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INVITED SPEAKERS ABSTRACTS

GENOME RESEARCH AND GENETIC IMPROVEMENT OF CITRUS DURING THE PAST YEARS IN THE P.R.CHINA

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Although the Huanglongbing disease has seriously affected citrus production in southern China, both the acreage and total production of citrus has been increasing during the past decade in this country. The total production has reached at more than 34 million MT in 2014. The improved variety structure, i.e. more late varieties or late harvest production by hanging the fruits on the trees in the Three Gorges area and the middle-upper reaches of Yangtze River, has benefited the supply and also the price during the past years. Now, the earliest mandarin from the natural production can meet the market in the middle of July, and the latest ones in the Three Gorges areas will be harvested until late of May or June.

Citrus variety improvement during the past decade has gained a great achievement in China. A total of more than 20 new cultivars including mandarin, navel orange, and tangor etc. have been released since 2005. Most of the cultivar are from the bud-sport selection, such as the early season ponkan in Hunan, early mature navel orange in Jiangxi, and seedless red tangerines in Hubei and in Guizhou, seedless sweet orange in Hunan, and late season seedless tangerine in Guangdong. It is worth pointing out that 'Jinshaju' tangerine, an early tangerine was released by CRIC in Chongqing, which is the first commercial cultivar from planed crossing breeding in China during the past 20 years. Another important progress worth mentioning is the seedless ponkan selection from Huazhong Agricultural University(HZAU). Ponkan is one of the key cultivars in citrus industry of China; it's easy peeling, crisp and tender flesh attracts consumers. Citrus breeders hare been pursuing the seedless cultivar for decades; A few selections were obtained, and little acreage was planted with them since the low production and small fruit. The new selection of seedless ponkan bears the normal size fruits and the production is near the same as the seedy parents. Another work should be highlighted is the protoplast fusion research in China, as has lasted for more than 20 years. A seedless cultivar 'Huayou No.2' pummelo from HZAU was registered in 2015. It is from fusing protoplasts of Satsuma mandarin with seedy pummelo; with the molecular marker selection and ploidy screening by flow cytometry, the diploid plants with the cytoplasm of Satsuma mandarin and nuclear of pummelo were obtained more than 10 years ago, and the cybrid with the pummelo nuclear and the cytoplasm of Satsuma mandarin was protected as a cultivar by MOA last year. Transgenic research of citrus with the aim to improve citrus resistance to bacteria such as citrus canker and Huanglongbing has also made good progress at CRIC of Chongqing; a few lines has gotten the permission of MOA to test the resistances in the controlled environment; results

seems very positive.

Five years ago, citrus genome work was listed on the agenda for Chinese citrus researchers. With the aid of DH line from the anther culture of Valencia sweet orange in 2010, the draft genome of this species was finished in 2011 and published online in the late of 2012. The assembled sequence covers 87.3% of the estimated orange genome. A total of 29,445 protein-coding genes were predicted; half of which are in the heterozygous state. This platform has served the citrus community; the website had been visited by more than 20 thousand times at the end of 2015. More citrus species including *Fortunella spp.*, *Poncirus trifoliata*, C. maxim, C. reticulata, and the relatives of *Citrus*, such as *Severinia buxifolia*, have been or are being sequenced and assembled. More information will be added in the platform. It will be sure that the genome information will benefit not only the citrus genetic improvement, but also the understanding of the citrus plant resistant/susceptible to pathogens such as HLB.

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Supported by NSFC and CARS (China Agricultural Research System).

USING THE CTV VECTOR TO ATTEMPT TO CONTROL HLB

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We created a transient-expression vector based on *Citrus tristeza virus* (CTV) to express foreign genes or silence endogenous genes in citrus. This vector can be graft transmitted and expressed in a range of citrus varieties of different ages. The original objective was to build a vector as a tool for citrus improvement. With the emergence of the citrus greening disease into Florida, we began screening genes for activity against the bacterial pathogen or the psyllid vector, with the intention of building transgenic citrus with the genes found to be effective. However, the spread of greening in Florida has been more rapid than expected and there is desperate need to find solutions quicker than transgenic trees. The CTV vector is now being considered for use in the field as an interim measure until transgenic citrus becomes available. A disadvantage of the vector is that it does not permanently retain foreign sequences, but a major advantage of the vector is that nothing is put into the environment permanently. Additionally, if we can find appropriate genes, it is possible that the CTV vector can be used to treat trees in the field after they become infected with HLB. We are screening for antimicrobial peptides to limit the pathogen and are using RNAi to limit production of the insect vector in citrus.

BREEDING OF SCION AND ROOTSTOCK HLB TOLERANCE THROUGH GENOME-BASED INFORMATION

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Huanglongbing is the most challenging citrus disease ever experienced globally because of the many biological complexities inherent to this pathosystem that make management and control difficult, and because of the severe economic consequences to the agricultural communities that produce citrus fruit. Various approaches are being explored to manage existing plantings, as well as to mitigate disease symptoms and severity in HLB-affected trees, to delay decline and extend productivity. The most desirable long-term control option is the development of highly resistant or immune trees and there are many programs across the globe pursuing several unique strategies to accomplish that goal. In the interim, new plantings utilizing more tolerant scion or rootstock cultivars may enable struggling citrus industries to remain economically viable for a sufficient time until the resistant trees of the future, developed through whatever means can succeed, are available for planting. Understanding the mechanisms of enhanced tolerance to HLB through genomic-based approaches can provide tools to accelerate not only the development and deployment of more tolerant cultivars, but also to provide the basis upon which more robust, highly resistant cultivars may be developed. Some current knowledge and information will be reviewed, and certain potential research directions that might lead forward to the development of resistance to HLB disease will be highlighted and discussed.

MOLECULAR MECHANISMS BEHIND THE HLB SYMPTOM VARIATIONS AND RAPID SELECTION FOR VARIANT CITRUS PLANTS WITH GREATER HLB RESISTANCE/TOLERANCE

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Huanglongbing (HLB) is a devastating bacterial disease of citrus worldwide due to its intracellular and systemic infection. Various HLB symptoms are observed in different species/varieties of citrus plants: from yellow shoots to blotchy mottles on the leaves, from vein yellowing/vein corky to mosaic/green island as zinc deficiency on the leaves, from whitish discoloration to small green leaves, etc. These variations of symptoms, resulted from the host-pathogen interactions along with the impact of environmental conditions, are not only present on individual plants from a variety but also exist on individual branches of an infected plant. Our results indicated that the adaptation of the bacterial populations, such as the dynamics of '*Candidatus* Liberibacter asiaticus' (*Ca*Las), plays an important role in induction of various symptoms, which was affected by the number and recombination of *Ca*Las prophages/phages. Meanwhile, the selection of the host plants (resistance/tolerance) for the bacterial populations is also critical for symptom expression during disease progression. Based on severity, we divided HLB symptoms into four grades. It is worth noting that our newly identified biomarker from host plants has shown a positive correlation with the grades of HLB symptom severity, and that gene expression profiling of different grades of infected leaves rationalized the differentiation based on the dynamics of this biomarker. In addition, three effectors that either target host mitochondria or chloroplasts may be involved with symptom induction. Based on the variations of the HLB symptoms, the bacterial populations/titers, and the dynamics of the biomarker, we propose new approaches that allow for rapid selection of variant citrus plants including bud sports with greater HLB resistance/tolerance together with protocols and criteria for their evaluation.

DEVELOPMENT OF GENOME-BASED THERAPEUTICS TO CONTROL HLB

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Huanglongbing (HLB) is a highly destructive citrus disease which threatens citrus production worldwide. The disease is associated with Gram-negative, phloem-limited bacterium named '*Candidatus* Liberibacter' in the members of the *Rhizobiaceae* α-Proteobacteria. There are three species of '*Ca*. Liberibacter'; '*Ca*. L. asiaticus', '*Ca*. L. africanus' and '*Ca*. L. americanus', each was named based on its presumptive origin from different continents. Currently, the strategies to curtail the spread of disease are limited due to the fact that all commercial citrus trees are susceptible and non-effective psyllid control measurement. Thus, methods to kill or suppress the HLB pathogens to prevent new infection and to restore tree growth and production are urgently needed. Since the causative agents are yet unculturable, Koch's postulates have not been fulfilled, knowledge and information regarding genetics and pathogenesis of the pathogens is limited. In spite of the challenges, we have successfully obtained complete genome sequences from three Liberibacter species. Comparative analyses of Liberibacter genomes provide unprecedented insights into the evolutionary history, phylogenetic and metabolic capacities in these pathogenic bacteria. Genomic information help identify the putative virulence genes/factors shared among three HLB Liberibacters. Orthologous gene replacement technique has been successfully developed to confirm the functions of key virulence genes in Liberibacters. These results facilitate development of antivirulence drugs that specifically target functional domains of the virulence genes and disarm pathogenicity. Unlike antibiotic treatments, antivirulence drugs exert no selection pressure on bacterial survival and therefore impose no selection pressure on pathogens for drug-resistant mutations. Here we represent research progress towards the development of a novel therapeutic strategy for HLB management.

GRAFT-TRANSMISSIBLE CITRUS DISEASES IN THE P.R. CHINA —RESEARCH DEVELOPMENTS

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In the P.R. China, nine graft-transmissible pathogens have been identified towards citrus, including *Candidatus* Liberibacter asiaticus, *Citrus tristeza virus* (CTV), Citrus tatter leaf virus (CTLV), *Citrus exocortis viroid* (CEVd), *Citrus yellow vein clearing virus* (CYVCV), *Satsuma dwarf virus* (SDV), *Citrus vein enation virus* (CVED), Citrus cachexia viroid (CCaVd) and *Citrus chlorotic dwarf virus* (CCDV). Of which, the first five cause damage towards field citrus trees, whereas the latter four were occasionally detected from the imported citrus material or field trees.

Since 2001, over 100 modern virus-free citrus nurseries have been established in 13 provinces through the implementation of virus-free scheme, therefore the loss caused by graft-transmissible citrus pathogens, especially for such the non-vector transmissible as CTLV, SDV, CEVd, has dramatically decreased. Since 2012, however, Huanglongbing (HLB) problem increased in severity in some fast growing citrus provinces such as Guangdong, Jiangxi and Hunan, for example, over 20 million citrus trees have been cut off due to HLB damage in south Jiangxi within recent three years. So, a series of research activities have been focused on the control of HLB, a few progresses have been made: 1) its putative prophage particles were observed in sweet orange; 2) the whole genome of three Chinese isolates were sequenced, bio-information accumulates quickly, focusing on the prophage genomic region with high genetic variation and recombination events, two HLB origin centers were proposed in the P.R. China; 3) transgenic citrus lines against HLB have been being addressed to field trials for safety evaluation; 4) a few effective combinations of pesticides have been selected to control citrus psyllids (*Diaphorina citri*); 5) natural thermotherapy towards citrus tree canopy by covering PVC mulch showed somewhat efficacy to reduce the symptoms.

Among the above mentioned citrus virus pathogens, a few vector-transmissible viruses such as CTV, CYVCV are of much importance for research due to the difficulty in provention in the field. Since severe stem-pitting type of CTV isolates is widely distributed in the P.R. China, a few mild isolates with potential protective capability, screened from thousands of field CTV isolates have been being applied in field trials under efficacy evaluation. Also the protein-protein interactions between CTV isolates and its sensitive hosts, CTV isolates and brown citrus aphids (*Toxoptera citricida*) have been addressed with somewhat progress. CYVCV is a new virus caused severe damage towards lemon industry in the P.R. China within

a few years, citrus whitefly (*Dialeurodes citri*) has been experimentally proved the vector of this virus. All viruses above mentioned have been sequenced, and approached for phylogenetic analysis. Furthermore, a few types of infectious viral vectors have been constructed. Although viroids are of less importance than other pathogens towards citrus industry, a few new variants such as *Citrus viroid V* and *Citrus viroid I-LSS* were detected in the P.R. China. The distribution of the above pathogens has been monitored.

A lot of attempts have been made for improving the diagnistic methods towards the above nine pathogens, some showed higher efficacy, and have been being widely applied for quick diagnosis.

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DIVERSITY OF VIRUSES ASSOCIATED WITH CITRUS LEPROSIS SYMPTOMS

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For decades, symptoms of citrus leprosis - chlorotic or necrotic localized lesions in leaves, stems or fruits - were associated with the presence of *Citrus leprosis virus* (CiLV), transmitted by *Brevipalpus spp*. mites. By mid-1990s, two types of CiLV were found: one that replicates in the cytoplasm of infected cells (*Citrus leprosis virus C*, CiLV-C), and another one that replicates in the nucleus (Citrus leprosis virus nuclear type, CiLV-N). Molecular biology tools and NGS have led to the identification of other leprosis-associated viruses (LAV) belonging to three distinct genera. The cytoplasmic type of LAV (LAV-C) are divided into Cilevirus (ss+RNA, bipartite) and Higrevirus (ss+RNA, tripartite), while the nuclear type (LAV-N) are members of the tentative genus *Dichorhavirus* (ss-RNA, bipartite). Recent studies have addressed not only variability among genera or species, but also within strains or isolates. What was considered *latu sensu* CiLV-N (syn. *Citrus necrotic spot virus*) found in Mexico and Colombia is likely a citrus strain of *Orchid fleck virus*. In herbarized material from Florida and from field samples from Brazil, two other strains (possibly species) of LAV-N have been identified, suggesting that at least three tentative dichorhaviruses are able to cause leprosis symptoms in citrus. The diversity of LAV-C is not less complex. There are reports of few volkameriana lemon trees infected by a higrevirus in Hawaii, and CiLV-C2 tentative species is prevalent in citrus orchards in Colombia. However, by far, CiLV-C is the main LAV in citrus worldwide. Data available on CiLV-C molecular variability suggest low spatial-temporal diversity among isolates, although a survey in Brazilian citrus orchards revealed the occurrence of a divergent clade of CiLV-C whose members share ~85% similarity with the type-member CiLV-C and are involved in putative interclade recombination events. Overall diversity of LAV and particular molecular variability of CiLV-C will be discussed.

Financial support: CNPq (375843/2012-4 and 401564/2012-6) and FAPESP (2014/08458-9).

CITRUS VIROIDS-RESEARCH DEVELOPMENTS

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In the 19th IOCV conference I had the honor to co-present with Dr. Nuria Duran-Vila the 80 years journey of the citrus viroids research (1934-2013). In this presentation,I will attempted to summarize the research developments on citrus viroids for the past three years. A general literature search for the key word "viroid" revealed 2190 articles, indicating an active international viroid research community. The more specialized literature search for the words "citrus viroid", in the articletitle, identified 22 publications. Seven articles reported citrus viroids for the first time in new areas or hosts. In North Africa, *Hop stunt viroid* (HSVd) was reported in Morocco and in Egypt in association with gumming and stem pitting of *Citrus volckameriana* while *Citrus viroid V* (CVd-V) was identified in Tunisia. In Asia, *Citrus exocortis viroid* (CEVd) and *Citrus dwarfing viroid* (CDVd) was reported in Syria, and *Citrus bent leaf viroid* (CBLVd) was identified in the United Arab Emirates. In Oceania, New Zealand reported the CDVd and Australia the CEVd in association with *Petunia spp*. In addition, two articles reported surveys and occurrence of citrus viroids in Korea and Greece. Four articles described interactions of host-viroid (*C.sinensis*-CEVd, HSVd and CDVd), virus-viroid (*Citrus tristeza virus*-CDVd),and viroid-viroid (CEVd-HSVd). Three articles presented molecular, structural and phylogenetic analysis of CVd-I-LSS and CVd-V in Pakistan and China and HSVd and CBLVd from *C.limettioides* and *C.sinensis* expressing"gummy stem blight"in Iran. Three articles described the development and application of quantitative real time PCR for the multiplex detection of CEVd and HSVd. Two articles reported the detection and identificationof HSVd and citrus bark cracking viroid in severely diseased *C.limon* in China and hops (*Humulus lupulus*) in Slovenia, respectively. Finally, Dr. Moshe Bar-Joseph published a review article for the history, emergence, and eradication of xyloporosis in the IOCV *Journal of Citrus Pathology*.

CITRUS PSOROSIS VIRUS: STATE OF ART

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Citrus psorosis disease was reported more than one century ago. This graft transmissible disease has caused serious economic looses in many citrus regions of the world. Since the 80s, *Citrus psorosis virus* (CPsV) was closely associated with psorosis disease and characterized, and currently studied. This virus has circular and filamentous particles of two different sizes with snaky appearance, leading to call the genus *Ophiovirus* and *Ophioviridae* to the family. We found that CPsV is tripartite, each particle composed by one ssRNA of negative polarity coated by the 48K protein or CP, which is encoded in RNA3. The 54K protein, encoded in the RNA2, is the movement protein or MP, and is also involved in the suppression of the antiviral silencing pathway. In the RNA1 two genes are located, the gene of the RNA-dependent RNA polymerase (RdRp) and the 24K protein, which interferes with the endogenous miRNA biogenesis and presents activity as viral suppressor of the post transcriptional gene silencing.

Over the years, we and other labs have developed rapid and sensitive protein- and RNA-based detection protocols, helping certification programs, and testing CPsV in many citrus-producing areas. Progress has been done in the identification of the two components (psorosis A and psorosis B) traditionally associated with non-scaled and scaled bark inoculum, respectively. Analysis of genetic variation and evolutionary forces shaping the CPsV populations, from psorosis-infected plants and study their interactions are important contributions of the knowledge of the virus and its behaviour in citrus and citrus relatives. Today, we have advanced in studies on the interactions between virus and host factors. We count with transgenic sweet orange lines resistant to both psorosis A and psorosis B, and also with lines with variable degrees of resistance.

