dsRNA extracts from a *Citrus tristeza virus*-infected plant. OBDV coat protein (CP) antibodies reacted very weakly with protein extracts from the same source and gave a specific band at approximately 28 kDa. Using the cDNA clone sequence as a probe in northern analyses from source plant total RNA and RNA extracted from virus purification fractions, the genomic RNA is approximately 7.5 kb with indications of at least two sub-genomic RNAs. Grapevine fleck virus (GFkV), an unassigned virus within the proposed new genus *Maculavirus*, which is phylogenetically related to OBDV, has a genomic RNA and major coat protein similar in size to those of the unknown entity. GFkV CP antibodies did not react with protein extracts from the source plant by ELISA.

Exploring Replicase-Mediated Resistance Against *Citrus tristeza virus*

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ABSTRACT. Replication-associated genes have been used for developing resistance against different groups of plant viruses. The replicase-mediated resistance (RMR) was achieved by transforming plants with sense, anti-sense, untranslatable, mutant or truncated RNA-dependent RNA polymerase RdRp genes. To explore the possibilities of RMR against *Citrus tristeza virus* (CTV), epicotyl segments of Duncan grapefruit seedlings were transformed with five different constructs of the RdRp gene of CTV, including a sense, anti-sense, untranslatable and two mutants with point mutations and a deletion at the GDD motif. A number of transgenic plants for each construct were regenerated and established in the greenhouse. The transgenic nature of most of these plants was confirmed by PCR amplification of the GUS and RdPp genes from their genomic DNA. The transgenic plants are currently being analyzed and evaluated for resistance to CTV.

Map-Based Cloning and Analysis of the *Citrus tristeza virus* Resistance Gene

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ABSTRACT. *Citrus tristeza virus* (CTV) can be a devastating disease of citrus, causing economic losses by killing trees or reducing fruit size. As aphids transmit the virus, control is difficult. All commercially grown citrus varieties are susceptible to CTV, but the level of damage

varies with cultivar and viral strain. Development and use of resistant varieties could minimize damage to new plantings. Two major approaches to development of virus resistant plants are resistance mediated by transformation with sequences derived from the virus, and resistance mediated by plant virus resistance genes. Both alternatives are being pursued in the *Citrus*/CTV system in our laboratories. With the latter approach, we are using positional cloning methods to isolate a dominant gene (*Ctv*) from *Poncirus* that causes resistance to the virus. The gene will then be transformed into CTV-susceptible citrus cultivars to produce virus-resistant plants. We have identified markers close to *Ctv* using ends of BAC clones from a 1.2-Mb contig that spans the *Ctv* region. This work delimited the region that must contain *Ctv* to a contig of four overlapping BACs that span only 250 kb, and sequencing of these four BACs has been completed. This 250-kb region contains at least 33 predicted and/or confirmed genes. Based on expression patterns between *Citrus* and *Poncirus*, we have identified several candidate genes for CTV in this region. The current major emphasis is the transformation of these genes into CTV susceptible cultivars.

Preliminary Evaluation of *uncp* Transgenic Rio Red Grapefruit Scions for Resistance to *Citrus tristeza virus*

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ABSTRACT. Transgenic Rio Red grapefruit scions can be generated efficiently using an *Agrobacterium*-mediated transfer system and binary vectors. Sixty-three transgenic scions representing separate transgenic events using an untranslatable coat protein sequence (*uncp*) from *Citrus tristeza virus* (CTV) isolate SY568 have been generated. Under controlled conditions in Texas with wild type and non-transformed scions as controls, 12 *uncp* events have been grafted to virus-free rootstocks in duplicate, and challenged with a severe CTV isolate. The plants have been evaluated over 11 mo post-inoculation using CTV coat protein ELISA. Five *uncp* sources have also been graft inoculated with a mild and a severe CTV isolate under quarantine conditions in South Africa. In Texas, for some of the transgenic scions, a relative decrease in CTV titer has been observed compared to infected controls. Northern hybridizations of total RNA to a probe made from the transgene also had weak signals for these samples. For other transgenic scions, there were apparently no differences in CTV infection relative to controls, with northern analysis giving strong hybridization patterns. Stem pitting occurrence from the South African isolates 6 mo post-inoculation was similar in transgenic and control plants. Since a predicted resistance mechanism would be post-transcriptional gene silencing-based, initial CTV infection followed by 'recovery' could be expected to occur in some of the transgenic tissues.

Coat-Protein Mediated Protection Against *Citrus tristeza virus* (CTV) in Transgenic Mexican Lime Plants Expressing the Viral p25 Gene

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ABSTRACT. *Citrus tristeza virus* (CTV), a member of the genus *Closterovirus*, is the causal agent of the most economically important viral disease of citrus worldwide. To develop resistance against CTV through genetic engineering, Mexican lime plants were transformed with the p25 gene, coding for the major viral coat protein (CP) of the virus. More than 40 transgenic lines were generated, 25 containing the p25 CP gene of the severe CTV strain T305, and 17 with that of the mild strain T317. Transgene integration patterns were usually complex with almost half of the plants showing T-DNA truncations. Copy number of the p25 CP transgene was also variable in the transgenic lines, ranging from one to six. Accumulation of the p25 CP protein was detected in most of the transgenic lines. When plants propagated from each transgenic line were graft-inoculated with T-305 or aphid-inoculated with T300, two types of response to viral challenge were observed: Some lines developed CTV symptoms similar to those of non-transgenic controls, whereas others exhibited protection against the virus. This protection consisted of a proportion of plants, ranging from 10 to 33%, that were resistant to CTV, and the rest of them that showed a

significant delay in virus accumulation and symptom onset. Protection was efficient against non-homologous CTV strains and was generally accompanied by high accumulation of p25 CP. This evidence suggests that a CP-mediated resistance mechanism was active in the protected transgenic lines. This is the first report demonstrating pathogen-derived resistance in transgenic plants against a *Closterovirus* member in a natural host.

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Immunochemistry Characterization of Mabs Against Recombinant Coat Proteins of the *Citrus tristeza virus* Capão Bonito Complex

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ABSTRACT. The Capão Bonito complex of *Citrus tristeza virus* (CTV) is still restricted in its distribution in Brazil, but it is efficiently transmitted by the brown citrus aphid, *Toxoptera citricida*. Sweet orange varieties on Rangpur lime are especially susceptible to it. Recently, two different proteins from the viral capsid protein (CP) of CTV present in Pera sweet orange 135/Cravo 507 which displayed symptoms of severe tristeza Capão Bonita complex, were cloned and expressed in *Escherichia coli*. These proteins, called CB-104 and CB-22, showed 93% homology in their amino acid sequence when used as antigen, and were used to produce three monoclonal antibodies. The immunochemistry characteristics of each (sensibility, specificity and affinity) were determined, and the antibodies were tested against samples from infected plants to standardize an efficient immunodiagnostic test.