GENOME-BASED MOLECULAR BREEDING OF VIRAL RESISTANT SILKWORM

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Bombyx mori is a common lepidopteran model and an important economic insect for silk production. Sericulture faces biological challenges from pathogenic viruses, fungi and bacteria, which cause losses of almost 20% of potential cocoon production each year. A few resistant silkworm strains have been bred by traditional methods and none have been applied in sericulture.

Following completion of the draft sequence, detailed sequencing and resequencing of the silkworm genome, researches in interaction of silkworm host and pathogen have been advanced, which including invasion of the host by the pathogen, host response, and enhancement of host resistance. The Toll and Imd pathways play crucial roles in activation of immune responses to invading microbes, of which the gene members have been identified from the silkworm genome. *Bacillus bombysepticus* α-toxin directly bound to G protein-coupled receptor kinase 2 (GRK2), activated cAMP/PKA signal transduction altered downstream effectors that affected homeostasis and fundamental biological processes, disturbing the structural and functional integrity of cells, resulting in death of silkworm. BmNPV activates ERK and PI3K/Akt signaling pathway via BmEGFR, this virus also induces the upregulation of EGF and downregulation of Spry to activate EGFR/ERK signaling pathway, which directly regualte the transcription of viral late gene. These studies have greatly promoted the molecular breeding for silkworm disease resistance.

Transgenic technology is an available tool for species improvement. According to the infection process of BmNPV, we selected different target genes to generate transgenic silkworms with high antiviral capacity, that suppress BmNPV at initial infection and affects virus mRNA, viral protein synthesis, and host immunity. Most studies revealed that the resistance was significantly enhanced while growth and economic traits were unchanged in the antivirus silkworms that created by molecular methods, which displayed greater applied prospect.

In summary, the molecular breeding of silkworm has been significantly promoted by the completion of silkworm genome project, which overcomes the problems associated with traditional breeding methods and would provide new strains for sericulture.

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APPLICATIONS OF NEXT GENERATION SEQUENCING IN PLANT VIROLOGY

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Next-generation sequencing (NGS) technologies provide a high throughput, efficient and fast DNA sequencing platform compared to the standard and traditional technologies. The technologies have been rapidly applied to several areas of plant virology including virus/viroid genome sequencing, discovery and detection, ecology, replication and antiviral mechanism since 2009. We report here the use of small interfering RNAs (siRNAs) and Hiseq RNA sequencing for the study of virus-host interaction and virus detection/diagnosis. Analysis of the siRNA sequencing data revealed the presence of a genetically distinct class of virus-activated siRNAs (vasiRNAs) in *Arabidopsis thaliana*. We propose that antiviral RNAi activates broad-spectrum antiviral activity via widespread silencing of host genes directed by vasiRNAs in addition to specific antiviral defense by viral siRNAs. Both siRNA and Hiseq RNA sequences were used to identify known and unknown pathogens from citrus, other fruit trees and sweet potato. The results showed that fast, accurate, and full indexing and identification of the pathogens were achieved by both methods. It is expected that NGS will play a very prominent role in fundamental and applied research areas of plant virology.

ORAL SESSION ABSTRACTS

MOLECULAR CHARACTERIZATION OF A GROUP 16SrDNA III PHYTOPLASMA ASSOCIATED WITH HLB-LIKE SYMPTOMS IN SWEET ORANGE IN BRAZIL

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Huanglongbing (HLB) was found in Brazil in 2004, with *Candidatus* Liberibacter americanus infecting most of the trees and *Ca*. L. asiaticus only a minor proportion. However, since 2006, *Ca*. L. asiaticus has been the predominant Liberibacter associated with HLB in citrus orchards in São Paulo (SP) and Minas Gerais (MG) States, the major citrus growing regions in Brazil. In 2007, for the first time worldwide, a phytoplasma, belonging to 16S group IX, was found associated with HLB symptoms in Brazil. Since 2014, sweet orange leaf samples were identified with 16S group III phytoplasma. Most of the affected leaf samples have asymmetric chlorosis, resembling blotchy mottle symptoms. However, clear-cut blotchy mottle was not as common as in the case of group IX phytoplasma. 16SrDNA from 22 samples were obtained and sequenced, confirming that a group III phytoplasma is found associated with HLB symptoms in SP and MG States. Eleven single nucleotide polymorphisms (SNPs) were found in the 1427 bp 16SrDNA sequences. Ribosomal protein genes (*rp*) were also sequenced from nine samples and assembled into two main groups based on eight SNPs. SNPs in 16S and *rp* gene sequences is common in 16S group III phytoplasmas and these phytoplasmas are widespread in South America and very common in *Melia azedarach*, a well common tree in Brazil and Argentina. A SYBR Green qPCR protocol was developed with primers based on the sequence of *rpsC_rplV* genes, which showed to be more sensitive than nested PCR for 16S and *rp* gene detection. An assessment of field samples with this qPCR protocol showed the 16S group III phytoplasma to be present in 10% of HLB-like samples from southwest of Minas Gerais State.

GENETIC VARIATION AND HIGH FREQUENCY TRANPOSITION OF NON-AUTONOMOUS TRANSPOSABLE ELEMENTS FROM CANDIDATUS LIBERIBACTER ASIATICUS

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Two miniature inverted-repeat transposable elements (MITEs), MCLas-A and MCLas-B, were recently identified from '*Candidatus* Liberibacter asiaticus' known to be associated with citrus Huanglongbing (HLB, greening disease). MCLas-A was suggested as an active MITE because of its mobility. The immediate upstream gene of the two MITEs was predicted to be a putative transposase. The main goal of this study is to analyze the sequence variation in the upstream putative transposase of MITEs and explore the possible correlation between sequence variation of transposase gene and MITE activity. The transposition frequency of the two MITEs in field samples was also evaluated through a large-scale survey. PCR and sequence analysis showed that 12 sequence types were found in six major amplicon types from 43 representative '*Ca*. L asiaticus' isolates from China, the United States and Brazil. Out of the 12 sequence types, three (T4, T5-2, T6) were reported for the first time. Recombination events were found in the two unique sequence types (T5-2 and T6) which were detected in all Brazilian isolates. Notably, no sequence variation or recombination events were detected in the upstream putative transposase gene of MCLas-A, suggesting the conservation of the transposase gene might be closely related with the MITE activity. Phylogenetic analysis demonstrated two well supported clades including five subclades were identified, clearly reflecting the geographical origins of isolates, especially that of Ruili isolates, São Paulo isolates and a few Florida isolates. 350 '*Ca*. L asiaticus' isolates newly collected from Guangxi, Guangdong, Jiangxi, Fujian, Hunan, Guizhou and Yunnan and 50 Guangxi samples DNAs extracted in 2011 and 2012 were amplified for evaluating the transposition status of MITEs. MCLas-A was only detected in ten samples (2.86%) from Guangdong and Guizhou and the putative transposition products were predominant in all samples newly collected, whereas MCLas-A (60.00%) was predominant in 50 previously collected samples, suggesting the possible high frequency transposition of MCLas-A recently.

ANTIBODY-BASED DIAGNOSIS OF CITRUS HUANGLONGBING AND STUBBORN USING PATHOGEN SECRETED PROTEINS AS DETECTION MARKERS

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Citrus industry is facing threats worldwide from insect-transmitted and phloem-colonizing bacterial pathogens associated with diseases such as Huanglongbing (HLB) and Citrus Stubborn Disease (CSD). Diagnosis of HLB and CSD is challenging due to the uneven distribution and variable titer of pathogens, the highly variable latency period, and symptoms that are easily confused with other diseases or nutrient deficiencies. We seek to develop serological detection methods using pathogen secreted proteins as markers. Methods based on pathogen-specific secreted proteins, which are readily distributed in the infected trees through vascular flows, could better cope with the erratic distribution of the pathogen; therefore, improving disease diagnosis. We have identified secreted proteins from the HLB associated bacteria *Candidatus* Liberibacter asiaticus and the CSD causative agent *Spiroplasma citri* and generated antibodies that can specifically bind to selected secreted proteins. Using these antibodies, specific signals were picked up from HLB and CSD infected plants using Direct Tissue Blot Immunoassay (DTBIA). For HLB, signals were also observed from plant tissue imprints of asymptomatic field samples, providing promise of early detection before the development of physical symptoms. A quantitative Enzyme-linked Immunosorbent Assay (ELISA) and Dot Blot Immunoassay (DBIA) is also under development using these antibodies. On-going efforts and progress on the development, evaluation and validation of these novel secreted protein-based detection methods will be reported.

RESEARCH PROGRESS OF THE INSECT VECTORS OF HUANGLONGBING IN CHINA

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Citrus is the most important fruit in South China. Huanglongbing (HLB), first reported in China in 1919, is the most devastating citrus disease. A dispute persisted over the cause of HLB between Horticulturists and Plant Pathologists until Prof. Lin published two papers on demonstrating that it was an infectious disease and possibly transmitted by insect vector. Although the Asian citrus psyllid (ACP, *Diaphorina citri*) was first found in 1934 in the mainland of China, little attention was paid until 1953 when Prof. Huang found that it was important pest of citrus young shoots and published results of his biological studies. ACP was proved to be the vector of HLB in China in 1977. Since then, studies have been focusing on the disease transmission mechanism and management. The pathogen was found widely distributed in the salivary glands, midgut, hindgut, and filter chamber digestive system of the psyllid under electron microscope in 1980s, and PCR was first used in the pathogen detection in host plants and ACP in 1996. ACP and HLB are now widely distributed in 11 of the 18 citrus plantation provinces. In 2012, the pomelo psyllid (*Cacopsylla* (*Psylla*) *citrisuga*), which was found in Yunnan province was proved to be another vector of HLB. This psyllid species is also very effective in HLB transmission, but the distribution is limited in high altitude area. Although greatly impacted by HLB, citrus production has been increasing in China since 1980s. One objective is to develop in areas where HLB is absent. In regions where HLB is present, free nursery stock, sanitation of the planting environment, improvement of tolerance and vector management are key strategies. Applying of organic fertilizers is believed to be able to reduce the psyllid population, and improve citrus tolerance to HLB. Dormancy spray by mineral oils is most effective for ACP control.

MONITORING CITRUS FLUSH SHOOT ONTOGENY AS A POTENTIAL STRATEGY FOR HLB AND PSYLLID MANAGEMENT

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Citrus flushing pattern greatly influences behaviour of *Diaphorina citri* in the field, which has been extensively studied regarding its host preference and sensory responses, but not the suitability of each flush stage during flush ontogeny for its biology. To assess this, we first describe developement of flushes from 2-year-old nursery Valencia trees (4.7L pot), to identify its ontogeny: bud emergence phase, with stage V1, from opening of the protective scales until the leaf primordia became visible; flush development, with V2 and V3, from early bud elongation to ending of leaf production; maturation, with V4 and V5, from ending of leave emission until shoot tip chlorosis and abscission; and dormancy phase, with V6, with hardened and dark green leaves. Then, two pairs of unmated 15-d-old *D.citri* were confined in a mesh sleeve cage for 72h on each flush stage (1 per plant, n-variable), first in acclimatized room (26℃, 70% RH, 12:12h L:D with 3750 lux) and after repeated in screen-house (T: 25℃, max. 42, min. 12; RH: 53%, max. 93, min. 15). In both ambient behaviour was similar. 100, 97, 94, 93 and 73% of the plants with V1, V2, V3, V4, and V5 shoots, respectively had oviposition, but none of V6. Psyllid took three days longer to begin oviposition on V5 shoots than on the others. Most suitable stages for oviposition and nymph survival (NS) were V2-V3 (eggs: 58.1±7, 49.7±7; NS: 83.2%±5, 81.8%±5), and the worst were V4-V5 (eggs: 24.9±8, 10.5±6; NS: 19.6%±6, 10.6%±2). Then, we compared flushing data taken weekly from 27/11/2012 to 02/07/2013 from Valencia plants (5 on dryland, 5 on irrigated area) planted in 05/2012 in a farm in Matão city (SP, Brazil), counting and classifying flushes in the whole canopy to analize it through area under the curve method. Dryland plants have+1.3% total flushes. But, regarding the biotic potential for psyllid, dryland plants had+24.5%. Thus, presence of more suitable flushes was higher on dryland area, despite the total amount have been almost the same. We think these findings could be useful as decision criteria in HLB and psyllid IPM's.

CONTROL PROGRESS OF CITRUS HUANGLONGBING IN GUANGXI, P.R. CHINA

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Huanglongbing is the most destructive disease in citrus industry in Guangxi, P.R. China. During the last thirty years, more than 67,000 hm^2 citrus groves had been destroyed by Huanglongbing, and the economic loss caused by citrus Huanglongbing (HLB) had reached more than 1.5 billion dollar. Since 2005, integrated control of HLB had been continuously carried out in Guangxi, P.R. China. To date, 612 integrated control demonstration regions of HLB with total area of 11,533 hm^2 had been built, and the techniques of integrated control of HLB had been applied to 160,000 hm^2 citrus orchads. More than 20 virus-free citrus nurseries had been set up in the last decade. Incidence of HLB infection on citrus trees had continuously decreased in the last ten years in Guangxi. The incidence of HLB infection was 6.45% in 2005, 3.90% in 2006, 2.16% in 2007, 1.80% in 2008, 1.50% in 2009, 1.20% in 2010, respectively, and less than 1.00% since 2011. Both cultivation area and yield of citrus had increased year by year after 2005 in Guangxi. By 2014, the cultivation area and yield of citrus had been reached up to 293,000 hm^2 and 4,695,700 tonnes, respectively, in Guangxi. Currently, Guangxi had become the second largest citrus cultivation province in P.R. China. Looking back on the cotrol progress of HLB during the last decade in Guangxi, some valuable experiences were worth summarizing and sharing.

ASIAN CITRUS PSYLLID AND HUANGLONGBING IN CALIFORNIA

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In August 2008, the California Department of Food and Agriculture and the United States Department of Agriculture announced the detection of Asian citrus psyllid (ACP), the vector of Huanglongbing (HLB), in San Diego, California (CA). Later that year ACP was also detected in the neighboring Imperial County. ACP has continued to spread north from San Diego into Los Angeles, Orange and Ventura Counties in 2009, Riverside and San Bernardino in 2010, Santa Barbara and Tulare in 2012, Kern and Fresno in 2013, San Luis Obispo, Santa Clara, Madera and San Joaquin in 2014, and San Benito, Stanislaus and San Mateo in 2015. The ACP quarantine zone in CA is over 53,000 sq miles and includes citrus producing areas at the south, desert, inland, coastal and central valley and areas as north as San Francisco and Sacramento counties. In April 2012, the first HLB positive tree, a lemon/pummelo tree with 23 graft unions, was confirmed in Hacienda Heights, Los Angeles CA. The HLB bacterium was originally detected in ACP collected from the tree. In July 2015, a second HLB positive tree was found in San Gabriel about 15 miles northwest from Hacienda Heights after a routine follow up to an inconclusive qPCR result from ACP collected on the property. The San Gabriel detection has led to 12 additional HLB positive finds within the quarantined area. HLB was detected on a kumquat tree followed by HLB findings in lime, mandarin, and calamondin trees. The San Gabriel HLB finds were the result of a risk based survey that takes in consideration not only citrus and ACP but census data about human activities such as traveling in HLB infested areas around the world. HLB finds trigger insecticide application and rigorous sampling of all ACP and HLB hosts in surrounding areas. The HLB trees in CA were tested positive for Candidatus Liberibacter asiaticus and were immediately removed with the consent of the homeowners. ACP and HLB funded research continues in all fronts and ACP trapping and treatments as well as HLB testing continues in collaborative efforts with the industry, state and federal agencies.

IMPACT OF THE ENVIRONMENT ON CANDIDATUS LIBERIBACTER ASIATICUS MULTIPLICATION IN YOUNG SHOOTS OF CITRUS TREES

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Fundecitrus

The major citrus growing area of Brazil occupies～500,000 ha between Triângulo Mineiro (TM) region of Minas Gerais State, bordering northern São Paulo State (SPS), to the extreme south of SPS. Significant climatic variation occurs seasonally within and between regions. Hot summers and mild and dry winters predominate in the northern regions, and milder summers and wetter, colder winters in the south. HLB was first reported in 2004 in the center of this large area. The disease subsequently spread in all directions, but spread towards and within the northern regions was slower. To investigate possible impacts of local climates on *Ca*Las, qPCR was used to assess pathogen titers in 25 to 50 young shoots (～10 cm long with soft immature leaves) sampled periodically over two years from naturally-infected mature trees within fine mesh cages on farms in Analãndia, central SPS, and in Frutal and Comendador Gomes, within the TM region. Data-loggers recorded local air temperatures and relative humidity hourly. During flush sampling, the shoots were placed in distilled water and transported in foam box to the laboratory where each shoot was placed in a separate plastic tube. On each occasion, five *Ca*Las-free *Diaphorina citri* adults were placed on each shoot for 48 hours. Flushes and insects were then individually processed for qPCR. Average *Ca*Las titers and *Ca*Las acquisition rates by psyllids were lower from flushes from Comendador Gomes (0.70 ± 0.34 log, 1.65 ± 1.29%) than those from Frutal (1.75 ± 0.28 log, 4.18 ± 1.03%) or Analândia (2.27 ± 0.28 log, 6.38 ± 1.06%). In attempts to identify the climatic factors that could be the responsible for *Ca*Las titer variations, titer data and climatic factors, registered during the last 7, 15 or 30 days prior evaluation dates (DPE), were submitted to multiple regression analysis. Climate data consisted of the number of hours at several temperature and relative humidity ranges, and the cumulated amount of rain. Stronger association was observed between *Ca*Las titer and the number of hours bellow 15℃ ($h < 15C$) or above 30℃ ($h > 30C$), and the cumulated amount of rain ($F = 21.64$, $R^2 = 0.82$, $P < 0.001$) registered during 15 DPE. *Ca*Las titers associated positively with $h < 15C$ and rain, and negatively with $h > 30C$. The implications for improvements in HLB management will be discussed. Preliminary results of this work were already presented (IRCHLB, 2015).

EFFECT OF BACTERIAL DNA CONCENTRATION IN DIAGNOSIS OF HLB USING CONVENTIONAL AND QPCR METHODS

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The disease Huanglongbing (HLB) is currently the most serious threat for citriculture worldwide. Due to the inability to grow any of the causal bacterium, *Candidatus* Liberibacter asiaticus (*Ca*Las), africanus (*Ca*Laf), and americanus (*Ca*Lam), early diagnosis of affected tissues by molecular methods as conventional Polymerase Chain Reaction (PCR) or quantitative real-time PCR (qPCR) are recommended. This work was initiated to determine the accuracy of HLB detection in samples with low bacterial concentration. Samples with decreasing amount of *Ca*Las DNA were used in working conditions for a member laboratory of the HLB Detection Network in Argentina. With conventional C-TAB extraction methods from 500mg of tangerine leaf-vein tissue an average yield of 330 micrograms of DNA was obtained. DNA from healthy citrus tissue was compared to positive control samples analyzed previously. The primers product used from conventional PCR detection (OI1/OI2) were run in agarose gel. For qPCR, specific set of primers was used according Taqman requirements (primers HLBas-HLBam). Serial dilutions were performed to experimentally reproduce decreasing numbers of HLB bacterium cells, and changes of qPCR curve shapes were observed of a symptomatic sample of HLB. The dilutions were 1:5; 1:50; 1:100; 1:250 and 1:500 which were made in two separated batches using different amounts of water, and DNA from the healthy sample. No differences in accuracy were observed between positive control and 1:5 dilutions for both methods. For conventional PCR method, dilution 1:50 gave more attenuated band, which became invisible at 1:100. The Ct values obtained for water dilutions were 21; 22.5; 24.20; 26.2; 28.3 and 28.3 respectively. For dilutions with DNA from the healthy sample, the Ct values were respectively: 21.9; 25.9; 26.8; 27.9; 29.5. Conventional PCR method was less precise for detection of low titer of bacterium. Due to the risk of false negative results the recommendation is to replicate every analysis, and do dilutions in unclear results. Still, new simple and sensitive methods are needed to keep the survey in a country or region where HLB is incipient or absent.

DIVERSITY AND VARIATION OF "CANDIDATUS LIBERIBACTER ASIATICUS" ASSOCIATED PHAGE IN SOUTHERN CHINA

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"*Candidatus* Liberibacter asiaticus" (*Ca*Las) is a psyllid-transmitted, phloem-limited, uncluturable α-proteobacteria bacteria, associated with a highly destructive worldwide citrus disease known as citrus Huanglongbing (HLB). The disease was first observed in Guangdong Province of southern China over a hundred years ago and it still remains endemic there. Due to the nonculturable of *Ca*Las, little information was known about the *Ca*Las biology before the whole genome sequence was available. Currently, two known prophage types, Type 1 (SC1-like) and Type 2 (SC2-like) was identified to can be both separately and simultaneously integrated into *Ca*Las chromosome. In this study, the prophage specific loci were selected as the marker to reveal the distribution and dynamic of prophage in *Ca*Las population in southern China. A total of 512 *Ca*Las-infected citrus and psyllid DNA samples were applied and the frequency of each prophage types were observed. Of 512 *Ca*Las strains (plant and psyllid origin) collected from southern China, 21.09% (108 strains) only harbored Type 1 prophage, 72.26% (370 strains) only harbored Type 2 prophage, 3.13% (16 strains) harbored both Type 1 and Type 2 prophage, and 3.52% (18 strains) harbored none of two prophages. The result also demonstrated that the diversity of prophage type in *Ca*Las population was highly associated with their geographical origins in southern China. Analysis of genetic structure based on prophage specific marker revealed the *Ca*Las population can be divided into three groups, with one including Guangdong, Fujian, Hainan, Jiangxi and Zhejiang, one including Guangxi and Guizhou and the other including Yunnan and Hainan. In addition, the intraspecific variation of prophage was also observed in the Type 2 specific loci (Type2-6 and Type2-8), indicating the possible horizontal gene transfer between prophage and *Ca*Las chromosome.

FIELD PERFORMANCE OF VARIOUS CITRUS TRISTEZA VIRUS CROSS-PROTECTION SOURCES TRIALED IN GRAPEFRUIT IN DIFFERENT CLIMATIC REGIONS

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The use of cross-protection in South Africa has significantly extended the tree life of grapefruit varieties sensitive to *Citrus tristeza virus* (CTV). Incidences of severe stem pitting in grapefruit, experienced since the implementation of cross-protection was correlated to segregation of strains within the original pre-immunisation source. A change in the pre-immunising source for cross-protection of grapefruit varieties was made and grapefruit mild strain 12 (GFMS12) was replaced with GFMS35. Indications of a possible severe CTV component in GFMS12 (Nartia A) cross-protecting source, necessitated the separation of the strain populations into sub-isolates by single aphid transmissions. Sub-isolates derived from two Nartia sources and a Mouton source which derived from sweet orange, were included in field trial evaluations together with GFMS12 and GFMS35 (current standard cross-protector for grapefruit). These sources include B389-1, B389-4, B390-3, B390-5, GFMS12/7 and GFMS 12/9. To assess the performance of these sources in different climatic conditions, trials were established in both hot and humid areas, and hot and dry areas respectively. Comparative results obtained between the trials regarding tree size, stem pitting development and yield are presented.

EVOLUTIONARY DYNAMIC OF A NEW CTV GENETIC LINEAGE: WHAT DOES THE HISTORY TELL US?

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Citrus tristeza virus (CTV) has been intensively studied since several decades ago, due to its capacity to cause one of the most devastating diseases in citrus industry worldwide. Nevertheless, few studies have been focused on its evolutionary history (Silva *et al*., 2012; Davino *et al*., 2013; Owen *et al*., 2013; Harper, 2014). In Uruguay, studies describing the presence of CTV have been done in the early 40's showing that this pathogen, along with *Citrus psorosis virus* (CPsV) and *Citrus exocortis viroid* (CEVd) are responsible of annual losses up to 30% of citrus national production. However, the prevalence and distribution of these pathogens in the country is yet not known, although studies in this matter have been already started by our group. In the past four years, we were been focused on the study of CTV genetic diversity in Uruguay based on the molecular analysis of *p*25, *p*20 and *p*23 genes. We describe the co-circulation of VT, T3, and T36 genotypes as well as a fourth lineage named NC, which is highly represented in Uruguayan citrus orchards (Benítez-Galeano *et al*., 2015). Sequences of this lineage share 99% of nucleotide identity with previously reported isolates, such as Taiwan- Pum/SP/T1 and the Hawaiian isolates HA16-5 and HA18-9. We also described the presence of mixed infections within the same host and the presence of some recombinant genomes (Benítez-Galeano *et al*., 2015). Nowadays, we continue with the surveillance of citrus orchards from all the country and the presence of this new lineage is growing as well as the presence of different genotypes, such as RB not founded before. With the aim to develop a long term cross protection program, to be incorporated on the ongoing National Sanitation Program, we are trying to get a deep knowledge about these variants that circulate in Uruguay. For this purpose, we want to unravel the biological, molecular and evolutionary traits of the NC lineage, not intensely studied so far. In the present work, we studied the evolutionary history of this NC lineage based on a Bayesian coalescent approach using genomic sequences of *p*25 and *p*20 genes. To do this, a large set of heterochronous gene sequences retrieved from GenBank and also Uruguayan sequences obtained by our group during the past few years were included in the analysis. Dated sequences from Uruguay, Argentina, Brazil, Greece, Portugal, China, United States, Angola, among others, from a time period from 1979 to 2015 were analyzed in this study. Recombinant sequences were discarded from the analysis in order to do not mix evolutionary histories and the best-fitting evolutionary model for each dataset was elucidated using jModel Test software. In this way, we could estimate the evolutionary rate for both genes as well as the time to the most recent common ancestor (tMRCA) of the NC lineage, showing consistent results in both cases either

for *p*25 and *p*20. We also analyzed the demographic behaviour of the population from its origin to the present, and we observed a consistent pattern of constant growing of almost 30 years, followed by an exponential growth of the effective population size in the last 5 years. Based on a phylogeographic approach, we could determine the movement around the world of this genetic lineage. Our results pointed that this NC lineage was origined from Uruguay, almost 35 years ago, and then a following radiation into two separate events leads one genetic group to Brazil and the Mediterranean region, and the other genetic group to United States, Argentina and Uruguay. Supporting our findings, Owen and co-workers described the recently introduction towards the Mediterranean region of a new strain highly similar to the previously reported Taiwan-Pum/SP/T1 strain (Owen *et al*., 2014). Understanding the evolutionary history of CTV variants circulating in the country could be of great importance to develop strategic control plans to manage this destroying pathogen. To our knowledge, this is the first comprehensive study about the evolutionary history of a CTV genetic lineage in Uruguay.

SURVEY AND MOLECULAR DETECTION OF CITRUS YELLOW VEIN CLEARING VIRUS AND CITRUS CHOLOROTIC DWARF ASSOCIATED VIRUS IN CITRUS NURSERIES AT EAST MEDITERRANEAN REGION IN TURKEY

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In order to assess the occurrence and distribution of two citrus vector transmitted diseases, *Citrus yellow vein clearing virus* (CYVCV) and *Citrus chlorotic dwarf associated virus* (CCDaV), extensive surveys were conducted in commercial citrus nurseries area of east Mediterranean region during 2012 and 2015. CYVCV was found in only one nursery but CCDaV was found in 23 nurseries. The varieties CCDaV affected were W. navel, Valencia sweet orange, Okitsu, Satsuma, Clementine, Fremont mandarin, Star Ruby grapefruit, Minneola tangerine, Enterdonate, Kutdiken, Meyer, Aydin lemon and sour orange which were used as rootstock. CYVCV was detected only in Kutdiken lemon variety. To analyze the molecular characterization of CCDaV, DNA extractions were made from 55 samples of different infected citrus varieties with leaf deformations, chlorotic lesions and yellow veins of leaves. PCR detection was performed using primer pair (sense 5'-GTTCTGTGTTTCGACCCTT-3' and antisense 5'-CGGATTCGCATGGATAGCTCATCCAA-3') designed from coat protein gene of CCDaV genome (GenBank Accession No. KF561253). With the aim of detecting CYVCV, A one-step RT-PCR using primer pair (sense: 5′-ACCTCACGATGGACCACGTT-3′ and antisense: 5′-CAGAAAATGGAAACTGAAAGCCTG-3′), designed from coat protein gene of CYVCV genome (GenBank Accession No. JX040635), was performed. All the symptomatic CCDaV samples yielded 444 bp PCR products. Blast analysis showed that these nucleotide sequences had greater than 98% nucleotide identity with the corresponding region of CCDaV reference genomes. Symptomatic samples of CYVCV yielded the expected cDNA fragment. Four of PCR amplicons were selected for sequencing. BLAST analysis showed that these nucleotide sequences had greater than 98% nucleotide identity with the corresponding region of CYVCV reference genomes.

THE VIRUS/VECTOR RELATIONSHIP IN THE CITRUS LEPROSIS VIRUS C PATHOSYSTEM

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Citrus leprosis, caused by *Citrus leprosis virus C* (CiLV-C) is one of the most destructive citrus disease, especially for sweet oranges, and is vectored by the mite *Brevipalpus yothersi*. Little was known about the virus-vector relationship until recently. CiLV-C is not vertically transmitted, but the mite at all the developmental stages is able to transmit the virus. The period of virus acquisition feeding and inoculation feeding is about four hours and the latent period is seven hours. Virus retention by the mite vector exceeded 12 days. *Brevipalpus* mites feed using stylet to pierce epidermal and parenchymal cells below, but not the vascular region. Saliva is injected starting a predigestion of cell contents. If the mite is viruliferous, CiLV-C is inoculated with the saliva. Apparently stylet is not used to suck cell sap since the salivar channel is very narrow. Probably mite retracts the stylet after the initial penetration and the sap may flow out from the hole by cell turgor and then sucked by the mite with the help of powerful faringeal pump. Several evidences suggest that CiLV-C has a persitent and circulative relationship with the mite vector: (a) the virus is transmitted still in the larval stage of the mite; (b) short latent period; (c) qRT-PCR did not indicate increase in virus titer after initial acquisition; (c) in viruliferous mites virions occur between cell membranes of adjacent cells in the prosome, but not within cells, and viroplasms have not been detected. Roy *et al*. (2015) suggested that CiLV-C might replicate in the mite since complementary vRNAs were detected in the mite following acquisition. However, the experiment did not totally preclude the detection of these molecules still present in the ingested sap. If CiLV-C replicates in the vector, virus/vector relationship in this pathosystem would be peculiar in the sense that the virus may circulate initially and then replicate.

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Financial support by FAPESP and CNPq

A NOVEL CITRUS VIROID FOUND IN AUSTRALIA, TENTATIVELY NAMED CITRUS VIROID VII

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A novel citrus viroid was discovered following routine biological indexing of a non-symptomatic Lisbon lemon field tree in Dareton, New South Wales, Australia. In February 2015, bark pieces from the Lisbon lemon were grafted onto 'Etrog' citron Arizona 861-S-1 and maintained in a temperature controlled greenhouse at 32°C. Subsequent 'Etrog' flushes expressed stunting and leaf epinasty symptoms indicating the presence of citrus viroid(s). Total RNA was extracted from the symptomatic 'Etrog' citrons. Known viroids were not detected using conventional RT-PCR assays for *Citrus bent leaf viroid*, *Hop stunt viroid*, *Citrus dwarfing viroid*, *Citrus bark cracking viroid*, *Citrus viroid V*, *Citrus viroid VI* and *Citrus exocortis viroid*. An unknown apscaviroid was detected in a SYBR green RT-qPCR assay designed for the universal detection of citrus viroids showing a different melting temperature peak from the known apscaviroid controls. The 281bp PCR product was sequenced and specific primers for the amplification of the full viroid genome were designed. The resulting 368bp RT-PCR product was cloned and several clones were sequenced. BLASTn analysis of the viroid genome revealed that the novel apscaviroid, tentatively named *Citrus viroid VII* (CVd-VII), is significantly different from all other known viroids, showing low similarity to *Australian grapevine viroid* and *Apple fruit crinkle viroid* (a tentative *Apscaviroid* species). The terminal conserved region and central conserved region present are characteristic of the *Apscaviroid* genus and it has a GC rich (52-53%) genome with 69% paired nucleotides. It is proposed that CVd-VII is a new species in the *Apscaviroid* genus. The new apscaviroid was directly detected in the Lisbon field tree and in 'Etrog' citron plants slashed inoculated with Lisbon lemon RNA extracts. To the best of our knowledge, this is the first formal report of a novel citrus apscaviroid, tentatively named CVd-VII.

ihpCP SWEET ORANGE TRANSGENIC LINES ARE RESISTANT TO PSOROSIS A AND PSOROSIS B

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Citrus psorosis is a serious disease caused by *Citrus psorosis virus* (CPsV). Two different psorosis syndromes have been described, called Psorosis A (PsA) and Psorosis B (PsB). The most common PsA affects only the trunk and main branches, showing bark scaling and gum accumulation, and PsB, the most aggressive, shows severe lesions in the bark causing its detachment and gum accumulation and pustules, even on young branches. Transgenic Pineapple sweet orange (*Citrus sinensis* L. Osb.) were generated expressing hairpin structures containing a fragment of the viral coat protein gene (cp) from CPsV 90-1-1 isolate. These transgenic lines, called ihpCP induced post transcriptional gene silencing for cp gene, and were resistant to the homologous isolate, which causes a PsA syndrome. IhpCP plants resulted asymptomatic and no virus was detected by RT-PCR and TAS-ELISA (Reyes *et al.*, 2011).

In this work we selected the lines ihpCP-10 and ihpCP-15, which were propagated on rough lemon and challenged with the heterologous CPsV 189-34 isolate from Concordia (Entre Rios, Argentina), which causes strong PsB syndrome. A local alignment between the sequences of CPsV 189-34 (PsB) and CPsV 90-1-1 (Ps A) was performed showing identity of 93%.

Plants were challenged with CPsV 189-34 by grafting, and the evolution of infection was analyzed through three successive flushes. The ihpCP-15 line resulted totally resistant in 8 of the 8 inoculated plants, without psorosis symptoms, and no virus was detected by molecular tests. The ihpCP-10 plants showed partial resistance, given that some individuals became infected from the second flush, but with lower viral titers and fainter PsB symptoms than those of non-transgenic control. These results show that the ihpCP transgene protects the plants against not only PsA, but also PsB. Besides the ihpCP-15 line is more efficient against a heterologous isolate than ihpCP-10 in the first three flushes.