Two SSCP Common Variants of the Minor Capsid Protein Gene p27 in Colombian Field Isolates of *Citrus tristeza virus*

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ABSTRACT. *Citrus tristeza virus* (CTV) populations are usually a mixture of variants which can induce differential symptom expression depending on the host. Early studies of Colombian CTV isolates showed, by serological analysis and/or biological characterization, that most of them were severe, except for a small fraction of mild isolates, some of them from trees on sour orange rootstock from the Mompox region. The minor coat protein gene (CPm) codes for the p27 protein, participates in virion assembly and is possibly involved in the movement of the virus within the host and in aphid transmission. In the present work, we studied the major variants of p27 of six field isolates of CTV by gene cloning, SSCP variant analysis and DNA sequencing. The six field isolates from four regions, including Mompox, were collected from different hosts and showed differed MCA13 reactions. They had between three and 11 SSCP variant, resembling quasi-species distribution, with one variant (A1 and/or T1) of greatest frequency. SSCP variant comparisons between the different isolates revealed that A1 is a common variant for all isolates, while T1 is present only in the Mompox isolates in high frequency. Isolates with A1 as the major variant were MCA13 positive, while those with T1 were MCA13 negative. The deduced amino acid sequence similarity of A1 with isolate T36 was 99.1%, while that between T1 and T30 was 97.9-98.3%. The presence of the A1 variant in different hosts and areas of Colombia suggests that it is a successful variant which, possibly because of the multifunctional role of its p27 protein, may give it an advantage under strong selection pressure.

Characterization of the HSP70 Protein Homolog (HSP70h) of *Citrus tristeza virus*

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ABSTRACT. *Citrus tristeza virus* (CTV), a member of the *Closteroviridae*, is the causal agent of one of the most destructive disease of citrus, causing a diversity of symptoms on various scion and rootstock combinations. The virus is a monopartite, single-stranded, positive-sense RNA

virus, with a genome of about 20 kb encapsidated by two capsid proteins. The *Closteroviridae* is the only family known to encode a homolog of the HSP70 family of cellular chaperones. The CTV HSP70 is a 65 kDa protein (p65) with high homolgy to cellular chaperones. The 3' end of the p65 gene of CTV was cloned with a histidine tag fusion and expressed in *Escherichia coli*. The purified protein was used to raise a polyclonal antibody in chicken. The carboxyl end of p65 (3' end of p65) was chosen for study because of its lower homology with cellular chaperones, to avoid cross-reactivity of the antibody with host proteins. Using this antibody, the CTV p65 gene product was specifically detected in CTV-infected plants, but not in healthy citrus. The localization pattern of the p65 and the viral coat protein were similar in direct print studies. The same antibody was used for immunogold labeling studies that revealed a close association of the HSP70h protein with the virion. This association was later confirmed by co-immuno-precipitation of the virion and the p65 protein. We are currently studying the presence of the p65 protein in the characteristic inclusion bodies present in CTV-infected tissue, and the intracellular localization of this gene product in infected cells.

Localization of the Capsid Protein (CP) and the Minor CP of *Citrus tristeza virus* in Relation to Genomic RNA

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ABSTRACT. *Citrus tristeza virus* (CTV), a member of the genus *Closterovirus* within the family *Closteroviridae*, causes one of the most devastating citrus diseases in tropical and semi-tropical regions worldwide. The CTV virions consist of flexuous particles about 2,000 × 11 nm with a capsid protein (CP) encapsidating 95% of the genomic RNA (*circa* 1,900 nm) and a minor CP, p27, at one end of the particle encapsidating the remaining 5% of the genome (75-85 nm). The association of the CP and minor CP in relation to the genomic RNA termini of purified CTV virions was examined by immunocapturing the p27 terminus with a p27-specific polyclonal antiserum. Immuno-capture reverse transcriptase-PCR (IC-RT-PCR) was optimized for the amplification of either 510 nt at the 5' terminus, or 899 nt at the 3' terminus of the CTV genome. CTV was purified, sonicated to fragment the virions, and then fractionated on a 10-40% rate zonal sucrose gradient. The fractions were first tested by ELISA to determine the presence of the virions, and the positive fragments were then used for termini amplification by IC-TR-PCR. The 5' terminus of CTV, but not the 3' terminus, was consistently amplified from samples immunocaptured using p27 antiserum. These results confirm that p27 is associated with the 5' terminus of the CTV genomic RNA.

Complete Genome Sequence of the *Citrus tristeza virus* 'Pera IAC' Protective Isolate

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ABSTRACT. *Citrus tristeza virus* (CTV), the causal agent of one of the most economically important diseases of citrus, occurs naturally in plants as a complex mixture of strains varying according to host and geographic localization. Cross-protection with mild isolates in susceptible sweet orange varieties has been used to control severe isolates causing stem-pitting and stunting in the scion and rootstocks in Brazil. The protective CTV-IAC isolate has been used for many years in the sweet orange cross-protection program in São Paulo State, Brazil, with suitable biological stability. The objective of this work was to sequence the genome of the CTV-IAC protective isolate. CTV ds RNA was isolated and used as a template for the first strand cDNA synthesis with random primers and Superscript™ II RNase H-Reverse transcriptase. After the second strand synthesis, cDNA was cloned into pUC18, according to the specifications of Amersham-Pharmacia for the Sure Clone Ligation Kit. The sequencing was done in an ABI Prism 377 (Perkin Elmer), and the sequences were analyzed using the Sequencher and PhredPhrap Consed software. The genome of the CTV-IAC isolate was completely covered and has the same organization of the other isolates already sequenced. The data also indicated that the homology with the genome of isolates varies according to the sequenced region, and is higher with the VT (Israel) and SY 568 (USA) isolates.

Molecular Analysis of Australian Isolates of *Citrus tristeza virus*

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ABSTRACT. In countries like Australia where *Citrus tristeza virus* (CTV) is endemic, the virus exists as a range of molecular variants ("quasispecies") that differ in the severity and type of symptoms expressed. Following molecular analyses of Australian isolates, and comparing them to the nucleotide sequence of isolates from other countries, we have developed methods to discriminate local isolates and assessed the molecular diversity of CTV isolates found in Australia. The p23 gene of selected isolates was amplified by RT-PCR and analyzed by RFLP and nucleotide sequencing. Molecular techniques were designed that will specifically detect and discriminate two sweet orange pitting isolates from Queensland, Australia. These methods can be used in potential incursions of orange stem pitting into southern states. Eight 'groups' of CTV isolates were defined based on RFLP analysis of the p23 gene, from local isolates and from database sequences for foreign isolates. Improved discrimination is particularly important in rapidly detecting new (exotic) strains of CTV and those variants that are "hidden" within mixtures. The genetic diversity of Australian CTV populations was assessed via a cloning analysis of the helicase region amplified from a range of CTV bud isolates, either known by RFLP and SSCP to contain diverse mixtures of strains, or of interest for other reasons (eg. the pre-immunizing isolate PB61). Phylogenetic analysis of the nucleotide sequences obtained from cloned amplicons identified 10 genotypes that fell into three distinct clades. This phylogenetic scheme may assist in predicting the capacity of particular isolates to provide effective cross protection against other strains. [This work was funded partly by the Australian Horticultural Research and Development Corporate (CT97009) and by a John Allwright Fellowship from the Australian Centre for International Agricultural Research.]