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GENOME SEQUENCE OF A NEW DICHORHAVIRUS ASSOCIATED TO CITRUS LEPROSIS NUCLEAR TYPE DISEASE

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Leprosis is a re-emergent citrus disease endemic of the Western Hemisphere and caused by a heterogenic group of ssRNA viruses. The disease produces necrotic and chlorotic symptoms in leaves, twigs and fruits leading to serious yield reduction in the citrus orchards. Leaf samples from three sweet orange (*Citrus sinensis*) trees showing typical leprosis symptoms were collected in Brazil in 2015, and their RNA extracts were analyzed by RT-PCR tests using specific primers for the detection of known leprosis causal agents: the cileviruses *Citrus leprosis virus C*, *Citrus leprosis virus C2* and a citrus strain of the dichorhaviruses Citrus leprosis virus Nuclear type (CiLV-N, *syn*. *Orchid fleck virus*, OFV). No amplicons were obtained, which appeared incongruent considering that the presence of bacilliform particles in cells from the infected tissues had been previously confirmed by transmission electron microscopy. After next generation sequencing of the three samples and bioinformatic analysis, the assembled bipartite genome resembled those described for dichorhaviruses and, globally, the deduced amino acid sequences from its six ORFs showed 50%-76% of similarity with those from OFV and Coffee ringspot dichorhavirus. Moreover, deduced amino acid sequence of L protein showed 61%-84% of similarity with fragments derived from a Citrus leprosis-associated virus recorded in herbarium specimens collected 50 years ago in Florida, USA (GenBank accessions AKO62450-AKO62452). Our results corroborate the increasing diversity of citrus leprosis associated viruses and lead to re-think the nomenclature of the dichorhaviruses infecting citrus, since the first two described, *Citrus necrotic spot virus* and CiLV-N, are now recognized as strains of OFV infecting citrus.

Financial support: CNPq (375843/2012-4 and 401564/2012-6) and FAPESP (2014/08458-9).

PLANT DEFENSE MECHANISMS DURING THE LOCALLY-RESTRICTED INFECTION OF CITRUS LEPROSIS VIRUS C

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Citrus leprosis virus C (CiLV-C), the type member of *Cilevirus*, is an atypical (+)ssRNA virus that does not spread systemically in its known hosts. Upon inoculation with CiLV-C viruliferous *Brevipalpus* spp. mites, only localized lesions are observed and viral infection remains restricted to cells around the inoculation sites. These characteristics resemble the outcome of host defenses such as hypersensitive response (HR). To investigate plant defenses during the unusual CiLV-C infection, we compared the expression of defense genes by RT-qPCR in the model plant *Arabidopsis thaliana* and the CiLV-C natural host *Citrus sinensis* after the infestation with non-viruliferous and viruliferous mites. In addition to the response to the mites, in CiLV-C infected plants the classical antiviral mechanisms i.e. RNA silencing and salicylic acid pathways were activated. HR marker genes were also identified. To evaluate the role of the activated defenses on CiLV-C infection, we assessed CiLV-C replication and movement in *Arabidopsis* mutants. Viral loads and symptom severity in the inoculated leaves of *dcl2/4* and *rdr2/6* mutants were higher than in wild type plants, although the systemic infection was not accomplished in the assayed genotypes. To support the involvement of HR in response to CiLV-C, we searched for ROS burst and cell death through histochemical analyses. H_2O_2 and dead cells were detected since early times of the infestation with viruliferous mites and were sustained along the infection, suggesting the activation of an HR-like response. Although the role of viral-derived factors cannot be precluded as the origin of the locally-restricted phenotype of CiLV-C infection, our results indicate that CiLV-C/plant interaction should be considered as an incompatible rather than a compatible interaction. Furthermore, induction of defense responses in plants challenged with non-viruliferous mites suggest that mite feeding might pre-induce host resistance against CiLV-C infection.

Support: FAPESP, INCT Citrus and NPq

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF WING DEVELOPMENT-RELATED GENES IN THE CITRUS TRISTEZA VIRUS (CTV) VECTOR TOXOPTERA CITRICIDA

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The brown citrus aphid, *Toxoptera citricida* (Kirkaldy) (Hemiptera: Aphididae), is the main vector of *Citrus tristeza virus* (CTV) worldwide. Like other aphids, *T. citricida* has apterous and alate wing dimorphisms. Having strong flight muscles, the alate morph can readily fly long distances with the wind spreading CTV in citrus growing regions. In this study, we are aiming at finding the key genes involved in the wing development by using RNAseq, digital gene expression profiling (DGE) and RNA interference (RNAi). The *de novo* assembly of transcriptome of the aphid was obtained through the short read sequencing technology (Illumina). A total of 44,199 unigenes were generated and 27,640 were annotated. The transcriptomic differences between the alate and apterous adults were examined by DGE. As the results indicating that 279 unigenes were highly expressed in alate adults, whereas 5,470 expressed significantly higher in apterous adults. Top 10 highly expressed genes in alate adults subjected to the qRT-PCR analysis showed the similar trends as the DGE results. Among them, several differentially expressed genes were further analyzed by dsRNA feeding mediated RNAi. For instance, silencing of lipid synthesis and degradation gene (3-ketoacyl-CoA thiolase, mitochondrial-like (*KAT*)) and glycogen genes (Phosphoenolpyruvate carboxykinase [GTP]-like (*PEPCK*) or Glycogen phosphorylase-like isoform 2 (*GP*)) resulted in the under-development wings. The results indicated that both lipid and glycogen metabolism provided the energy for wing development in this aphid. Further researches are needed to reveal how they are involved in the wing development. The large number of sequences and expression data produced from the transcriptome and DGE sequencing, respectively, will greatly improve our understanding of this important insect at molecular level. Furthermore, the RNAi system we built would contribute to the in-depth research on wing development mechanisms as well as exploring the potential target for the control of this important vector.

VARIABILITY AND SEQUENCE DIVERSITY OF CITRUS TRISTEZA VIRUS ISOLATES FROM PAKISTAN

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Citrus tristeza virus (CTV) is one of the major threats to citrus production and fruit quality worldwide. In Pakistan more than 95% of the citrus trees are grown on sour orange rootstock which is highly susceptible to CTV. We studied the genetic variability of four genomic regions (*p*18, *p*20, *p*23 and *p*25) of 21 CTV isolates collected from the citrus orchards in Pakistan. High divergence was revealed among the isolates from Pakistan and also with reference isolates. An inter-isolate identity ranges from 93.1% to 100% at the nucleotide level and from 89.8% to 100% at the amino acid level were found. Phylogenetic analysis of the predominant sequence variants of each isolate revealed almost similar grouping of isolates for each genes. The groups revealed by phylogenetic trees include sequences of severe quick decline, seedling yellows and stem-pitting (SP) and also mild isolates. The high percentage of mixed infections is alarming for further diversification and spread of severe variants into new citrus growing areas of Pakistan and the neighboring countries.

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QUANTIGENE PLEX: A NON-PCR, HIGH THROUGHPUT, MULTIPLEX DETECTION ASSAY FOR CITRUS PATHOGENS

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High throughput multiplex diagnostics are critical for the early detection and eradication of citrus pathogens. PCR based assays are limited in their multiplex capacity (3-5 targets). A QuantiGene Plex-Luminex based assay was developed for the robust, sensitive, and simultaneous detection, identification and quantification of multiple citrus pathogens. The developed assay is the first to successfully detect 14 targets in one reaction. The reaction includes one housekeeping citrus gene (internal control), two universal citrus viroid probes (detection of seven citrus viroid species and their variants) and 11 species specific citrus pathogen probes for 9 viruses (tristeza, psorosis, tatter leaf, leaf blotch, leprosis, vein enation, variegation, yellow vein clearing, and Satsuma dwarf) and two viroids (citrus exocortis and hop stunt). The QuantiGene Plex assay is performed in 96-well format and can be manipulated to detect and quantify up to 80 RNA or 33 DNA targets. There is no need for reverse transcription reaction or DNA amplification because the assay is based on hybridization using magnetic beads with specific probes and sequential branched DNA signal amplification. The target detection is achieved in three major steps, namely target hybridization, signal amplification, and Luminex analysis. First the target of interest is hybridized to the magnetic capture beads, capture extender, label extender, and capture probe. Second, the signal is amplified by adding the Pre-amplifier, Amplifier, Label Probe solution, and streptavidin phycoerythrin (SAPE). Finally, the hybridization complex signals are detected and targets are quantified on a Luminex 200 (flow cytometry-based) or Luminex MAGPIX (LED-based) instrument. The detection values are expressed as net Median Fluorescence Intensity. In our laboratory, the QuantiGene Plex is paired with a high throughput, semi-automated, robotic nucleic acid extraction procedure optimized for citrus tissues for increased results uniformity, efficiency, and cost effectiveness.

CITRUS VIRUS DETECTION IN NGS DATA USING E-PROBES

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The development of high-throughput sequencing (HTS) applications to detect viruses in plants shifted the focus of detection assays away from multiple assays for the detection of different viruses, to the simultaneous detection of multiple viruses within a single assay. In a conventional HTS-based virus detection approach, data will be trimmed and filtered to retain high quality reads and then de novo assembled into contigs. These contigs are then screened for homology to known and unknown viruses. Conventional HTS data analysis has extensive computational requirements during contig assembly and homology searching steps, which prolongs the time required for a diagnostic result. In this project we set out to explore an alternative method for the detection of nine known citrus viruses using virus specific electronic probes (e-probes) in order to minimise the amount of bioinformatic processing time and resources needed. E-probes were designed to be unique for a specific virus and the optimal minimum length and E-value threshold were determined. The e-probes were able to accurately detect their respective viruses in simulated data that contained less than 1% viral reads. Multiple citrus viruses within a given dataset can be identified as long as the probe set for each virus is used. To add confidence to detection calls additional statistical methods were used, in which target e-probe signals were compared to signals produced by a decoy set of e-probes. The efficiency of the e-probe based approach was evaluated with different input HTS datasets: 1) dsRNA HTS data of Mexican Lime singly infected with different *Citrus tristeza virus* (CTV) genotypes, 2) dsRNA HTS data of Delta Valencia field samples of unknown virus status, 3) sRNA HTS data of CTV (T3) infected grapefruit cv Marsh and 4) RNA-Seq HTS data of grapefruit cv Marsh. Irrespective of the input dataset the e-probe based assay was able to detect the appropriate viruses without false negatives or positives. The results were confirmed by RT-PCR and conventional bioinformatic virus detection. In this study we were able to demonstrate that an e-probe based detection assay for known viruses can be efficiently implemented for different citrus HTS datasets for at least nine known citrus viruses. This assay can be carried out on a personal computer faster than a traditional metagenomic analysis, a feature highly desirable within a diagnostic setting.

USA CITRUS CLEAN PLANT NETWORK

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The availability of pathogen-tested propagative material is critical for the production of citrus and other vegetatively propagated crops. In the United States, several programs at the national, state or regional level have addressed clean plant needs for citrus such as the California Citrus Clonal Protection Program (CCPP) and the Florida Citrus Budwood Registration Program (CBRP). However, a comprehensive national effort did not start until 2005. Then, three Agencies of the United States Department of Agriculture (Animal and Plant Health Inspection Service, Agricultural Research Service, and National Institute for Food and Agriculture) collaborated to create a national network to produce, disseminate and preserve clean propagative materials. This network, the National Clean Plant Network (NCPN), was authorized and funded by the U.S. Congress under the Farm Bill of 2008 and the program was re-authorized and made permanent under the subsequent Farm Bill of 2014. The establishment of the CCPN has allowed smaller producing areas to join California and Florida in the network and to operate cooperatively at the national level. The CCPN currently includes centers in California, Florida, Arizona, Texas, Louisiana, Alabama, Hawaii, Maryland, and Puerto Rico. In a typical year, NCPN Citrus centers conduct over 75,000 diagnostic tests, distribute over 600,000 clean plant materials, perform therapeutics on hundreds of plants, and maintain hundreds of foundation plantings. NCPN Citrus has established and enhanced quarantine, germplasm, and extension/education programs in all of the major and minor citrus producing regions to facilitate the importation, testing, therapy, establishment of foundation plantings, and release of pathogen-tested citrus to nurseries, growers, and the public regionally, nationally and globally.

CALIFORNIA'S CITRUS NURSERY STOCK PEST CLEANLINESS PROGRAM: A SUCCESS STORY OF THE COLLABORATIVE POWER OF INDUSTRY, UNIVERSITY, AND REGULATORY AGENCIES

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In June 11-12, 2009, the workshop "Meeting the Challenge of the Asian Citrus Psyllid in California Nurseries" was organized by the Citrus Industry, University of California, and United States Department of Agriculture. Fifteen speakers representing growers, nurseries, regulators, scientists, greenhouse manufacturers and financial institutions from around the world shared their experience with the Asian citrus psyllid, Huanglongbing (HLB) and other devastating citrus diseases. The clear message of the workshop was that the basis for any successful management program for HLB and other graft-transmissible diseases of citrus is the mandatory propagation of nursery stock under protective structures using pathogen-tested germplasm. Since 1962, the California Department of Food and Agriculture (CDFA) registration program for citrus germplasm sources included mandatory testing for tristeza and voluntary testing for psorosis and citrus viroid diseases by bioindexing. Following the 2009 workshop, on May 17, 2010, regulations for the mandatory "Citrus Nursery Stock Pest Cleanliness Program" were filed as an emergency action by CDFA. The program included the use of protective nursery structures and the testing for HLB, tristeza, psorosis, and citrus viroid diseases. On May 21, 2012, a protocol for the high throughput nucleic acid extraction from citrus tissues and the RT-qPCR universal detection of citrus viroids, developed with industry, federal, and state funds, was submitted to the CDFA by the Citrus Clonal Protection Program. On September 10, 2012, CDFA issued the "QC 1354, Permit For PCR Protocol For Viroid Testing" and on January 14, 2014, issued the "NO. QC 1388, Permit For PCR Protocol For Virus Testing". Since HLB was never present in California nurseries the infection rate of citrus viroids was used as an indicator for the success of the new program. Bioindexing could test a limited number of samples per year. As a result, in the 2005-10 period, the viroid infection rate was 4.3%-9.5%. Since 2010 the program has tested over 10,000 samples reducing the viroid infection rate at 0.5%.

VIRUS IDENTIFICATION IN CITRUS RED MITES (*PANONYCHUS CITRI*)

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The citrus red mite, *Panonychus citri* (McGregor), is an important pest that devastates both deciduous and evergreen fruit trees such as citrus, pear, peach, and holly. In addition to the damage to plants, the incidence of mites may cause dermatitis and allergic reactions in people who are directly involved in the production process, especially during the fruit harvest. Mite is hosts and vectors of, a great variety of known and unknown viruses. Some of these viruses most likely have the potential to be important fundamental and/or practical resources. We used high-throughput next generation sequencing technology and bioinformatics analysis to identify putative viruses associated with *P. citri*. Our analysis revealed a diversified viral species, which may belong to *Vesiculovirus*, *Bunyavirus*, *Chuvirus*, *Negevirus*, *Picornavirus*, *Secovirus*, *Tobamovirus*, *Nudivirus*, and *Birnavirus*, in our field collected samples. Then, two approaches were used to further analyze these viral-related sequences. Firstly, the presence of these putative viruses was screened in extra field collected samples by PCR. Secondly, in the positive samples with viruses, the replication of the identified positive and negative RNA viruses was analyzed through detection of their replication strand. These results would provide valuable information on putative viruses in the major citrus pests, some of which may have the potential as biocontrol agents. In the view of citrus ecology, the possible interaction of these viruses and citrus viruses vectored by these pests are proposed.

POSTER SESSION ABSTRACTS

BACKYARD HOSTS OF ASIAN CITRUS PSILLID AND CANDITATUS LIBERIBACTER ASIATICUS

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Citrus is an important industry in Mexico and is threatened by Huanglongbing (HLB) and its vector the Asian citrus psillid (ACP), *Diaphorina citri* Kuwayama. The ACP damages shoots of plants in the family *Rutaceae* and is notable as the vector of *Candidatus* Liberibacter asiaticus, causal agent of the disease known as HLB, yellow shoot, or greening. HLB is currently in the process of dissemination in Mexico and in their main Tahiti lime production area, located in the Gulf of Mexico. We investigated the hosts of ACP and HLB among wild and garden plant species in the family *Rutaceae* present in the state of Veracruz, Mexico. On three different dates during the year (winter, summer and autumn), ten species were sampled in Northern, Central and Southern regions of the state and the characteristic symptoms of HLB were determined. The material was then examined under a stereomicroscope to confirm the presence of ACP on leaves or branches. In addition, conventional and real time PCR analysis was carried out on three different dates to determine the presence of the bacterium in the plant species collected. Infestation of *D.citri* on *C.limetta* Risso, *C.limonia* Osbeck, *C. sinensis* (L.) Osbeck (pro. sp.), and *Murraya paniculata* (L.) Jack, was observed on the three dates sampled. In addition, the presence of '*Candidatus* Liberibacter asiaticus' was confirmed in *Trichilia havanensis* Jacq (Meliaceae), and *Casimiroa sapota* Oerst (Rutaceae). Leaf mottling was observed in *T.havanensis*.

INFECTION ROUTE OF CANDIDATUS LIBERIBACTER ASIATICUS IN THE BODY OF ITS INSECT VECTER, DIAPHORINA CITRI (HEMIPTERA:PSYLLIDAE)

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The bacterium "*Candidatus* Liberibacter asiaticus" (*Ca*Las) is the pathogen of Asian-form Huanglongbing (HLB), which is one of the most devastating citrus diseases in Asia and America. *Ca*Las is transmitted by the Asian citrus psyllid (ACP) *Diaphorina citri* (Hemiptera: Psyllidae) in a persistent manner. In this study, immunofluorescence microscopy was used to investigate the infection route of *Ca*Las in the internal organs of ACP after acquiring the bacteria by feeding on HLB-infected citrus plants. The sequential infection study revealed that *Ca*Las initially infected the midgut epithelium of ACP, then crossed the basal lamina into the midgut visceral muscles, from where apparently spread into the hemolymph, then into the salivary glands and other organ. Our result suggests that *Ca*Las may direct spread from the initially infected epithelium to the salivary glands after accumulation in the internal midgut in ACP, contributing to efficient transmission of HLB by its insect vector.

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FIRST REPORT OF HUANGLONGBING DISEASE ON MEXICAN LIME IN IRAN

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Huanglongbing (HLB), also known as greening, is a destructive disease of citrus worldwide. HLB was first reported on orange in southern Iran in 2009. During the 2012 surveys of citrus growing areas of Sistan-Baluchestan province, leaf samples were collected from several Mexican limes (*Citrus aurantifola*) which showed severe infestation of Asian citrus psyllid (*Diaphorina citri*) as well as asymmetrical yellow mottling symptoms. Moreover, leaf sample was taken from a healthy lime tree in psyllid free region. Total DNA was extracted from 0.2 g of midrib tissue using the Hung *et al*. (1999) protocol. Nested-PCR using primer pairs OMP5F/OMP3R (first round) and Omp1218f/Omp2026r (second round) was conducted to detect *Candidatus* Liberibacter asiaticus (*Ca*Las) in DNA samples. Target DNA (800 bp) was amplified in 4 of 30 DNA samples but not in DNA from healthy Mexican lime and sterile water. The amplified fragment obtained from a Mexican lime sample was directly sequenced (GenBank Accession No. KJ765855). BLAST search showed 100% identity with corresponding sequences of *Ca*Las (KC411983, KC357752 and JX121096). This is the first report of the natural occurrence of HLB disease on Mexican lime in Iran. Regarding high population of the Asian citrus psyllid on Mexican lime as its preferred host plant in Iran, it could be an appropriate host for increasing vector population as well as survival of *Ca*Las. In conclusion, it could not be denied the significant role of Mexican lime in epidemiology of the Huanglongbing disease in Iran.

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STUDY OF THE NATURAL HOST RANGE OF CANDIDATUS LIBERIBACTER ASIATICUS IN SEVERAL HERBACEOUS PLANTS AND SHRUBS IN SOUTHERN IRAN

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In order to determine the natural host range of *Candidatus* Liberibacter asiaticus (*Ca*Las), the causal agent of citrus Huanglongbing in Iran, a survey was conducted especially in citrus orchards of southern provinces of Iran during 2012－2013. Several samplings of main herbaceous plants and shrubs were carried out. Total DNA was extracted from tissues of plants using CTAB protocol. The samples were analyzed for *Ca*Las DNA by direct PCR using A2/J5 primer pair and nested-PCR using primer pairs OMPF/OMPR (first round), omp1218/omp2024 (second round) and OI1/OI2c (first round), CG3F/CG5R (second round). The examined plants included *Cynodon dactylon*, *Alhagi camelorum*, *Solanum nigrum*, *Descurainia Sophia*, *Reseda aucheri*, *Echinochloa crus-galli*, *Alopecurus myosuroides*, *Plantago major*, *Cyperus rotundus*, *Amaranthus retroflexus*, *Sorghum halepense*, *Galium aparine*, *Polygonum pensylvanicum*, *Chenopodium album*, *Abutilon theophrasti*, *Rumex crispus*, *Bromus* spp., Convolvulus arvensis, *Acroptilon repens*, *Papaver rhoeas*, *Arctium lappa*, *Agropyrum repens*, *Malva* spp., and *Lactuca serriola*. No *Ca*Las DNA was detected in experimented plants by direct and nested-PCR assays. Based on the obtained results the mentioned herbaceous plants and shrubs in citrus orchards could not be the sources of the inoculums and fail to have a significant impact in disease development. Hence, significant sources for propagation and survival of *Ca*Las are the members of the family *Rutaceae* as well as *Diaphorina citri* as vector of the disease.

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TRANSCRIPTOME ANALYSIS OF PERIWINKLE TO INFECTION WITH *CANDIDATUS* LIBERIBACTER ASIATICUS

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Huanglongbing threatens the global citrus industry. In this study, the presumptive pathogens '*Candidatus* Liberibacter asiaticus' (*Ca*Las) was transferred from rough lemon to periwinkle using the holoparasitic dodder plants. Transcriptome comparison was applied to describe the response of periwinkle leaves infected with *Ca*Las at the early stage using deep sequencing technology. 7702 differentially expressed genes (DEGs) were identified, among which 3365 (43.7%) genes were up regulated while 4337 (56.3%) were down regulated. These DEGs were assumed to be involved in a variety of different biological processes, including cellular process, metabolic process, catalytic activity, photosynthesis, transport, carbohydrate metabolism and plant hormones. 36 DEGs were selected based on the function and fold change amplitude and assayed by real time qPCR. The gene expression levels of photosynthesis, plant hormones, carbohydrate metabolism and zinc transport proteins pathways of both *Ca*Las-infecting and healthy periwinkle leaves were performed and analyzed by real time qPCR method. More DEGs in response to stress in plant hormone and plant-pathogen interaction pathways were up regulated in the periwinkle leaves infected with *Ca*Las, but few DEGs related to carbohydrate metabolism was identified, which might be the result of the early infection stage in periwinkle by *Ca*Las.

FIRST REPORT OF CANDIDATUS LIBERIBACTER ASIATICUS ASSOCIATED WITH HLB DISEASE IN GRAPEFRUIT FROM SOUTH OF IRAN

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Kerman province located in the south of Iran is a third citrus production area of the country. Grapefruit (*Citrus paradisi*) is a major citrus fruit of this region. Huanglongbing (HLB), also known as citrus greening, caused by a sieve tube-restricted bacterium, *Candidatus* Liberibacter spp., is one of the most destructive diseases of citrus in the world (Bove 2006). It can be spread efficiently by the psyllid vectors *Diaphorina citri* and *Trioza erytreae* and through infected plant materials (Li *et al*., 2007). The disease was reported in sweet orange and *D. citri* from Iran (Faghihi et al., 2008). Inspection of citrus trees in southern of Iran revealed nearly more than 50 grapefruit trees with yellowish and mottling of the mature leaves and small, malformed fruits with aborted seeds. Three symptomatic samples were analyzed from a red blash grapefruit on Bakraee (a natural hybrid) rootstock in Jiroft of Kerman Province. Total DNA was extracted from leaf midrib and fruit by CTAB method with little modifications (Murray and Thompson, 1980). The DNA samples subjected to conventional PCR using the primer pairs A2/J5 and OI1/OI2c that amplify the ribosomal protein gene in the rplKAJL-rpoBC operon and the 16Ss ribosomal respectively, of '*Ca*. L. africanus' and '*Ca*. L. asiaticus'. Positive PCR reactions were obtained for all three symptomatic samples with both primer pairs. XbaI digestion of the amplicons obtained with OI1 and OI2c yielded approximate 520 and 640-bp fragments, characteristic of *Candidatus* Liberibacter asiaticus. PCR amplicons of 1077 bp (OI1/OI2c) recovered from two of these samples were purified, and sequenced. BLAST analysis showed that the nucleotide sequences obtained for the ribosomal protein (GenBank Accessions No. JN049632) had 100% identity with sequences of '*Ca*. L. asiaticus' from China (DQ431997), Taiwan (AB555707), Indonesia (AB480102), Florida (CP001677), and Brazil (AY91933). This is the first report on the occurrence of HLB disease on citrus grapefruit in Iran.

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PREDOMINANCE OF SINGLE PROPHAGE CARRYING A CRISPR/CAS SYSTEM IN "CANDIDATUS LIBERIBACTER ASIATICUS" STRAINS IN SOUTHERN CHINA

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"*Candidatus* Liberibacter asiaticus" (*Ca*Las) is an uncultureable α-proteobacterium associated with citrus Huanglongbing (HLB, yellow shoot disease), a highly destructive disease affecting citrus production worldwide. HLB was observed in Guangdong Province of China over a hundred years ago and remains endemic there. Little is known about *Ca*Las biology due to its uncultureable nature. This study began with the genome sequence analysis of *Ca*Las strain A4 from Guangdong in the prophage region. Within the two currently known prophage types, Type 1 (SC1-like) and Type 2 (SC2-like), A4 genome contained only a Type 2 prophage, CGdP2, namely. An analysis on *Ca*Las strains collected in Guangdong showed that Type 2 prophage dominated the bacterial population (82.6%, 71/86). An extended survey covering five provinces in southern China also revealed the predominance of single prophage (Type 1 or Type 2) in the *Ca*Las population (90.4%, 169/187). *Ca*Las strains with two and no prophage types accounted for 7.2% and 2.8%, respectively. In silico analyses on CGdP2 identified a CRISPR (clustered regularly interspaced short palindromic repeats)/cas (CRISPR-associated protein genes) system, consisting of four 22 bp repeats, three 23 bp spacers and 9 predicted cas. Similar CRISPR/cas systems were detected in all 10 published *Ca*Las prophages as well as 14 *Ca*Las field strains in southern China. Both Type 1 and Type 2 prophages shared almost identical sequences in spacer 1 and 3 but not spacer 2. Considering that the function of a CRISPR/cas system was to destroy invading, it was hypothesized that a pre-established *Ca*Las prophage could use its CRISPR/cas system guided by spacer 1 or/and 3 to defeat the invasion of the other phage/prophage DNA. This hypothesis explained the predominance of single prophage type in the *Ca*Las population in southern China. This is the first report of CRISPR/cas system the "*Ca*. Liberibacter" genera.

GENETIC DIVERSITY OF CANDIDATUS LIBERIBACTER ASIATICUS IN IRAN

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Citrus Huanglongbing (HLB) has been reported in recent years in various areas of southern Iran. The genetic diversity of Iranian isolates of *Candidatus* Liberibacter asiaticus (*Ca*Las) was carried out by PCR amplification and sequencing four DNA fragments including 16S rRNA gene, 16S/23S rRNA intergenic spacer region, the *rplKAJL-rpoBC* operon and outer membrane protein (*omp*) gene. Sequence analysis of the 16S rRNA gene, and the 16S/23S rRNA intergenic spacer region did not reveal any differences between various Iranian isolates of *Ca*Las. Partial sequence analysis of the *rplKAJL-rpoBC* operon and *omp* gene showed that identity level among Iranian isolates of *Ca*. L. asiaticus was 99.8% to 100% and 99.2% to 100%, respectively. Phylogenetic trees based on sequences of 16S rRNA gene, 16S/23S rRNA intergenic spacer region, *rplKAJL-rpoBC* operon and *omp* gene showed that the Iranian isolates of *Ca*Las with other *Ca*Las isolates of different origins were in one group, and distinct from *Candidatus* Liberibacter africanus, and *Candidatus* Liberibacter americanus. A number of nine microsatellite markers were used to study the genetic diversity of nineteen *Ca*Las isolates from Iran, Taiwan, Japan and Brazil. All nine primer pairs showed polymorphism among the isolates. A total of 38 alleles were detected using nine SSR markers. The number of alleles per locus ranged from 2 to 7, with an average of 4.22 alleles per locus. The average of the haploid genetic diversity (H) was 0.5819 for all SSR markers. Cluster analysis of the *Ca*Las isolates using the UPGMA method based on Jaccard coefficient clearly separated the isolates into four clusters including Iranian, Japanese, Taiwanese and Brazilian isolates. Iranian isolates of *Ca*Las were clustered in two major groups. UPGMA dendrogram separated the isolates according to their geographical origins. SSR markers showed the genetic diversity between and within the *Ca*Las populations. Using nine SSR markers among 19 studied isolates 18 haplotypes were identified.

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MULTIPLEX QPCR DETECTION OF CANDIDATUS LIBERIBACTER SPP. AND SPIROPLASMA CITRI

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A single real-time multiplex quantitative polymerase chain reaction (qPCR) assay for the simultaneous detection of *Candidatus* liberibacter spp. and *Spiroplasma citri* was developed and validated using two different fluorescently labeled minor groove binding qPCR probes. The qPCR assays have been designed to detect all *Candidatus* liberibacter spp. (asiaticus, americanus and africanus). The capacity of the multiplex qPCR assay in detecting the pathogens was compared to singleplex qPCR designed specifically for each pathogen and it was assessed using multiple pathogen isolates from diverse geographical regions and citrus species. No significant differences in detection limits were found and specificity was not affected by the inclusion of the two assays in a multiplex reaction. Comparison of the pathogen load for each pathogen using singleplex and multiplex qPCR assays, revealed no significant differences between the two assays. Optimizing the DNA extraction technique for citrus tissues and testing the quality of the extracted DNA using qPCR targeting the cytochrome oxidase citrus gene as a specific internal control proved to generate better diagnostic assays. Results showed that the developed multiplex qPCR can streamline pathogen testing by replacing four separate singleplex assays, thus reducing time and labor while retaining the same sensitivity and specificity. Adopting a compatible multiplex qPCR testing protocol for these pathogens as well as other RNA and DNA regulated pathogens will provide a valuable tool for pathogen detection in programs such as California's "Citrus Nursery Stock Pest Cleanliness Program".

FALSE POSITIVES IN MOLECULAR DETECTION OF CANDIDATUS LIBERIBACTER IN CITRUS ASSOCIATED TO ENDOPHYTIC BACTERIA

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Citrus are severely affected by Huanglongbing (HLB) in several countries of South America, Central America and Florida (USA). The disease is caused by three *Candidatus* Liberibacter spp. It is considered one of the most destructive diseases affecting citrus worldwide, considering the severity of the symptoms, its rapid spread and that up to date there is no curative control. Early detection is difficult because symptoms can take a year to be evident and can be confused with other diseases or nutrient deficiencies. Bacterial development is sensible to specific temperatures, pathogen load, its latent period and symptom manifestation. In Chile HLB has not been detected, but it is necessary to implement high reproducible methods for the early detection of this pathogen. With the objective to verify and validate the absence of HLB in plants of the citrus germplasm of the Experimental Station of La Palma, plants of 10 different accessions were kept in greenhouse under two different temperatures (20± 3℃ and 25±3℃) for a six-month period. Later these plants were evaluated to detect the presence of HLB using a commercial kit for *Candidatus* Liberibacter spp. (Plant Print, Diagnostic, Spain). The plants analyzed were all symptomless, but two of them gave positive PCR results. Barks from positive plants were grafted onto healthy (tested to be HBL free) citrus plants and were grown for 10 months in a greenhouse at 25± 5℃, using 6 replicates. Three of the replicates gave positive results using the commercial kit; negative controls showed negative results. Endophytic bacteria were recovered from vascular tissues; 16SrRNA tests showed the presence of *Ochrobacterium pseudogrignonense*, *Acinetobacter johnsonii* and *Sphingomonas paucimobilis*. This will be useful to avoid the eradication of positive symptomless citrus plants in nurseries or commercial orchards.

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Funded by: Vicerrectoría de Investigación y Estudios Avanzados VIREA-PUCV Proyecto DI 037.450/2013.

ANATOMICAL RESEARCH ON TOLERANT CITRUS VARIETY INFECTED WITH HUANGLONGBING

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Huanglongbing (HLB) is a wide-spread disease causing severe impact to the citrus industry around the world. HLB could infect almost every variety of citrus, with which susceptible varieties would typically show yellowing symptoms. The tolerance of pumelo varieties has been noticed in the field over the past decade in the P.R. China and pumelo affected by HLB could remain similar production as the non-infected. However, the mechanism of such tolerance is still under poor knowledge.

This study focused on the anatomical response of pumelo to the HLB. Symptomatic leaves of pumelo trees were collected from the field and qPCR was conducted to detect *Candidatus* Liberibacter asiaticus (*Ca*Las). Slices of midribs of HLB-affected leaves were prepared using paraffin sectioning and ultrathin sectioning method followed by investigation under light microscopy and transmission electron microscope (TEM). Foliar samples of healthy pumelo were used as control. Mandarin samples were also examined because of its susceptible characteristic to HLB.

The common morphological features of pumelo and mandarin were listed as below: starch accumulation in parenchyma tissues, disorder arrangement of phloem cells, thickening intercellular layer and cell wall. These results were in accordance with previous studies.

Different features were also discovered between pumelo and mandarin. The thickening growth of intercellular layer of pumelo was relatively mild, the phloem organization was less misshapen, primary and secondary phloem parts were more distinguishable compared to mandarin. While the great starch granules disrupted inner structure of the companion cells in mandarin midribs, the organelles of pumelo companion cells were still recognizable. The results of TEM showed that the sizes of starch granule in pumelo were larger than those in mandarin. Transverse section of single pumelo starch could reach 2.848 μm^2, some even 6.0 μm^2, while single mandarin starch could just reach 1.924μm^2 with maximum size as 3.3 μm^2. Pumelo also carried more vigorous ability of differentiation in the phloem. In the same secondary ray, the numbers of cell layer of the phloem in healthy pumelo were 8-12, but those of HLB could reach 19-30 layers, the numbers in infected mandarins were below 20. At last, unique "loops of thickening" were discovered in the phloem parts of infected pumelo midribs. These types of loops were seemed to be extensively thickening of dead phloem tissues and such feature could not be found in all healthy pumelo and HLB-mandarin.

In conclusion, some unique anatomical features on the pumelo could play some role in its tolerance to HLB. But it's necessary to commit further investigation for a complete understanding.

MOLECULAR IDENTIFICATION OF CITRUS TRISTEZA VIRUS ISOLATES FROM CITRUS GROWING REGIONS OF NIGERIA

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Field surveys were conducted in twelve citrus growing locations of Nigeria and a total of 120 samples were obtained from symptomatic and asymptomatic citrus trees. Total RNA was extracted and the presence of *Citrus tristeza virus* (CTV) was confirmed in 41 samples using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with virus-specific primers. Twenty isolates from different locations were selected and characterized by bi-directional PCR (BD-PCR) analysis of their coat protein (*CP*) and *P*23 genes. Results of the CP gene showed amplification of a 320 bp fragment in all the 20 isolates which is specific for severe CTV strains. For the *P*23 gene, fragments of 239 bp and 450 bp were simultaneously amplified in 12 isolates, indicating mixed occurrence of mild and severe strains. Eight isolates however produced only a 450 bp fragment which is representative of severe strains. These results indicate the predominant occurrence of severe CTV strains in Nigeria which occurred both in single and mixed populations. This might be as a result of several years of strain accumulation in infected trees. Full length sequence analysis of the *CP* and *P*23 genes are ongoing to reveal their relationship with other CTV isolates worldwide. This study reveals the potential of severe CTV strains to undermine citrus orchards as a source of income for many rural households in Nigeria.