Population Structure of the p23 Gene Within Argentine *Citrus tristeza virus* Isolates

N. G. Iglesias, S. Gago-Zachert, N. Costa, M. I. Plata, and L. Semorile

ABSTRACT. *Citrus tristeza virus* (CTV) isolates are complex populations of genetically related variants that vary greatly in biological characteristics such as aphid transmissibility or symptoms induced in host species. Knowledge of CTV population structure, the genetic relationship between intra-isolate variants, and population dynamics is crucial to understand the complexity of CTV and therefore, to design effective strategies to control the resulting disease. In this work, we have studied the population structure of 11 CTV isolates by SSCP analysis of the p23 gene which encodes an RNA-binding protein. Also, we have estimated the genetic diversity within and between isolates by analysis of variant nucleotide sequences. Eleven Argentine isolates from different geographical regions and citrus hosts were selected, and biologically characterized by inoculation into four indicator citrus species. Each field CTV isolate was assigned to one of the five principal biogroups defined for this viral complex. For each isolate, 10 clones of the p23 gene were obtained. From each clone, the cDNA fragment corresponding to p23 gene was digested with NdeI, and two fragments of 396 and 340 bp were obtained. A variable number of SSCP patterns and percentage of the major variant were observed for each CTV isolate. Nucleotide sequences of the major variants were determined in both directions by using a DNA sequencer. Multiple alignments of these sequences were realized with the CLUSTAL X program and phylogenetic relationships were inferred using PAUP*. From the results, several interesting observations were made: i) p23 showed higher heterogeneity than p25 or p27 genes of the same isolate; ii) both p23 gene fragments analyzed were involved in the heterogeneity observed; iii) seven isolates showed five or more different SSCP patterns indicating a population structure with no predominant variant; iv) the population structure in sever CTV isolates was more heterogeneous than in mild isolates. The exception was the isolate C257-7 (biotype V) with four SSCP patterns and the major variant representing 70% of the viral population; v) the major variant sequences from the CTV isolates analyzed showed a nucleotide identity varying between 88 and 99%.

Biological Characterization of Isolates of *Citrus tristeza virus* in Grapefruit in Argentina

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ABSTRACT. Tristeza and *Toxoptera citricida* have been present in Argentina since 1930, and since then more than 14 million trees have died. Argentinian citriculture uses the *Citrus tristeza*

virus (CTV)-tolerant rootstocks, trifoliate orange, Cleopatra mandarin, Swingle citrimelo, Volkamer lemon, Rangpur lime and rough lemon. Grapefruit is mainly produced in the northwest of Argentina where, since 1990, young red grapefruit trees have been affected with sever stem pitting in trunk and branches, with deformed fruit and reduced production. The objective of this study was to characterize the CTV isolates present in these grapefruit. Thirty-three CTV isolates from the northwest and also the northeast regions were collected and analyzed by direct immunoprinting-ELISA and on indicator plants under greenhouse conditions. The following conclusions were drawn: 1) All isolates were positive for CTV by direct immunoprinting-ELISA; 2) different pathotypes were found among the isolates; 3) all of the isolates studied induced stem pitting on Duncan grapefruit; and 4) some differences in symptom expression were observed.

Typing of *Citrus tristeza virus* Variants by Cleavase Fragment Length Polymorphism Analysis

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ABSTRACT. A panel of *Citrus tristeza virus* (CTV) isolates from different origins and displaying different biological properties were compared for polymorphisms in their coat protein (CP) gene by Cleavase Fragment Length Polymorphism (CFLP) analysis. This method has been used recently for differentiation of animal viruses and other organisms, but not for plant viruses. CFLP is based on the ability of the enzyme Cleavase I to cut dsDNA at the base of hairpins formed as a result of self base pairing of the DNA strand during the temperature decrease following denaturation. This results in a pattern of single stranded fragments that are visualized by electrophoretic separation on a denaturing gel. This pattern is usually composed of 15-20 bands. The cloned CP gene of 17 isolates was analyzed by CFLP and the patterns compared using the Pearson similarity coefficient. The results were compared with SSCP and with nucleotide sequences. Five clusters could be distinguished based on sequence data of the isolates. With the exception of one isolate, the same pattern of grouping was obtained by CFLP. SSCP analysis was shown to be much more limited in ability to group isolates. Sequence data and CFLP analysis depicted a significant linear correlation. Additionally, the CP gene of 12 isolates obtained from infected plants by immunocapture-RT-PCR was also analyzed to infer on the ability of CFLP to directly analyze biological samples which may be composed of a mixture of genomic variants and which may differ in their biological properties. In most cases, the isolates were typed in their correct cluster. Thus CFLP analysis of the CTV CP gene is a rapid and reproducible technique which enables identification and differentiation with accuracy and precision.

Citrus tristeza virus Resistance Breakdown Occurring in *Poncirus trifoliata*

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ABSTRACT. *Poncirus trifoliata* is presumed to be resistant to *Citrus tristeza virus* (CTV), but allows short distance passive movement. In this study *P. trifoliata* cv Pomeroy plants, known to possess the resistance gene, were inoculated with strains of CTV that had previously been transmitted through *P. trifoliata* cv Rubidoux. Reverse transcription polymerase chain reaction (RT-PCR), using primers specific to the sequence of the coat protein gene, was performed on the extracted dsRNA or RNA from bark obtained from Pomeroy plants. Several PCR products of the coat protein gene were obtained. Stem pitting has been observed in some Pomeroy plants that had been inoculated with the transmitted CTV strains. Eight of the original 15 transmitted strains have been transmitted and proved to be infectious in Madam Vinous sweet orange as evidenced by DAS-ELISA.

The Effect of Temperature on the Detection of *Citrus tristeza virus* by DAS-ELISA

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ABSTRACT. *Citrus tristeza virus* (CTV) has become a major concern for citriculture in Cyprus during the last decade. A survey conducted since 1992 has shown a CTV incidence of about 5% among 180,000 trees indexed by ELISA. While conducting the survey, we noticed that during

summer, infected trees often tested negative in ELISA tests. An investigation was therefore undertaken to examine the effect of temperature and/or time of year on DAS-ELISA detection of CTV. Samples from infected trees were collected at weekly intervals from May to December 1998, from three different areas, two inland and one coastal, with variable temperature regimes. For each tree, four young fresh shoots were obtained, one each of the four sides of the tree, plus one fruit pedicle. The samples were transferred to the Virology Lab of the Agricultural Research Institute in Nicosia, and tested by ELISA (antisera from Volcani Center, Israel) the same or the next day. Temperature readings of the three areas were obtained from the Cyprus Meteorology Service. Results showed that the high summer temperatures had an adverse affect on virus detection. CTV detection in summer was better in the coastal area than inland. Temperatures above 30°C appeared less favorable for ELISA detection of CTV. Fruit pedicles had higher OD_{450} values than young shoots from the same tree.