MULTIPLE GENES EXPRESSION FROM CITRUS TRISTEZA VIRUS BASED VECTORS

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Viruses transform infected cells into virus protein producing factories. Yet modification of the compact genome of most plant viruses to express a foreign gene results in recombination and elimination of the inserted sequence relatively quickly. In contrast, *Citrus tristeza virus* (CTV), the positive sense single stranded RNA virus with the largest reported genome, has expressed a single foreign gene from different locations within the T36 strain genome for several years. To further our understanding of the limits of CTV as a transient expression vector, we created a series of vectors to express two and three different genes from different locations within the T36 CTV genome using two different strategies. The first strategy depended on using a single controller element through the gene fusion strategy and post-translational processing. The second strategy depended on using multiple controller elements to drive two or three different foreign genes by combining the different positions previously identified. For building the new series of vectors we utilized genes that express the green fluorescent protein (GFP) and red fluorescent protein (RFP), bimolecular complementation (BiFC) comprised of a divided yellow fluorescent protein with interacting transcription factors, β-Glucuronidase (GUS) and two *Tobacco etch virus* proteases (helper component protease (HC-Pro) and nuclear inclusion a (Nla). Initially, we tested our vectors in the experimental host *Nicotiana benthamiana* before selecting which constructs to move to citrus. Both strategies enabled the expression of foreign genes from CTV based vectors albeit with different efficiency and stability. Expression of large gene products by post-translational processing tended to express the foreign sequence much less efficiently based on the number of cells infected, the amount of fluorescence and GUS expression, especially when inserted at the position between p23 and 3'UTR of the genome. On the other hand, expression of the different genes from different positions within the CTV genome enabled a larger number of cells to be infected and better expression of the reporter genes.

APPLICATION OF RNAI TO IMPROVEMENT OF CITRUS TO ACQUIRE RESISTANCE TO CITRUS TRISTEZA VIRUS

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Citrus tristeza virus (CTV) was one of the most destructive and economically important diseases of commercial citrus worldwide. It is necessary to exploit other control methods, while it is difficult to make control effect persistent on CTV by application of virus-free trees because of the aphid transmission. Various studies revealed RNAi as gene silencing technology was a viable tool to achieve control of plant viruses. Here, we tried to use this technology to acquire citrus resistant to CTV. An expression vector was designed to express a hairpin RNA (hpRNA) homologous of CTV p23 which is an important CTV pathogenicity determinant. Based on the transient expression technology established in citrus, we investigated the ability of dsRNA construct to initiate RNAi, and forecast the feasibility of dsRNA construct in making citrus resistant to CTV before genetic transformation. Then, the p23-RNAi vector was transferred into 'DA HONG' sweet orange via *Agrobacterium* mediated transformation. There were 7 transgenic plants (named A, B, C, E, F, G and H) confirmed by PCR test. The real-time PCR results indicated that these transgenic plants had different ability to express dsRNA. After generations of transgenic plants were inoculated with CTV, generation-C was found to have significant resistance to CTV by the detections of ELISA and qPCR.

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LATERAL FLOW IMMUNOASSAY FOR THE RAPID DETECTION OF *CITRUS TRISTEZA VIRUS*

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A lateral flow methodology was developed using gold nanoparticles for rapid detection of *Citrus tristeza virus* (CTV). The test strip was based on a sandwich immunoassay and could be accomplished within 10 minutes. A sample was considered negative for CTV when only the control line appeared; whereas, a sample was considered positive when both control and test lines appeared. A single test required 0.1－0.2 mL of plant extract prepared using coating buffer (pH 9.6). Colloidal gold was prepared by a reduction method using tetrachloroauric acid with trisodium citrate. Purified polyclonal antibodies were developed using a concentrated preparation of CTV virions generated in *Nicotiana benthamiana* plants inoculated with infectious cDNA clone of CTV. The optimum quantity of antibodies that avoided aggregation of colloidal gold solution by NaCl was chosen for gold conjugation. Affinity purified IgG to CTV and Mouse IgG were conjugated with gold nanoparticles and coated onto a glass fiber membrane to serve as the conjugate pad. The nitrocellulose membrane was coated using CTV IgG (1mg/mL) and anti-mouse IgG (1mg/mL) as test line and control line, respectively. The sensitivity of the lateral flow test was up to 1:160 dilution of clarified plant extract. The methodology detected different strains of CTV such as T30, T36, VT and RB. The lateral flow strip provides a rapid and cost effective instant field CTV detection method usable by non-skilled personnel.

MOLECULAR CHARACTERIZATION OF PERUVIAN CITRUS TRISTEZA VIRUS ISOLATES BASED ON 3' UNTRANSCRIBED REGION SEQUENCES

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Citrus in Peru was decimated by quick decline and stem pitting strains of *Citrus tristeza virus* (CTV). Commercial citrus production in Peru is being restored by use of CTV cross-protection. To characterize the predominant CTV strains involved, Peruvian CTV isolates from "protected" and "non-protective" trees were imported and propagated in a quarantine facility in Beltsville, MD. Isolates were examined by serology, multiple molecular marker, single-stranded conformational polymorphism, and Taqman-based Revere Transcription quantitative PCR assays but strain differentiation was unclear. Therefore, 14 Peruvian CTV isolates were screened based on sequences derived from 3' Untranslated Region (UTR) degenerate primers since 3'UTR sequences are conserved in the CTV genome. Phylogenetic analysis of Peruvian isolates with 56 known CTV isolates from NCBI based on the 3'UTR region showed that 11 Peruvian isolates were in the VT clade and 3 were in the RB clade. This data was confirmed with additional phylogenetic analysis of selected Peruvian isolates based on 9 ORFs (p6 to p23). In summary, screening of CTV genotypes using 3'UTR sequences was more precise than other techniques and is being used to characterize putative CTV cross-protective isolates and its phenotype.

INFLUENCE OF THE QUANTITY OF CITRUS TRISTEZA VIRUS ON TRANSMISSIBILITY BY DIFFERENT APHID SPECIES

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Citrus tristeza virus (CTV) is transmitted in a semi-persistent manner by several aphid species. In this study, eight CTV isolates were detected in single aphid of *Toxoptera citricida*, *Aphis gossypii*, *T.aurantii* and *A. citricola* with frequency of 76%-82% by nested RT-PCR after 24h acquisition access period. CTV detection rates in *A. gossypii* was the highest, followed by *A. citricola*, *T. citricida* and *T. aurantii*, respectively. These results suggested that CTV was acquired by different aphid species regardless of their transmissibility. Analysis by real-time RT-PCR showed the amount of CTV in Jincheng sweet orange was similar and no obvious relationship between transmissibility and CTV accumulation in Jincheng sweet orange was found. The results also showed the number of CTV-targets in aphid ranged from $5.36 \times 10^2 \pm 2.33 \times 10^2$ to $2.01 \times 10^6 \pm 3.67 \times 10^5$. As the most efficient vector to CTV, most copies of CTV-targets were detected in single viruliferous aphid of *T.citricida*, followed by *A. gossypii*, *T. aurantii* and *A. citricola*, respectively. Furthermore, CTV isolates with high transmissibility were present with much more virus particles in single aphid of *T.citricida* than isolates with low transmissibility. These results indicated that the quantity of CTV in aphid may play a role in aphid transmission.

This work was supported by Chongqing Research Program of Foundation Research and Frontier Technology (cstc2015jcyjBX0043).

GENOME SEQUENCING THROUGH VIRAL SMALL RNAS OF A CITRUS TRISTEZA VIRUS ISOLATE FROM HUNAN PROVINCE REVEALS THE PRESENCE OF MULTIPLE STEM PITTING STRAINS

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The biological and genomic characterization of a *Citrus tristeza virus* (CTV) isolate detected in Chenzhou, Hunan was completed. It induces severe vein corking and stem pitting on Mexican lime; severe seedling yellows on sour orange, and yellowing, short internodes and stem pitting on Duncan grapefruit and Hamlin grafted on sour orange. CE-SSCP profiles of p23 and p25 revealed a multiple peaks pattern, indicative of the presence of mixed infections with several sequence variants. According to highly conserved p25, the main variant clustered close to recombinant isolates H16-5 and H18-9 from Hawaii and the VT-like Kpg3 from India, while the minor variants were located close to the severe VT.

In order to complete the genome characterization, deep sequencing analysis was carried out by the high-throughput Illumina technology (HiSeq 2000) on the small RNA fraction isolated from sour orange bark inoculated with the source plant. The sRNA reads were aligned to a set of 17 reference sequences of CTV isolates representative of the six genotypes described. Seven VT-like isolates showed the highest mapped read count and combined consensus nucleotide identity (96-98%). The consensus obtained by alignment with T318A isolate, showed the highest number of reads with genome coverage of 100%. This was considered the main isolate of CTV population infecting the natural hosting plant. It was named CTV-HU and was submitted to GenBank under the accession number KU720382. Subsequent bioinformatic analysis showed a smaller read count with reference isolates belonging to T3 and T68 genotypes, responsible of severe stem pitting. Since stem pitting was not shown in the field tree it appears that multiple strains infections occurred along the years likely affected the phenotype expression of the tristeza disease.

INTERACTIONS BETWEEN STRUCTURAL AND NONSTRUCTURAL PROTEINS OF CITRUS TRISTEZA VIRUS

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Citrus tristeza virus (CTV), a member of the genus *Closterovirus* in the family *Closterovidae*, possess a highly complicated genome encoding at least 19 proteins, of which, the ten open reading frames (ORFs) at the 3′ terminal of its genome have multiple biological functions. Specific protein-protein interactions (PPIs) among viral proteins play important roles for its successful infection, replication and movement in hosts. Recently, we have identified the key sequences responsible for interactions between CP and P20, and for self-interactions of the two proteins, respectively. In this study, we further investigated the PPIs between three virus proteins (CPm, CP and P20) and other seven nonstructural proteins (P33, P6, P65, P61, P18, P20, and P23) at the 3′ terminal of the viral genome by using a GAL4-based yeast two-hybrid (Y2H) system. When these complete proteins were used as baits or preys, there was any positive interaction identified by Y2H. Then the possible transmembrane domains predicted using a software were deleted from these nonstructural proteins. Y2H assays revealed consistent interaction signals from reaction combinations CPm-△P61-N (aa 1-200th), CPm-△P65-N (aa 20-109th), P20-△P61-N, P20-△P65-N, and P20-△P65-M (159-386th). However, the reactions were direction depended, and positive reactions were happened only when CPm and P20 served as baits. CTV P20 is a major component of inclusion bodies, and P61, P65 along with CP and CPm are the capsid components of CTV virons. Our findings indicate that these PPIs may play important roles in CTV virion assembly. In addition, further experiments will be conducted to analyse the possible new functions of the interactions in CTV infection.

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This study was financially supported by the National Natural Science Foundation of China (grant no: 31272145 and 30871684).

CITRUS LEPROSIS AND BREVIPALPUS IN THE LOWER RIO GRANDE VALLEY-TEXAS

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Citrus leprosis virus (CiLV) causes one of the most economically damaging diseases for the citrus industry worldwide. The flat mite *Brevipalpus phoenicis* is one of the vectors of CiLV. The fairly recent discoveries of CiLV in Chiapas and Queretaro in Mexico have increased the possibility that the disease and the mites carrying CiLV could reach the United States. Since 2006, 15,000 citrus fruits and leaf samples from dooryards and groves throughout the Lower Rio Grande Valley-TX (LRGV) have been collected and examined for the diagnostic symptoms of citrus leprosis. *Brevipalpus* mites were also collected from citrus and other plants for identification and determination of its geographical distribution. Thus far, citrus leprosis has not been detected, but *Brevipalpus* mites were widely distributed in LRGV, and they were more frequently found in citrus in dooryards than in groves. Based on DNA sequence similarity, members of the *B. californicus*, *B. phoenicis*, and *B. obovatus* species groups were found. Two of these species groups, *B. californicus* and *B. phoenicis*, appear to be widespread occurring from Brownsville to McAllen and north to Raymondville. Further, *B. californicus* members occurred on every sampled host except lemon and wild olive, whereas *B. phoenicis* and *B. obovatus* members appeared to be more restricted in their distribution and host plants. Currently, fast and more efficient methods of detection for CiLV and better identification of the vector are being pursued to limit CiLV spread if it is found.

CITRUS PSOROSIS VIRUS IN TUNISIA: PREVALENCE AND MOLECULAR CHARACTERIZATION

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The total citrus growing area in Tunisia is about 24.000 ha mainly located in Cap Bon (Northwest region) with an average yield ranging from 15 to 18 tons. This yield decrease is attributed to abiotic and/or biotic constraints. From these, viral diseases are known to seriously reduce the potential of the most cultivars. Psorosis is a severe disease of citrus with a worlwide distribution. it is is caused by *Citrus psorosis virus* (CPsV), genus *Ophiovirus* (Milne et al; 2000). In order to study the incidence of CPsV, an extensive survey in commercial orchards in Cap bon region was conducted in 2013/2014 that included 575 samples trees of many cultivars of sweet orange (*Citrus sinensis*), mandarin (*C. reticulata*) and clementine (*C. clementina*). The contamination rate of CPsV was determined by serological test (ELISA-DAS) using specific monoclonal antibody (Agritest, Italy). 29.7% of samples tested were positive. To investigate genetic variability and population structure of CPsV in selected Tunisian isolates, we focused on coat protein gene (RNA 3). cDNA encompassing the full-length RNA 3 from Tunisian isolates was synthesized by RT-PCR using specific primers CPV1/CPV2, ligated into the pGEM-T plasmid vector (Promega Corp.) and cloned in Escherichia coli DH5α cells. DNA PCR amplified from individual clones was SSCP analysed. The nucleotide sequence of selected inserts having different profiles was determined. Two full length sequences of CP gene were obtained and deposited in GenBank NCBI under accession numbers KT989886 and KT989887. A low level of variability into coat protein gene of Tunisian isolates was shown by SSCP analysis and confirmed by sequencing. In fact, sequence identity among these new isolates shared 99% with only 9 variables sites. Multiple alignment performed with 8 other CP gene sequences from Spain, Algeria, Italy, Egypt and USA shows a sequences identity ranged between 95% and 99% with Tunisian isolates.

DISTRIBUTION OF CYVCV AND CELL STRUCTURAL CHANGE IN INFECTED EUNEKA LEMON LEAVES

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Citrus yellow vein clearing, caused by *Citrus yellow vein clearing virus* (CYVCV), is a serious threat to the development of lemon and sour orange. This disease results in severe symptoms on Eureka lemon leaves, including chlorosis, lateral vein yellowing and clearing, leave crinkle or curl-up. Previous studies showed that CYVCV was a filamentous virus with 13 nm-14 nm×685 nm particles in width and length. In this study, CYVCV distribution and cell structural change was conducted by using the real-time fluorescence quantitative RT-PCR (qRT-PCR) and light/electron microscopy. More CYVCV particles, gathered or dispersed, were found in some inner layer cells around mesophyll secretory cavities of Eureka lemon leaves besides in phloem sieve tube cells, which was further confirmed by virus quantitative analysis using qRT-PCR. The morphological structure changes induced by CYVCV were observed, including more cell density in mesophyll, phloem collapse, the more and larger starch grains in chloroplasts, chloroplast deformity, osmiophilic granules accumulation, numerous small vacuoles, multivesicular bodies, fibril-containing vesicles, mitochondria disintegration, chromatin condensation, endoplasmic reticulum expansion, golgi structure fuzzy, etc.

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DEEP SEQUENCING AND CHARACTERIZATION OF CITRUS YELLOW VEIN CLEARING VIRUS ISOLATES FROM CHONGQING AND YUNNAN PROVINCE, CHINA

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Citrus yellow vein clearing was an important vector-transmitted disease that was found in Pakistan, India, Turkey and China. Here small RNA (sRNA) deep sequencing method was performed to explore the potential pathogens of the yellow vein clearing lemon samples collected from Chongqing and Yunnan. After sequence trimming, assembly and BLAST, it was generated a population of *Citrus yellow vein clearing virus* (CYVCV) derived contigs. In addition to RT-PCR and 5'/3' RACE, two full-length genome sequences were obtained as well as several putative ORFs of variable length were also predicted within their genomes, analysis such as BLASTs and SDT alignments showed that these two complete genomes shared 97.2% of nucleotide similarity with that of the isolate Y1 (No. JX040635.1) deposited in Genbank. Our results indicated that both CYVCV isolates CQ (KP313240.1) and YN (KP313242.1), along with isolate PK (KP313241.1) collected from Pakistan and Y1, belong to the genus *Mandarivirus* in the family of *Alphaflexiviridae*, they not only shared highly genetic stability from different geographic regions, but also indicated diversification in their separate evolution history.

CURRENT STATUS OF CITRUS CHLOROTIC DWARF ASSOCIATED VIRUS DISEASE IN THE EASTERN MEDITERRANEAN REGION OF TURKEY

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A survey program was conducted to determine the ultimate rate of Citrus chlorotic dwarf disease in East Mediterranean Region of Turkey. The disease is very important for this region and firstly was observed in the eastern Mediterranean region of Turkey in the mid- 1980s. The disease has spread pretty much since the eighties in this region. *Citrus chlorotic dwarf associated virus* (CCDaV) was found only in the eastern Mediterranean region of Turkey where 85% of the Turkey citrus production is carried out in this area and the disease has not yet spread to other çitrus production regions. According to survey results, the infection rates were observed 36% in lemons, 25,3% in mandarins, 17,6% of oranges and 17,5% in grapefruit in total at the Eastern Mediterranean Region of Turkey. The survey of CCDaV disease was made macroscopically with the disease symptoms. 50 samples were collected from virus infected orchards and analyzed with PCR. In Conventional PCR studies, the forward (5′-gttctgtg tttcgacccgtt-3′) and the reverse (5′-gggattcgcatggatagctcatccaa-3′) primers were used for CCDaV and 444 bp bands were seen in agarose gel. The amplicons sequencing confirmed CCDaV infection of the disease for samples. CCDaV is carried by *Parabemisia myricae* (Kuwana) (Insecta: Hemiptera: Aleyrodidae: Aleyrodinae). There is no whitefly for along time in East Mediterranean Region of Turkey but the disease continues to spread in the region.