Comparison of Methodologies for Psorosis Indexing

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ABSTRACT. Psorosis is the main citrus phytosanitary problem in Argentina and Uruguay. In Argentina, citrus is affected by a severe type of psorosis with reports of natural transmission. The recommended assay for this disease in certification programs and research is biological indexing. With the development of several new methodologies such as ELISA and PCR, it was necessary to compare biological indexing with these methods. Of 172 samples from various sources assayed using biological indexing, TAS-ELISA, and hemi-nested RT-PCR, 102 were negative and 37 positive in all tests, nine were positive in biological plus at least one other assay, 15 positive by RT-PCR only, and nine positive in biological assay only which may possibly be due to misreading of symptoms or infection by non-psorosis agents such as concave gum. Other assays with smaller sample numbers also showed inconsistencies between samples and assays. While biological indexing gives the highest number of positives, some are non-psorosis and PCR can detect some non-symptomatic samples. We conclude that at present there is no single, rapid assay for use in massive indexing in commercial nurseries.

Genetic Variability of the RNA3 of *Citrus psorosis virus* (CPsV) in Campania, Italy

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ABSTRACT. The genome of *Citrus psorosis virus* (CPsV) consists of three negative sense ssRNAs. RNA2 and RNA3 present single open reading frames (ORFs) encoding a 53 kDa protein of unknown function and the 48 kDa coat protein. RNA1 has only been partially sequenced. Biological variability of CPsV isolates has been documented, but data on genetic variability are not available. To compare the nucleotide sequence of the major component of RNA3 from 15 CPsV isolates, total RNA from infected plants was extracted, reverse transcribed (RT) and PCR amplified using two pairs of primers, which yielded cDNA fragments of 599 and 675 nucleotides, located in the 5' and 3' halves of RNA3 respectively. Nucleotide sequences were obtained from RT-PCR products. The phylogenetic relationships of CPsV isolates were inferred from amino acid sequences by the Neighbor-Joining method, using MEGA program. To evaluate node values of phylogenetic trees, the "bootstrap" test was used. The phylogenetic tree of the 599-nt fragment showed that all isolates clustered in a single population, whereas two major groups were observed in the tree corresponding to the 675-nt fragment. Our data indicate that most genetic variability of RNA3 is accumulated in the 3' half.

Variability Amongst *Citrus psorosis virus* (CPsV) Sources in Campania, Italy

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ABSTRACT. *Citrus psorosis virus* (CPsV), genus *Ophiovirus*, has a genome of three negative-starnded ssRNAs. It is presumed to be the causal agent of psorosis, one of the most economically important graft-transmissible diseases of citrus. Biological behavior and serological and molecu-

lar characteristics suggest the existence of several strains of the virus. To investigate this variability, we analyzed 53 sources from several citrus species and cultivars from different areas of Campania, Italy. We used a panel of monoclonal antibodies to detect variation in the coat protein, and SSCP analysis to detect variation in the coat protein gene contained in RNA3. Serological analysis revealed nine patterns, seven of them different from those previously reported. Four patterns accounted for 22, 13, 10 and 3 virus sources, while the remaining five patterns appeared only once each. SSCP analysis of the PCR products of one fragment of RNA3, nt 65-740, gave specific profiles for each serotype, and sometimes different profiles within the same serotype, giving 14 profiles in all. Another fragment, nt 719-1318, gave only three profiles. The results indicate that the serological and SSCP analyses were in excellent agreement in differentiating CPsV strains, although the latter detected variability at higher resolution.

Indian Citrus Ringspot Virus: Genome Sequence and Taxonomic Position

G. Rustici, R. G. Milne, and G. P. Accotto

ABSTRACT. The genome of the K1 isolate of Indian citrus ringspot virus (ICRSV) from Kinnow mandarin in northern India has been sequenced. It is a single-stranded positive-sense RNA of 7,560 nt, excluding the poly (A) tail. It contains six ORFs which potentially encode proteins of 187.3, 25, 12, 6.4, 34 and 23 kDa respectively. ORF 1 codes for an RNA-dependent RNA polymerase (RdRp), ORFs 2,3 and 4 compose a triple-gene block, a common feature of several genera of filamentous viruses. ORF 5 encodes the coat protein (CP), and ORF 6 encodes a nucleotide-binding protein. The amino acid (aa) sequence of the CP and that of a 300 aa tract of the RdRp were compared with the respective sequences of representative filamentous viruses, using CLUSTALW, and displayed using TREEVIEW. Closest similarities within the RdRp were found with several potexviruses (identities 54-60%) and allexiviruses (43-45%). The CP was most closely related to some potexviruses (30-36% identity) and foveaviruses (25-27%). The presence of ORF6 places ICRSV near carla- and allexiviruses. The sequences of ORFs 1,2 and 5 are closest to potexviruses, ORFs 3 and 4 to carlaviruses and ORF 6 to allexiviruses. The virus differs from potex and serologically to *Potato virus X*. Thus ICRSV does not fit into any recognized genus, and a new genus should be created for it.

Purification of Virus-like Particles from Citrus Chlorotic Dwarf-Infected and Healthy Citrus Tissues

R. H. Brlansky, D. S. Howd, J. S. Hartung, S. M. Garnsey, and S. Korkmaz

ABSTRACT. Citrus chlorotic dwarf (CCD) was first found in Turkey in the mid 1980s, several years after the introduction of the bayberry whitefly, *Parabemisia myrica* (Kuwana). The disease affects nearly all citrus cultivars, although sweet orange is less susceptible. It is vectored by the bayberry whitefly and is graft and mechanically transmitted. The etiology is presumed to be viral, but no virion has been associated with the disease. CCD was established in the USDA, ARS exotic citrus quarantine facility in Beltsville, MD, USA, and virus purification was attempted. Young flush, new leaves, and bark tissue from CCD-infected citrus were homogenized in phosphate buffer, extracted through cheesecloth, clarified using butanol/chloroform, and PEG-precipitated overnight. The resuspended PEG pellet was layered on a sucrose cushion and centrifuged. The resulting pellet was resuspended and centrifuged overnight on a cesium sulfate gradient.Light-scattering bands were collected and examined by transmission electron microscopy. A slightly flexuous filamentous particle was seen in some of the bands. This material was slash-inoculated into healthy *Citrus macrophylla* plants, which produced non-persistent symptoms. Similar virus-like particles were found in healthy *C. macrophylla* plants. Further research is needed to resolve the etiology of CCD.

Citrus Viroids: Concepts and Considerations*

J. S. Semancik

ABSTRACT. The cataloguing of citrus viroids introduced at the 10th and 11th conferences of the IOCV served as a basis for the characterization of citrus viroids. From the definition of five "Groups" has evolved the complex collection of five different viroids, *Citrus exocortis viroid* (CEVd), Citrus viroid I (*Citrus bent leaf viroid*), Citrus viroid II (CVd-II) (*Hop stunt viroid*), *Citrus*

viroid III (CVd-III) and *Citrus viroid IV*, with the occurrence of dominant genome variants. Application of technical advances in sPAGE, RT-PCR, hybridization and molecular modeling has facilitated detection as well as an appreciation of biological potential and diversity of viroid quasi-species and multiple molecular forms. Unusual variants of CEVd have been detected in herbaceous hosts, some with greatly enlarged genomes constructed of terminal repeated sequences. The recently described Citrus viroid-OS with only 68% homology to the most similar citrus viroid, CVd-III, may represent a new citrus viroid. A clearer definition of the relationship of viroids to citrus diseases and responses of indexing cultivars has been made. Together with the description of the locus for pathogenicity for cachexia as a 5-6 nucleotide site, identity with xyloporosis has been demonstrated. Although new symptoms on citrus cultivars have been associated with different viroids, no other viroid-induced disease of economic importance has been added to exocortis and cachexia. Evidence has been introduced for a possible viroid etiology for gummy bark, gum pocket and yellow corky vein diseases. In contrast, efforts in different regions of citriculture have been directed to the application of the non-disease inducing viroids for citrus management and enhanced performance. The dwarfing of *Poncirus trifoliata* and related hybrid rootstocks with variants of CVd-II and CVd-III is most notable.

*Invited presentation

Host Directed Processing of *Citrus exocortis viroid* (CEVd)

J. A. Szychowski, and J. S. Semancik

ABSTRACT. Clones of *Citrus exocortis viroid* (CEVd)-related variants were recovered from a hybrid tomato (*Lycopersicon esculentum* X *L. peruvianum*) infected with CEVd-D92, a 463 nt variant containing a repeated sequence of 92 nt from the V-T2 domains of CEVd. The clones, designated as CEVd "D" forms, ranged in size from 372 to 459 nt. The nucleotide deletions detected in the progeny corresponded to specific locations occurring in the upper and/or lower T2 domain of CEVd. The seven different variants detected were transcribed and inoculated to a variety of herbaceous hosts and citrus species. Alternate hosts included chrysanthemum, cucumber, datura, eggplant, gynura, Rutgers tomato and the hybrid tomato source. Citron, sweet orange, kumquat and trifoliate orange seedlings were tested as citrus hosts. All RNA transcripts were transmissible in some but not all hosts. Either the "D" form and the CEVd or CEVd alone were detected as progeny when nucleotide changes occurred in either the upper or lower region of the T2 domain. The infecting CEVd "D" form was recovered as progeny when equivalent segment deletions were present in both upper and lower strands. Specific locations in the sequence within the T2 domain may suggest possible sites for initiation of replication and/or processing of multimers.

Detection of Citrus Viroids and *Apple stem grooving virus* in Citrus Trees in Japan Using Multiplex RT-PCR

Takao Ito, Hiroyuki Ieki, Katsuma Ozaki, and Tsultae Ito

ABSTRACT. Multiplex reverse transcription and polymerase chain reaction (RT-PCR) to simultaneously detect *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid*, *Hop stunt vi*roid (cachexia), *Citrus viroid III*, *Citrus viroid IV,* Citrus viroid OS, Citrus viroid I-LSS and *Apple stem grooving virus* (Citrus tatter leaf) has been developed. The results of multiplex RT-PCR were very consistent with those of other diagnoses such as ELISA, sPAGE and uniplex RT-PCR to detect each of the pathogens. Over 200 trees were diagnosed by multiplex RT-PCR. No CEVd was detected, but other viroids were detected in trees showing severe bark scaling in the trifoliate orange rootstocks, although combinations of viroids causing the scaling were not specifically identified.

Cachexia-Related Viroids Associated with Citrus Gummy Bark

N. Önelge, A. Çinar, J. A. Szychowski, and J. S. Semancik

ABSTRACT. A viroid etiology has been suggested for citrus gummy bark (CGB) disease of sweet orange based on the similarity of symptoms with cachexia. The consistent detection of *Hop stunt viroid*-related Group II citrus viroids (CVd-II) found in CGB-infected Washington navel and

the Turkish cultivar, Dortyol, while not in asymptomatic controls, further supports this view. A total of 46 clones constructed from RT-PCR products employing CVd-II specific primers were analyzed by sequence homology. RNA transcripts of representative clones from the different homology clusters were tested for transmission to citron. Infected citron budwood was used as an inoculum for CGB bioassay and testing of possible indexing hosts including Orlando tangelo, Parson's special mandarin, Rangpur lime, Palestine sweet lime and alemow in Turkey. All six clones derived from the asymptomatic Washington navel were closely related in size (302 nt) and sequence to the non-cachexia variant CVd-IIa found in the parent Washington navel in Riverside, CA. The 22 clones produced from CGB-infected Washington navel were almost equally divided between the CVd-IIa class of 301-304 nt (9 clones) and the CVd-IIc class of 296 nt (11 clones), with only single clones of 295 and 299 nt related to the CVd-IIb class. Among the 19 clones derived from CGB-infected Dortyol, three clones of the CVD-IIa class were found among the predominant cachexia-related CVd-IIb class of 11 clones (298-300 nt) and CVd-IIc class of five clones (296 nt). Transmission to citron by transcripts of representative clones from all clusters described by homology has been successful with CGB bioassay and indexing tests currently in progress.

Application of Hybridization Techniques for Viroid Indexing in Citrus Mother Trees in São Paulo State, Brazil

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ABSTRACT. Among the citrus viroids, *Citrus exocortis viroid* (CEVd) and Citrus cachexia viroid [*Hop stunt viroid*; Citrus viroid IIb (CVd-IIb)], are considered the most important economically. Due to their long latent periods, and their transmissibility by infected budwood and mechanically, it is necessary to control budwood production and nursery practices to prevention infections. Biological indexing in citron for CEVd and Parson's special mandarin or Clemelin 11-20 for CVd-IIb is commonly done, but these procedures take 3-6 mo for the former and 6-28 mo for the latter. In São Paulo State, Brazil, we have established fast and accurate methods of viroid detection using specific cDNA probes in imprint hybridization and dot blot assays for these two viroids to test mother trees at the Sylvio Moreiro Citriculture Center.