INVESTIGATING THE TSNRNA-IIIB-INDUCED CITRUS DWARFING MECHANISM

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The term 'Transmissible small nuclear Ribonucleic acids' (TsnRNAs) is used to describe well-characterized viroid RNA species that do not induce disease in specific citrus hosts, but rather act as regulatory genetic elements modifying tree performance. TsnRNA-IIIb (syn. citrus dwarfing viroid), reduced the canopy volume of navel orange trees on trifoliate rootstock, increased the yield per canopy volume, and concentrated fruit in the optimum canopy zone for harvest, without affecting fruit quality. On the other hand, TsnRNA-IIIb did not modify tree performance of other scion/rootstock combinations. These findings gave raised questions regarding the mechanism of this plant-TsnRNA interaction, and the different effects observed depending on the TsnRNA/scion/rootstock combination, which suggests the existence of a translocatable element and/or a fine-tuning mechanism depending on the citrus and RNA species involved. To understand the plant cellular and physiological mechanisms that are modulated by TsnRNAs and result in citrus tree dwarfing, we initiated a physiological study of a 1997 field trial. Our initial measurements showed that the TsnRNA-IIIb reduced canopy volume of parent navel trees on trifoliate rootstock is the result of reduced apical shoots development. Contrary to untreated trees, we also observed that the length of apical shoots is not significantly different between the north and south tree sections. The possible involvement of phytohormones, key regulators of growth and development, will be discussed in the context of the observed dwarfed trees. The importance of elucidating the TsnRNA dwarfing mechanism lies in the potential commercial applications without the use of a transmissible agent. Dwarfed trees are fundamental for high-density plantings that will be critical for meeting challenges posed by water shortages, diseases spread (e.g. Huanglongbing), farmland reduction, labor cost and the potential for mechanized harvesting.

ONE-STEP MULTIPLEX QUANTITATIVE RT-PCR FOR THE SIMULTANEOUS DETECTION OF THREE REGULATED CITRUS VIROIDS FROM DIFFERENT GENERA

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A one-step multiplex reverse transcription (RT) real-time quantitative polymerase chain reaction (qPCR) based on minor groove binding (MGB) probes was developed for the simultaneous detection and quantification of three citrus viroids belonging to different genera. *Citrus exocortis viroid* (*Pospiviroid*), *Hop stunt viroid* (*Hostuviroid*), and *Citrus bark cracking viroid* (*Cocadviroid*) are viroids with regulatory and disease significance. Singleplex qPCR primers and MGB probes for specific detection of individual viroids were designed individually, then multiplexed together. Single, double and triple infections were detected in 20.2%, 11.7% and 8.5% of the tested citrus samples, respectively. The newly developed MGB based multiplex RT-qPCR method was compared to the SYBR green based RT-qPCR assay for the universal detection of citrus viroids approved for official use in California's "Citrus Nursery Stock Pest Cleanliness Program".

IDETIFICATION OF VIROIDS IN CITRUS ORCHANDS AT CUKUROVA REGION IN TURKEY

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A survey was made in the most important citrus growing regions of Turkey, including Adana, Mersin and Hatay cities, for the presence of viroids. Citrus viroid surveys conducted from 2012 to 2016 revealed 45 samples from 25 sweet orange (*Citrus sinensis*), 10 mandarin (*C. reticulata*),10 from lemons. To identify specific viroids, RT-PCR analysis was carried out with specific primers for *Citrus exocortis viroid* (CEVd), *Citrus cachexia viroid* (CCaVd), *Citrus bark cracking viroid* (CBCVd) and CVd-V. 30 samples were infected by CEVd, 10 with CCaVd, 6 CVd-IV and 4 with CVd-V. Viroids were detected in ten citrus samples, mostly as complexes consisting of two to four different viroids. Since commercial cultivars are usually grown on sour orange rootstock in Çukurova region, exocortis symptoms have not been observed. Typical gum spots of CCaVd were discovered on mandarin trees associated to reduction of canopy and fruiting. Nucleotide sequence analysis of these viroids revealed more than 97% identity with GenBank reference sequences.

SIX-YEARS EXPERIENCE WITH THE HIGH THROUGHPUT ROBOTIC NUCLEIC ACID EXTRACTION AND PURIFICATION PROTOCOL FOR CITRUS DIAGNOSTICS

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A semi-automated, high-throughput, and economical (US $ 4.03/sample) total nucleic acid extraction and purification procedure was developed for citrus tissues by the Citrus Clonal Protection Program (CCPP) in 2010. The system utilizes the SPEX SamplePrep's Cryostation and Geno Grinder 2010 and the Applied Biosystems' MagMAX™ Express-96 (MME-96) along with a modified 5x MME-96 Viral RNA Isolation Kit. High quality RNA extracts, defined by concentration, purity, and integrity, are required for the detection of viral RNA by reverse transcription quantitative real time polymerase chain reactions (RT-qPCR). RNA concentration and purity were assessed by spectrophotometry at 260 nm and the 260/280 ratio, respectively. The RNA concentration of approximately 1000 samples had an average value of 118.9 ng/μL (n=968). The majority of the samples (82.6%) had concentrations ranging from 50-400 ng/μL. The RNA purity ratio (260/280) was higher than the desirable 1.8 (i.e. low protein contaminants) for the majority of the tested samples (98.7%) with a mean value of 2.30 (±0.24, n=968). The RNA integrity was also high, as evaluated by RT-qPCR targeting the mRNA of the NADH dehydrogenase citrus gene. The mean Cq value was 21.4 (±2.8, n=439) with maximum and minimum values of 29.5 and 16.2, respectively. For the past six years, CCPP has utilized this RNA extraction procedure for over 10,000 citrus samples from nurseries, orchards, budwood tree sources, and germplasm introductions for the detection of graft-transmissible pathogens using newly developed universal, single- and multi- plex qPCR assays. This procedure was also recently adopted for RNA extractions from avocado tissues. Paired with a newly developed individual RT-qPCR assay targeting the *Avocado sunblotch viroid* (ASBVd) and the avocado Cytochrome oxidase (COX) gene as an internal control, this has become the new ASBVd diagnostic standard in California.

DIGITAL PCR FOR DETECTION OF CITRUS PATHOGENS

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Citrus trees are often infected with multiple pathogens of economic importance, especially those with insect or mite vectors. Real-time/quantitative PCR (qPCR) has been used for high-throughput detection and relative quantification of pathogens; however, target reference or standards are required. In addition, sensitivity to inhibitors in perennial plants can lead to lower quantification accuracy. Recently, droplet digital PCR (ddPCR) has been proposed as an alternative method to overcome these drawbacks. Absolute quantification by ddPCR does not rely on standards and is reported to show high tolerance to inhibitors. Therefore, the performance of ddPCR method was tested to detect and absolute quantify multiple citrus pathogens. A one-step reverse transcriptase (RT) ddPCR system optimized for TaqMan probes for *Citrus tristeza virus* (CTV) and *Spiroplasma citri* was tested. Taqman probes were designed for CTV coat protein (CP), VT3 and T30 genotypes, and *S. citri* phage ORF1. Using a standardized nucleic acid sample at 200 ng/μL, ddPCR detected 6,890 copies (c) /μL CP; 6,690 c/μL of VT3; 6,530 c/μL of T30; and 141 c/μL of *S. citri*. These data showed that ddPCR directly quantified mixed pathogen samples by partitioning each of 96 wells into up to 20,000 partitions with each partition constituting an individual real-time PCR reaction. This procedure had excellent reproducibility and, thus, showed great promise to examine citrus pathogen populations in citrus trees infected with multiple pathogens or strains.

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF CITRUS VIROID VI ISOLATES FROM CHINA

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Citrus viroid VI (CVd-VI) is a viroid originally found from citrus and persimmon in Japan. We report here the identification and molecular characterization of CVd-VI from four citrus growth regions of China. A total of 90 cDNA clones from nine infected citrus cultivars were sequenced. Sequence analyses revealed the presence of 13 predominant variants of CVd-VI, one to two from each of the nine cultivars. These variants shared sequence homologies of 94.2%-97% to the Japanese reference sequence. Phylogenetic analysis of the 13 Chinese variants and 9 Japanese variants showed that they were grouped into two clades, one with 19 citrus variants and another with three persimmon variants, indicating a strong correlation with host species. Multiple sequence alignment showed that most nucleotide changes between the two clades occurred in the P, V and TL domains, and analysis indicated that these mutations influenced the predicted secondary structures under minimum energy.

THE MOLECULAR VARIATION OF P23 POPULATIONS OF CITRUS TRISTEZA VIRUS IN SWEET ORANGE AND GRAPEFRUIT

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Citrus tristeza virus (CTV) belongs to *Closteroviridae* (*Closterovirus*), and is the devastating pathogen that causes economic losses in the world. CTV is transmitted by a few aphid species, and has a complex genetic population with highly divergent pathogenicity and strain variants in the field. The post-transcriptional gene silencing (PTGS) has recently arisen as a more likely mechanism to explain plant defence against viral infections and cross protection between closely related virus strains. It was reported that both P20, P23 and P25 proteins of CTV act as RNA silencing suppressors in *Nicotiana benthamiana* and *N. tabacum* plants. However, the role of these suppressors in CTV biological variability is still largely unknown. In this research, the molecular variation among *p*23 genes of CTV mild strains and severe strains was analyzed to interpret the mechanism of mild strain cross protection (MSCP) against CTV.

The populations of CTV *p*23 were established by RT-PCR, cloning and sequencing technologies. Using specific primer pairs of P23r/P23f, *p*23 genes of CTV, amplified from 4 severe strains (TR-514Y, Ziyuanguanxi, Santai and Nanbucui) and 3 mild strains (CT31, Perq and 99) of CTV in sweet orange and grapefruit by RT-PCR, were analyzed by sequence comparison. The results showed that the *p*23 populations were composed by 165 sequences. The levels of genetic variation of *p*23 populations were 1.76×10^{-4} to 9.52×10^{-4}, which is comparable to that reported for plant viruses. There were 25 kinds of haplotypes in all of the *p*23 populations, and severe strains in grapefruit had complex population structures. The analysis of the obtained 165 sequences revealed that the mutation frequency of *p*23 populations of severe strains is 4.63×10^{-4} in sweet orange and 6.84×10^{-4} in grapefruit, which is more than that of mild strains. *p*23 population of Santai had the highest level of mutation frequency among all of *p*23 populations of CTV, in which 12 nucleotides mutations occurred. Alignment the nucleotide sequences corresponding to *p*23 genes indicated that substitution, insertion and deletion mutations were all found in *p*23 populations of CTV, and their frequencies of occurrence are similar. Conversions are significantly more than transversions in sweet orange and grapefruit. The most substitution mutation in sweet orange was from G to A and from A to G, while from T to C was found in grapefruit. Comparison of molecular variation

between severe and mild strains of CTV indicated that all the insertions detected in grapefruit occurred in the populations of severe strains and most of insertions were T. No insertion was found in sweet orange. Severe strains in sweet orange had a significant influence on the substitutions of CTV *p*23 gene. But the biological significance of these mutations and their role in pathogenicity divergence of CTV remains unclear.

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This research work was supported by the Special Fund for Agro-scientific Research in the Public Interest (Grant No. 201203076-04).

THE OTHER ABSTRACTS

HUANGLONGBING (HLB) DIAGNOSIS IN CITRUS USING FIBROUS ROOT TISSUE

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Huanglongbing (HLB) is a destructive disease in citrus that is caused by unculturable, phloem-limited Gram-negative Alpha-proteobacteria that belongs to the genus '*Candidatus* Liberibacter'. It has been reported that three species of *Candidatus* Liberibacter, *Ca*. L asiaticus (*Ca*Las), *Ca*. L. americanus (*Ca*Lam) and *Ca*. L. africanus (*Ca*Laf), are causing HLB in different part of the world. While *Ca*Laf and *Ca*Lam, as their name suggested, are geographically limited to Africa and America, respectively, *Ca*Las is the most widely spread and virulent strain causing serious economical loss in citrus-growing regions around the world. Two citrus psyllids, *Diaphorina citri* and *Trioza erytreae* are vectors of *Ca*Las and *Ca*Lam in Asia and America and *Ca*Laf in Africa Since there are no known resistant commercial citrus cultivars available, the early HLB detection is critical to minimize any potential economical loss caused by HLB. Currently, the most effective HLB pest management strategy is the use of pesticides against citrus psyllids. Symptomatic citrus leaves that sometimes show symptoms similar to those induced by other biotic/abiotic stresses, are source materials for the detection of HLB-causing bacteria in citrus by qPCR. However, the uneven distribution of HLB-causing bacteria in the aerial parts of a plant can lead to a misdiagnosis. Previous work conducted in our lab indicated that HLB-positive plant exhibits more uniform *Ca*Las distribution in the root system suggesting that the root tissue can be an alternative source material for more reliable HLB diagnosis in citrus. We surveyed one hundred young grapefruit trees for HLB by qPCR once a month for three months period using leaf and root samples. The results showed that HLB diagnosis using root tissue is as sensitive as the one obtained with leaf samples. This indicated that root tissue is another good source material for HLB diagnosis especially when no visible symptoms are present on the leaf tissue.

SEASONAL EFFECTS ON CANDIDATUS LIBERIBACTER ASIATICUS TITERS IN GRAPEFRUIT TREES IN TEXAS

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Citrus Huanglongbing is a serious disease affecting citrus production worldwide. Inoculum removal is one of the major methods of disease control and this requires reliable early detection. We explored the impact of seasonality on *Candidatus* Liberibacter asiaticus (*Ca*Las) detection in grapefruit trees in Texas by testing foliar samples monthly from infected trees over two years. We found that there were both increased bacterial titer and detection in symptomatic tissue in winter as opposed to summer. This pattern was mirrored and stronger in tissues showing early disease symptoms. This involved several thousand leaf samples. In addition, *Ca*Las titers in Asian citrus psyllids over time were also determined, and full results will be presented. This work has implications for the implementation of the diagnostic methods in Texas throughout the year.

FIELD STUDY OF THE EFFICACY OF PVC MULCH COVER AGAINST HUANGLONGBING

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Huanglongbing (HLB) is one of the most destructive diseases of citrus worldwide. No effective control option against the causal pathogen-*Candidatus* Liberibacter asiaticus (*Ca*Las), and no resistant (or tolerant) citrus cultivars are available so far. Current HLB control strategies focus on the vector insect prevention. We demonstrated that heat treatment can significantly reduce *Ca*Las population in HLB-affected plants under a laboratory condition. The objective of this study was to test the efficacy of heat treatment under a field condition. The study was carried out during August to September in Shunchang county, Fujian Province in 2012 and 2013, respectively. Forty 5-to-8-year-old *Citrus reticulata Ca*Las-positive trees, confirmed by nested-PCR, were subject to the heat treatment each year. The plants were randomly divided into two groups (treated and untreated control), with five plants/replicate (rep) and four reps/treatment. The treated plants were completely covered by using Polyvinyl chloride (PVC) mulch 10:00 to 17:00. A PVC mulch cover was supported with two pieces of bamboo crossing each other. The top of the cover was about 30-50 cm from the top of the plants. Ten 2-cm-in-diameter holes were made on the top of the each cover. The plants were treated three times in intermittent each year. The plants in control group were not covered by PVC mulch. Three HOBO temperature sensors (U23-001) were attached 1.25 m above the ground on the north side of the trunk of three treated trees (i.e. one sensor/tree), and other three sensors attached to three untreated control in the same way. The sensors recorded temperature data every five min. HLB symptom evaluations were made immediately before treatment, one and three months after treatment, respectively. A total of 20 plants, ten from the treated group, ten from untreated, was used for the evaluation. Ten mature leaves were randomly selected from a test plants, recording disease severity, based on pre-defined index, for later statistical analysis. Five leaves were collected from each plant before treatment, one, and three months after treatment, respectively. Total genomic DNA was extracted by CTAB (Cetyltrimethyl ammonium bromide) extraction method. The concentration of *Ca*Las was detected through quantitative real-time PCR (qRT-PCR). The results indicated that the lowest, and average temperatures inside of the PVC cover (the treated group) were 39.0 and 40.4, 44.8 and 45.2℃ in 2012 and 2013, respectively. HLB disease symptoms in the treated groups were significantly mitigated in both years - new flushes were

abundance three months after the last heat treatment, healthy green leaf color without typical HLB symptoms such as yellow or mottled. The disease index was reduced statistically significantly different, from 0.894 and 0.819 to 0.456 and 0.463 three month after the last treatment in 2012 and 2013, respectively. HLB symptoms in untreated control plants worsened, HLB disease index of three month after the last heat treatment increased from 0.763 and 0.781 to 0.844 and 0.875 in 2012 and 2013, respectively, but no statistically different. The concentrations of *Ca*Las were decreased by 71.77% and 49.22% one month after the last treatment in the treated group in 2012 and 2013, respectively, and HLB concentrations in 19 plants were reduced more than 90%. The concentrations of *Ca*Las were decreased by 85.36 and 81.67% three months after last treatment in 2012 and 2013, respectively, with a total of 27 plants having more than 90% *Ca*Las reduction. The concentrations of *Ca*Las of the untreated control plants increased 22.86 and 25.50 times three months after the last treatment in 2012 and 2013, respectively, with the highest reduction of 94-fold. The result of this study demonstrated that PVC mulch cover can significantly reduce *Ca*Las bacteria in infected plants. HLB disease symptoms were also dramatically reduced by the treatment. This study provides a solid foundation for future further work in finding effective option for the disease management.