Detection of Citrus Viroids in Croatia

D. Škorić, J. A. Szychowski, M. Krajačić, and J. S. Semancik

ABSTRACT. A survey was made in the most important citrus growing regions of Croatia, including Opuzen, Split and the Adriatic islands, for the presence of viroids. Samples were taken from a number of citrus scions displaying no symptoms, or with bark cracking on trifoliate orange rootstock, stunting and reduced yield. Buds from lemons, mandarins and sweet oranges were budded onto citron seedlings for viroid amplification. Citron apex tissues were used for nucleic acid extraction and viroid RNA enrichment, and sequential-PAGE was used for detection. The identity of *Citrus exocortis viroid* (CEVd), Citrus viroid II (CVd-II; *Hop stunt viroid*), and *Citrus viroid III* (CVd-III) variants were confirmed by RT-PCR using viroid specific primer pairs. Viroids were detected in seven citrus samples, mostly as complexes consisting of two to four different viroids, the exception being Washington navel orange which harbored only CVd-IIa. Interestingly, all lemon samples produced a consistent viroid profile of CEVd, CVd-II and CVd-III. One tree, an Owari mandarin on trifoliate orange, was found to be viroid-free.

Protein-Protein Interactions Between *Spiroplasma citri* and its Insect Vector, *Circulifer haematoceps*

S. Collette, A. Boutareaud, M.-P. Dubrana, and M. Garnier

ABSTRACT. *Spiroplasma citri*, a sieve tube-restricted pathogen, is transmitted in a persistent manner by the phloem sap-feeding leafhopper, *Circulifer haematoceps*. After ingestion by the vector, the spiroplasmas have to cross the gut barrier, multiply in the hemolymph, and invade the salivary glands, before they can be injected by the feeding leafhopper, into the sieve tubes of a new host plant. There are thus two physical barriers, the gut barrier and the salivary gland barrier.

The successful passage of *S. citri* through these barriers is probably mediated by receptor ligand protein interactions. However, receptors on the insect organ surfaces and ligands on the *S. citri* surface, have not yet been identified. In order to identify the spiroplasma protein ligands and corresponding insect organ receptors, strategies based either on protein overlay (Far-western blots) or transposon mutagenesis have been developed. Protein homogenates of whole *C. haematoceps* bodies or of excised salivary glands were subjected to SDS-PAGE and blotted onto a membrane. Part of this membrane was incubated with solubilized *S citri* proteins. *S. citri* proteins linked to insect receptors bound to the membrane were detected with polyclonal antibodies against *S citri*. In this way, five protein-protein interactions were detected with the proteins from whole insect bodies, and seven with proteins from salivary glands. These data suggest adhesion related proteins may be involved in spiroplasma-insect interactions, and that *S. citri* invasion of salivary glands may require interaction with multiple receptors. Mutagenesis of *S citri* strain GII3, with transposon Tn *4001* allowed the selection of mutant G76, with the following properties: It multiplies and reaches high titers in *C. haematoceps* as does the wild strain GII3; it is transmitted to periwinkle by the insect vector but the number of *S. citri* cells transmitted is 50 times lower than the wild strain. Hence, symptom expression is delayed by 4 weeks. We have shown that symptom development is not the result of reversion to wild type spiroplasmas, indicating that G76 is pathogenic. The transposon disrupted gene, *sc76*, has been cloned and sequenced. It codes for a putative product sharing appreciable homology with a surface lipoprotein.

Cell Shape Determination in *Spiroplasma citri*: Organization of *mre*B Genes and Effect of *mre*B1 Disruption on Insect Transmission and Pathogenicity

W. Maccheroni, J-L. Danet, S. Duret-Nurbel, J. M. Bové, M. Garnier, and J. Renaudin

ABSTRACT. In bacteria such as *Bacillus subtilis*, determination of cell shape is due in part to the existence of a cytoskeleton close to the cell surface. This complex structure is constituted of actin-like helical filaments formed by the products of two related genes, *mre*B and *mbl*. Disruption of either of these genes causes alterations in cell morphology and may lead to cell death. During the Genome Sequencing project of *Spiroplasma citri*, we have found five distinct homologs of genes *mre*B/*mbl*, four of them organized in tandem and transcribed as two separated operons (two genes each). The fifth homolog was not physically related to the operons and showed the highest transcription level among all. We have therefore disrupted this gene, designated *mre*B1, by a single crossing-over recombination strategy in an attempt to gain more information on the cell shape determination in *S. citri*. On solid medium, mutants harboring an inactivated *mre*B1 showed colonies with reduced size as well as less diffused due to reduced motility. In liquid medium, mutant cells appeared more elongated as compared to the wild-type strain, and they also aggregated more readily during growth. Leafhoppers (*Circulifer haematoceps*) microinjected with *mre*B1 mutants, were colonized by the bacteria at high titers, but transmission to periwinkle plants was reduced. Nevertheless, once infected by the mutants, plants developed typical symptoms, although delayed by a couple of weeks.

Fructose and Trehalose Greatly Enhance Transcription of Their Respective Operons in *Spiroplasma citri*

P. Gaurivaud, W. Maccheroni, J. Renaudin, J. M. Bové, and M. Garnier

ABSTRACT. Recently, fructose utilization, as directed by genes belonging to the fructose operon, was shown to be involved in *Spiroplasma citri* phytopathogenicity. The fructose operon comprises three genes: *fruR*, *fruA* and *fruK*. The first gene, *fruR*, codes for a protein showing high similarity with transcriptional regulators of sugar catabolic operons. The second, *fruA*, codes for the fructose permease, allowing uptake and concomitant phosphorylation of fructose-1-phosphate, and *fruK* codes for an ATP-kinase phosphorylating fructose-1-phosphate into fructose-1,6-biphosphate. We have now further investigated the role of *fruR* in the transcription regulation of the fructose operon. The trehalose operon, the organization of which is very similar to that of the fructose operon, has also been studied. In *S. citri* wild type strain GII-3 (phenotype $fruR^{+}A^{+}K^{+}$), *in vivo* transcription of the fructose operon was greatly enhanced more than ten-fold by the presence of fructose in the growth medium, while glucose had no effect and did not exert catabolic repression.

With *S. citri* mutant Fru36 (phenotype *fruR*A^+K^+) in which *fruR* is not present, fructose did not stimulate transcription of *fruA-fruK*. Fructose utilization by various strains of *S. citri* was also studied. As expected, strong fructose utilization occurred with strain GII-3, and no utilization with mutant GMT 553 (phenotype *fruR*$^-$*fruA*$^-$*fruK*$^-$). Only weak fructose utilization occurred with strain Fru36, i.e. mutant GMT 553 complimented with *fruA* + *fruK*. These experiments indicate that the product of *fruR*, FruR, appears as an activator of the fructose operon. The fructose operon promoter was characterized by primer extension, and the –35 and –10 boxes of the promoter identified. A direct repeat of 9 bp (5'-TTTTTGGTT-3') overlapping the –35 box was observed. This T-rich repeat is similar to the binding site of the *lol*R repressor of the *Bacillus subtilis* inositol operon, and could represent the binding site of FruR. Similar results have also been obtained with the *S. citri* trehalose operon. Trehalose is an important sugar in insects.

Short Sequence Repeats in the Genome of *Xylella fastidiosa* and Their Use in the Analysis of the Genetic Diversity of Related Strains

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ABSTRACT. In Brazil, *Xylella fastidiosa* has been responsible for causing diseases in both citrus and coffee. Methods for distinguishing between bacterial strains are important for detection of disease outbreaks, epidemiological analysis and also for understanding the genetic structure of microbial populations. Short sequence repeats (SSRs) with a potential variable number of tandem repeats (VNTR) within prokaryotic DNA, have been used as markers for strain discrimination with advantages in relation to other PCR-based techniques. The complete sequencing of the genome of *X. fastidiosa* strain 915c has allowed the identification of repetitive DNA motifs. In the present study, we conducted a search for SSR size variation in different strains of *Xylella*, comparing the results with the frequently used RAPD method. The genomic DNA sequence of *X. fastidiosa* strain 9a5c was screened for repetitive DNA with the Tandem Repeat Finder version 2.02 software. Sets of primers with potential for locus-specific amplification were designed from sequences bordering the repeat and used to amplify the DNA. Sixty-seven regions were found to have perfect repeats. Although mono- and di-nucleotides were absent, we found several intermediate length repeats containing 7, 8 and 9 nt, which we examined for allelic polymorphism using PCR. Five genuine VNTR loci were highly polymorphic within a set of 27 *X. fastidiosa* strains from different hosts. The average genetic diversity (H) value estimated for VNTR loci was H = 0.51, while random amplified polymorphic DNA (RAPD) loci gave H = 0.17. For *X. fastidiosa* strains from citrus, some specific VNTR loci gave H = 0.83, while the maximum value given by specific RAPD loci was H=0.12. These results show that VNTR markers provide a high-resolution tool for epidemiological, genetic and ecological analysis of citrus-specific *X. fastidiosa*.

Fast and Accurate Quantification of *Xylella fastidiosa* from Citrus Plants Through Real-time Quantitative PCR

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ABSTRACT. *Xylella fastidiosa* is the causal agent of citrus variegated chlorosis (CVC), a destructive disease of sweet orange varieties in Brazil. PCR-based assays constitute the principal diagnostic method for detection of the bacteria in asymptomatic trees. However, PCR allows only qualitative detection. In the present study, we established a real-time quantitative PCR (r-t qPCR) to quantify *X. fastidiosa* in citrus tissues. The nucleotide sequence from a CVC *X. fastidiosa* strain (GenBank – AE003946) was used for the design of the primers and TaqMan™ probe. Known amounts of *X. fastidiosa* DNA, ranging from 1.09×10^0 to 1.09×10^5 copies, were used to make standard curves (i.e., mean C_T values x $\log_{10}$ of the initial quantity of DNA), that presented a high linear relationship ($R^2 = 0.973$) and reproducibility (coefficient of variation from 0.84 to 3.2% and 1.13 to 4.21% in intra- and inter-assay variability, respectively). R-t qPCR containing DNA from 12 endophytical citrus bacteria showed no increase in the fluorescence signal during PCR ($\Delta R_n < 0.2$ U after 40 PCR cycles), indicating high specificity of this r-t qPCR. The *X. fastidiosa* cell number detected in the foliar xylem increased according to the age of the leaf (R^2=0.944), not detecting the bacteria in the upper section of midribs in young leaves, indicating timewise and spacewise distri-

bution patterns of *X. fastidiosa*. In addition, the number of *X. fastidiosa* cells was quantified in petioles of Pera sweet orange leaves (783 and 2,528 copies per ng/µl of citrus DNA, infected by needle procedure and natural inoculation, respectively) and Murcott tangor (none, C_T = 40), confirming the susceptibility and resistance status respectively, of these citrus varieties to the bacteria. To our knowledge, this is the first report of an r-t qPCR approach described for a citrus pathogen. The success of our r-t qPCR provides a tool for studies of different aspects of the *Xylella*-citrus pathosystem, and can be incorporated into a breeding program to select plants resistant to CVC.

Phytopathogenicity of *Xylella fastidiosa* CVC Strain: Genetic Analysis by Gene Inactivation

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ABSTRACT. *Xylella fastidiosa* is a gram-negative, xylem-restricted, sharpshooter-transmitted bacterium that causes economically important diseases such as citrus variegated chlorosis (CVC). The genome sequence of the CVC strain has revealed several genes that may be involved in phytopathogenicity, such as those involved in exopolysaccharide production, toxin synthesis, host cell wall degradation, adhesion, aggregation and iron uptake. Even though the putative systems have been identified in the *X. fastidiosa* genome, their role in plant colonization and symptom development has to be assessed. A classical approach to identify genetic determinants involved in pathogenicity is to produce and characterize mutants that are non-pathogenic or less virulent than the wild type strain. The transformation of *X. fastidiosa* with an artificial plasmid (p16Kdb) carrying the chromosomal origin of replication (*OriC*) and the kanamycin-resistance gene driven by an *rRNA* promoter of *Xylella* was previously achieved. This plasmid was found to be integrated into the chromosome at the *rRNA* promoter site by homologous recombination involving one crossing-over. This result indicates that disruption of genes by homologous recombination is possible in *X. fastidiosa*. We propose to use this approach to produce mutants disrupting 27 different genes involved in the above mechanisms. Initially we will disrupt the gene coding for β-galactosidase, which will be used as a phenotypic marker gene since we have detected the expression of β-gal in several strains. Plasmids containing a truncated copy of each of these candidate genes were constructed based on a plasmid we developed named pDT8 which contains the *OriC* and the tmRNA tag of *X. fastidiosa*, the kanamycin and chloramphenicol resistance genes. Transformants of the bacterium were obtained with these recombinant plasmids indicating that pDT8 is able to transform *Xylella*. The analysis of the integration of the recombinant plasmids by PCR, Southern blot and chloramphenicol resistance is in progress. The clones in which the plasmid is integrated into the candidate gene will be isolated and their phenotype characterized. The pathogenicity of each mutant will be evaluated on sweet orange seedlings and in periwinkle and tobacco. We also constructed a plasmid named pDTGpoly based on pDT8, containing the GFP reporter gene and the *pglA* gene of *X. fastidiosa* for the study of plant colonization and insect localization.

Differential Expression Profile of *Xylella fastidiosa* Cells Grown Under Aggregating and Non-Aggregating Conditions Revealed by Microarray

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ABSTRACT. Plant-associated bacteria produce extra-cellular polysaccharides (EPS) that allow adhesion within hosts and to other bacterial cells, playing important roles in bacterial survival and pathogenicity. Ultrastructural studies of *Xylella fastidiosa* strains in xylem vessels have shown bacterial cells immersed in electron dense regions, probably composed of EPS. The aggregated cells appeared to attach to the xylem vessels by extra-cellular strands produced by the bacteria. Primary *X. fastidiosa* isolates obtained from infected plants develop into small, solid and compact colonies. These primary isolates display typical colony morphology with a ring around them, where new adherent micro-colonies can be observed. After several subcultures, the bacteria lose the ability to develop such colony morphology. This situation can also be observed when *X. fastidiosa* is grown in liquid medium, since after isolation from sweet orange trees, the cultured cells attach to the flask walls, building aggregates that can cover a great part of the available sur-

face. The more passages in liquid medium, the more reduced the ability of the bacterium to form such aggregates. Research elsewhere has established a link between the loss of aggregating ability and decreased pathogenicity in Pierce's disease *X. fastidiosa*. The main goal of our work is to evaluate and compare the gene expression profile of aggregating and non-aggregating cells grown in both liquid and solid PW media, with the aid of microarray hybridization experiments. For this purpose, a complete *X. fastidiosa* biochip, carrying ~2,500 ORFs found in the bacterium's genome, was hybridized with Cy3 and Cy5-dCTP-labeled cDNA obtained from aggregating and non-aggregating cells, respectively. Analysis of this composed array image revealed groups of genes that were specifically regulated under such conditions, indicating a complex regulatory circuitry in both the liquid and solid media-grown cells.

Citrus Variegated Chlorosis Distribution in Northwestern Paraná State, Brazil

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ABSTRACT. Citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa*, is one the most important diseases of citrus in the northwestern region of Paraná State, Brazil. The objective of this research was to evaluate the temporal progress of CVC in commercial orchards of citrus. For this, CVC progress was assessed monthly during a 14-mo period in five orchards of Pera, Valencia, Folha Murcha, Natal and Lapar73 sweet oranges. CVC was assessed in plants of 10 rows in each orchard through a 0-3 rating scale, where 0 = no symptoms, 1 = few symptomatic leaves in few branches, 2 = several symptomatic leaves in several branches with some dead fruit and branches, 3=declining trees with small fruits. It was observed that the Natal orchard had the highest percentage of plants having rating 2 (38%). In Pera, Valencia and Folha Murcha, the highest number of trees were rated 1, with 44.7, 58.6 and 20.1% respectively.

Behavior of Mandarin Varieties Under Pressure of Leprosis and Citrus Variegated Chlorosis Pathogens in São Paulo State, Brazil

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ABSTRACT. There is a growing interest in mandarin production in São Paulo State, Brazil due to an increase in both internal and export demands. It is currently estimated that there are approximately 10 million mandarin trees in the state, comprising about 5% of the state's citrus production. Mandarin and mandarin hybrids grown mainly include Ponkan, Murcott, Cravo and Willow leaf, with smaller numbers of Satsuma, Dancy and Orlando tangelo. Field surveys and research studies have shown that mandarins and most of their hybrids are less affected by Citrus leprosis virus (CiLV) and the citrus variegated chlorosis (CVC) bacterium, *Xylella fastidiosa* which are prevalent in the state. A number of growers in areas with high CVC inoculum pressure are replacing orange trees with Orlando and other tangelos. Amongst tangors and mandarins, most varieties that have been evaluated have shown resistance to CiLV, while some others did develop severe symptoms.

The Role of the FAO in Rehabilitation of the Lime Industry in Oman and the Development of a Certification Program

C. N. Roistacher, M. Taher, and R. F. Lee

ABSTRACT. The comprehensive role of the Food and Agricultural Organization of the United Nations (FAO) in implementing programs of assistance to citrus producing countries in overcoming problems caused by virus and virus-like diseases has never been presented to the IOCV. Using witches' broom disease of lime (WBDL) in Oman as an example, we present the role of the FAO in attempting to overcome this devastating disease which has wiped out most of the lime trees in the northern Batinah coastal region. Based on a request from the Omani government, the FAO extended its Technical Cooperation Program assistance to 'Control virus and virus-like diseases of citrus in Oman'. After fact-finding visits, during which, in addition to WBDL, symptoms of stubborn, gummy

bark, psorosis, concave gum, a decline of Tahiti lime and a decline of sweet lime, were observed, it was recommended to revive the lime industry in the remote southern Dhofar region, enforce strict quarantine regulations, and establish a certification program for all citrus grown in Oman.

Current Situation of Systemic Citrus Pathogens and Vectors in Mexico

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ABSTRACT. The citrus industry of Mexico is of major economic and social importance. At present, there are approximately 495,000 ha of citrus and 53,000 growers. Most of the industry consists of sweet orange (about 70%), Mexican lime (19%) and Tahiti lime (5%). Mandarins and mandarin hybrids, grapefruit and lemon are planted to a much lesser extent. Sour orange is the predominant rootstock for sweet orange, grapefruit, Tahiti lime and mandarins. Approximately 45% of the Mexican lime trees are grown from seed; the rest is predominantly on *Citrus macrophylla*, with some on sour orange, volkamer lemon and *C. amblycarpa*. *Citrus exocortis viroid* (CEVd) occurs in a symptomless condition in most old orchards of sweet orange on sour orange. Analysis by RT-PCR of Tahiti lime samples revealed the common occurrence of both CEVd and Citrus cachexia viroid in mixed infections. *Citrus viroid III* was also found in a few samples. *Citrus tristeza virus* (CTV) was detected for the first time in 1983 in the northeastern state of Tamaulipas, and it has been found additionally as symptomless infections in other states; in no case have disease symptoms been observed in any field trees. The aphid species capable of CTV transmission prevalent in most citrus areas are *Aphis gossypii*, *A. spiraecola* and *Toxoptera aurantii*. *T. citricida* has been established in the Yucatán peninsula since early 2000. Symptoms of bark scaling typical of psorosis are common in old groves of sweet orange, grapefruit and mandarins, but at a low incidence throughout the country. There are a few sweet orange trees on rough lemon in Yucatán with blight-like symptoms. Other graft-transmissible agents have not been detected at this time.

Efficacy of Citrus Indexing Reactions with Mixed Infections

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ABSTRACT. The success of any program for importation, maintenance and release of certified budwood for propagation of clean nursery stock, is dependent upon the accuracy and reliability of disease diagnosis. In the case of citrus, most of the important diseases are carried by budwood and can only be diagnosed using indicator hosts grown under proper environmental conditions. Often budwood of imported varieties entering the California Citrus Clonal Protection Program (CCCPP) have been collected from field trees and can contain one or more of the following disease agents: *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), viroids, vein enation and Citrus tatter leaf virus (CTLV). Interactions among these pathogens can affect the accuracy and reliability of the indexing test. Symptoms of vein enation in Mexican lime and sweet orange do not develop if CTV or CTLV is also present. However, vein enation is clearly expressed in sweet orange when CPsV or viroids are also present, even though sweet orange is not typically used as an indexing host for vein enation. Similarly, psorosis symptoms are enhanced in Mexican lime when viroids are present.