TRANSCRIPTOME PROFILING OF ATALANTIA BUXIFOLIA RESPONSE TO 'CANDIDATUS LIBERIBACTER ASIATICUS' INFECTION

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Citrus Huanglongbing (HLB) is the severely destructive disease of citrus, induced by unculturable bacteria '*Candidatus* Liberibacter asiaticus' (*Ca*las). Breeding cultivars resistant to bacterial '*Ca*las' would be the best method for dealing with the disease. In our prophase research, *Atalantia buxifolia* have no typical syndrome after grafted with HLB-positive citrus branch. In order to explore the mechanism of this, the transcriptome profiling difference between healthy leaves (control) and affected (treated) *Atalantia buxifolia* leaves was conducted with RNA-Seq. 67080 unigenes were identified between treated and control after grafted two and three months. A total of 328 genes were identified after 2 months, there were 236 up-regulated and 92 down-regulated genes. After 3 months, there were 63 genes up-regulated and 30 down-regulated genes. KOG function classification involved in many classes including signal transduction mechanisms, defense mechanisms, transcription and general function. Gene ontology annotation analysis identified many pathogenesis-relevant categories, including 'ATP binding', 'oxidation-reduction process', and 'integral to membrane'. Based on qRT-PCR results, we studied some unigenes about disease-resistance such as BED finger-nbs-lrr resistance protein, probable disease resistance protein At4g27220 and putative disease resistance protein RGA4-like, and confirmed the accuracy of transcriptome data. The dynamic expression changes reflect transcriptome regulation of the networks in the HLB-infection response of *Atalantia buxifolia*.

COMPARISONS OF MIRNA PROFILES AND MIRAN TARGET GENE EXPRESSIONS IN RESPONSE TO HUANGLONGBING DISEASE IN CITRUS ROOTS

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MicroRNAs (miRNAs) play a critical role in plant-pathogen interactions. Profiling miRNAs involved in the responses of Citrus to Huanglongbing (HLB), a destructive disease caused by *Candidatus* Liberibacter spp., should provide clues to the understanding of HLB pathogenesis. In this study, solexa sequencing was used to reveal the changes in small RNAome profiles in roots of mock-inoculated (M) and Huanglongbing bacterium-inoculated (H) 'Sanhu' red tangerine (*Citrus reticulata* blanco cv. 'Sanhu') trees. A total of 186 known miRNAs from 63 miRNA families were identified. Thirty-seven of them exhibited a significant difference in expression between M and H samples. In addition, 71 novel miRNAs, among which 18 were differentially expressed, were also identified. The expression levels of 8 miRNAs and their putative target genes were analyzed by quantitative real-time PCR, and the expected inverse correlations were confirmed. MapMan analysis revealed that most of the genes putatively targeted by differentially expressed miRNAs were implicated in stress response, plant growth and development, transcription and metabolism. A large number of these genes were previously reported to be regulated by HLB, and their regulation, as discussed in this study, could be explained mostly by the regulatory effects of corresponding miRNAs.

QUANTITATION OF CTV IN VIRULIFEROUS APHIDS WITH VARIOUS INOCULATION ACCESS PERIODS

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Citrus tristeza disease caused by *Citrus tristeza virus* (CTV) is of economical importance, which is spread mainly by grafting and several aphid species in semi-persistent manner. *Toxoptera citricida* (Kirkaldy) is the most efficient vector. Factors affecting aphid transmission of CTV are various, including CTV strains, virus source plants, host palnts, aphid population densities, acquisition access period and environmental conditions. But population of CTV in viruliferous aphids with varous inoculation access periods has not been reported yet.

In this study, aphids after 6 h acquisition period were transferred onto healthy sweet orange seedlings, for various periods of 0.5-48 h and then the real-time quantitative RT-PCR was used to detect CTV in that viruliferous aphids using EF-1α and RAPAO as reference genes. The results indicated that the relative quantity of CTV significantly reduced in viruliferous aphids with CT11, CT14 and CT_{Lijian} after feeding on healthy citrus for 0-3 h. Though the initial quantity of four CTV isolates in viruliferous aphids was different, the final quantity of those was similar in the period of 3-9 h or 12-48 h inoculation access.

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SUPPRESSION AND SEQUENCE VARIATION OF CITRUS TRISTEZA VIRUS GENOTYPES BY CITRUS CULTIVARS

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For the preliminary study on the suppression of *Citrus tristeza virus*(CTV) genotypes by different citrus cultivars. Four genotypes of CTV were graft-inoculated to Symons sweet orange, Mexican lime, Duncan grapefruit and Chandler pummelo. The results of RT-qPCR showed that Mexican lime was most suitable for the survival of these four CTV genotypes. The copy levels of VT, T3 and T36 genotypes in Duncan grapefruit were much lower than in other citrus cultivars. Furthermore, T36 genotype was reduced significantly by all of the citrus cultivars. Sequence analysis indicated that CP gene of VT genotype had frequent mutation in sweet orange.

This work was partially supported by Chongqing Research Program of Foundation Research and Frontier Technology (cstc2015jcyjBX0043).

CHARACTERIZATION OF CITRUS TRISTEZA VIRUS-RESPONSIVE MICRORNAS IN SWEET ORANGE LEAVES BY SMALL RNA AND DEGRADOME SEQUENCING

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Citrus tristeza virus (CTV), a member of the genus *Closterovirus* in the family *Closteroviridae*, has been divided into six genotypes: T3, T30, T36, VT, B165, and resistance breaking (RB) based on symptoms, host range, and genomic sequence. The RB genotype has been identified a novel group that can overcome *Poncirus trifoliata* resistance. MicroRNAs (miRNAs) are small, non-coding RNAs, typically functioning by guiding cleavage of target mRNAs. They play important roles in responses to pathogens. In this research, we deep sequenced the sRNA libraries and degradome libraries constructed from the leaves of healthy Sweet orange and plants infected with CTV isolate S45-2 (RB genotype). Analysis of the deep sequencing results showed that the expression patterns of 25 miRNAs in leaves changed significantly in response to CTV infection. By using degradome sequencing, a total of 13 target transcripts for 9 conserved miRNAs were shown to be responsive to CTV infection. The 13 targeting of these transcripts by RNA silencing was validated by RNA ligase-mediated rapid amplification of cDNA ends (5′ RLM-RACE). 5 cleavage of transcripts were confirmed by 5′ RLM-RACE. The results provide new insights into the regulatory networks of miRNAs and their targets in response to CTV infection.

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This study was financially supported by the National Natural Science Foundation of China (grant no: 31272145 and 30871684).

PROTEIN-PROTEIN INTERACTIONS BETWEEN A SEVERE ISOLATE OF CITRUS TRISTEZA VIRUS AND MEXICAN LIME

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Citrus tristeza virus (CTV) is considered as the most devastating virus in citrus industry. The successful infection of a plant virus depends on the interaction between the virus and its host. The accumulation of virus in the process of infection disturbs the normal metabolism of host and results in biological phenotypes. The mechanism underlying the interactions between CTV and its host citrus plants is still poorly understood. In this research, cDNA plasmid libraries of Mexican lime (*Citrus aurantifolia*) and *P. trifoliata* cDNA libraries were constructed by RT-rPCR in combination with in vitro fusion technology. Ten genes (ORFs 2-11) and the HEL domain in ORF1a of a CTV severe isolate S4 were cloned and used for the constructions of bait vectors in yeast two hybridization (Y2H) tests. The CTV gene bait and citrus library prey plasmids were co-transformed into yeast strain Y2HGold. The interactions between virus proteins and host proteins were evaluated on medium QDO/X/A. Totally 28 clones which showed positive reactions with P20 and P23 of CTV were identified from cDNA plasmid libraries of Mexican lime. Plasmid rescue and sequencing showed that these clones were from eight genes of Mexican lime. After re-construction of the prey and bait vectors of these host genes and yeast hybridization tests with P20 and P23, it was found that Carbon-sulfur lyases showed positive interaction signals with both P20 and P23, and Homeodomain leucine zipper family IV protein (HD-ZIP) and Probable protein phosphatase 2C interacted with P20 and P23, respectively. The interactions between P20 and HD-ZIP, and between P20 and Probable protein phosphatase 2C were confirmed by BiFC tests. These results provide primary clues for in sighting into the interactions between CTV isolates and their sensitive hosts.

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This study was financially supported by the National Natural Science Foundation of China (grant no: 31272145 and 30871684).

DIRECT TISSUE BLOT IMMUNOASSAY FOR DETECTION OF CITRUS YELLOW VEIN CLEARING VIRUS

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A Direct Tissue Blot Immunoassay (DTBIA) was developed to detect *Citrus yellow vein clearing virus* (CYVCV) by exploring the optimal dilution of rabbit-anti-CYVCV polyclonal antibody (first antibody) and Alkaline phosphatase (Ap) conjuagted goat-anti-rabbit IgG (secondary antibody). The DTBIA was the most effective when the first antibody and secondary antibody diluted in 8 000 and 2 000 separately. Using the method, only CYVCV infected samples showed positive from detected samples associated with different citrus diseases; CYVCV could be effectively detected within 90 days after the tissue blotting membranes kept at 4℃. The consistency between DTBIA and one-step RT-PCR was 97.92% in CYVCV detection of citrus varieties. The results showed that the DTBIA method is rapid, simple, stable, specific and reliable, and can be applied for rapid detection of CYVCV in the field, and play an important role in control of the transmission of *Citrus yellow vein clearing virus* and spread of the disease.

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DEVELOPMENT AND APPLICATION OF A QUANTITATIVE RT-PCR APPROACH FOR QUANTIFICATION OF CITRUS VEIN ENATION VIRUS

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Citrus vein enation virus (CVEV) is the presumptive causal agent of citrus vein enation/woody gall disease, occurring in Argentina, Australia, Brazil, China, Japan, India, Peru, South Africa, Spain, United States and Turkey. In order to fast detect the virus with sensitivity and specificity, a Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) assay was developed with selective primer pairs (EVqF4/EVqR4) and optimized conditions. Using the method, only samples infected with CVEV showed positive, while those with other five citrus pathogens were negative. The sensitivity of the method was 100 times higher than the conventional RT-PCR. There was a good linear ($R^2 = 0.992$) relationship between the threshold cycle and CVEV template concentration, while the amplification efficiency of the RT-qPCR was 101.8%. The coefficients of variation of the intra- and inter-assay were both within 2.85%, indicating a good reproducibility of the method. The results also showed that CVEV was uneven distributed in sour orange plants and hybrid citrus plants, and the amount of the virus in rootlets was the highest, followed by that in barks (81261 copies) and leaves (22660 copies).

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SIMULTANEOUS DETECTION OF CITRUS VIRUSES BY A NEW MULTIPLEX PCR

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A new multiplex PCR assay was developed for its effectiveness as a means to simultaneously detect 4 citrus virus, *Citrus tristeza virus* (CTV), Citrus tatter leaf virus (CTLV), *Satsuma dwarf virus* (SDV) and *Citrus mosaic virus* (CiMV). For this, we developed PP2-1 primer pairs to easily detect SDV and CiMV based on *PP2* gene full sequence of our isolates. The SDV and CiMV were detected more easily by using the PP2-1 primer pair than other primer set. CTLV-2013 and CTV-po primer sets were developed for simultaneous detection of CTLV and CTV, respectively. The results of the multiplex PCR were consistent with those of other diagnoses, such as uniplex RT-PCR, to detect each of the virus. The new multiplex PCR provides an efficient method for detecting four citrus virus, which will help diagnose many citrus plants at a time.

Appendix：

FULL PAPERS

INFLUENCE OF THE QUANTITY AND VARIABILITY OF CITRUS TRISTEZA VIRUS ON THE TRANSMISSIBILITY BY SINGLE TOXOPTERA CITRICIDA (KIRKALDY)

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ABSTRACT

In fields, *Citrus tristeza virus* (CTV) is transmitted in a semi-persistent manner by several aphid species, among which *Toxoptera citricida* is the most efficient vector. After 24h acquisition access period, eight CTV isolates were detected in single aphid of *T. citricida* at various frequencies of 63.3%-91.1% and 71.1%-91.1% by nested RT-PCR and real-time RT-PCR, respectively. Most copies of CTV-targets were detected in single viruliferous *T. citricida* that acquired CTV isolates with high transmissibility. Further analysis on the individual of viruliferous *T. citricida* that acquired CTV isolates ST9, GS14, CT11, HH12, and CTV-infected receptor plants by Single Strand Conformation Polymorphism (SSCP) showed that most of the CTV-infected receptor plants and individual of viruliferous aphids carried one haplotype of CTV, and CTV in the receptor plants showed less haplotype diversity than that in the aphids.

Key words: *Citrus tristeza virus* (CTV); aphid transmissibility; quantity and variability of CTV

INTRODUCTION

Citrus tristeza virus (CTV), one of the most destructive citrus diseases in the world, causes various disease syndromes such as stem pitting, decline and small fruit (Bar-Joseph *et al*. 1989). Economic losses caused by CTV have been recorded in some areas of China (Zhao *et al*., 1979; Zhou, 1997), and it has become more harmful to Chinese citrus production in recent years (Xu *et al*., 2006; Zhou *et al*., 2007). Tolerant rootsocks are extensively used to control tristeza decline syndrome, and Mild Strain Cross Protection (MSCP) has played an important role on controlling stem pitting in grapefruit and sweet orange (Lin *et al*., 2002; Powell *et al*., 2003).

CTV is disseminated by grafting and some aphid species in a semi-persistent manner. Toxoptera *citricida* (Kirkaldy) is known as the most efficient vector (Bar-Joseph *et al*., 1989), which can spread different CTV strains, including the strains occasionally transmitted by other aphid species (Rocha-Pena *et al*., 1995). *T. citricida* has widely distributed in

Southern China, Southeast Asia, South America, and Africa (Bar-Joseph *et al*., 1989). In recent decades, *T. citricida* has spread from South America into Caribbean, Central America and Florida. It has caused the breakdown of cross protection in grapefruit and sweet orange (Powell *et al*., 2003).

About the molecular interactions between CTV and *T. citricida*, Cambra *et al*. (2000) found that there was a correlation between RT-PCR detection of CTV in the single *Aphis gossypii* and the transmission to Mexican lime seedling. Thereafter, Satyanarayana *et al*. (2001) hinted that the quantitation of CTV in the aphids is important to assess the epidemiology of CTV. Moreno *et al*. (2009) found that the amount of *Plum pox virus* (PPV) plays a role on its aphid transmissibility. Currently, more sensitive techniques are available to detect CTV in the aphids, the relationships among the transmission efficiency of *T. citricida* and the rate of viruliferous *T. citricida* or the amount of CTV that acquired by single aphid could be further studied.

In this study, real-time RT-PCR was used to estimate if the number of CTV-targets in the single *T. citricida* was contribute to its transmissibility, and the variability of CTV during the single aphid transmission was analyzed by Single Strand Conformation Polymorphism (SSCP).

MATERIALS AND METHODS

Maintenance of CTV. CTV isolates tested are part of a collection kept at Citrus Research Institute, Chongqing. Isolates SS7, HH12, GS14 and HB1 were severe isolates, which caused severe stem pitting in Symons sweet orange (*Citrus sinensis*), Fenghuang pummelo (*C. grandis*) and Duncan grapefruit (*C. paradisi*), isolates CT11, ST9, HH3 and LJ1 were mild ones, which did not cause apparent symptoms in above indicators. All of the CTV isolates were graft-inoculated onto Jincheng sweet orange (*C. sinensis*) seedlings in the greenhouse at 15℃ to 27℃. The virus infection was confirmed at 90 days post-inoculation by direct tissue blot immunoassay (DTBIA) (Garnsey *et al*., 1993).

Single aphid transmission. A colony of CTV-free *T. citricida* was obtained using the method reported by Broadbent *et al*. (1996). CTV-free colonies were maintained on young flushes of CTV-free Jincheng sweet orange seedlings in insectaries. CTV-infected Jincheng sweet orange seedlings were used as donor plants to feed of 100 to 200 CTV-free apterae adult aphids. After 24h acquired period, the aphids were singly placed on young flushes of CTV-free Jincheng sweet orange seedlings for 24 h. The receptor plants were then transferred into an insect proof greenhouse at 18℃ to 25℃.

Thirty apterae adult aphids were used to transmit each CTV isolate in single aphid transmission. These measures were repeated thrice to insure consistency in transmission rate. Four months later, the receptor plants were detected by DTBIA (Garnsey *et al*., 1993).

RNA extraction. Total RNA extracts were obtained from donor plants and individuals of aphid, which fed on the CTV-infected donor plants for 24h by Trizol reagent (Invitrogen). RNA extracts were resuspended in 25μl of RNase-free water, and treated with RNase-free DNase (TaKaRa). After ST9, GS14, HH12 and CT11 were transmitted by single apterae adult

aphid, total RNAs from the CTV-infected receptor plants were extracted according to the method reported by Zhou *et al*., (2001). All of the RNA extractions were stored at - 80℃.

Detection and characterization of a conserved 3′ UTR fragment from CTV isolates in aphids and receptor plants. Nested PR-PCR amplification was according to Olmos *et al*., (1999). Nested PR-PCR products of CTV RNA from single viruliferous aphid that acquired isolates ST9, GS14, HH12, CT11 and the CTV-infected receptor plants were examined by SSCP analysis in 8% polyacrylamide gel at 4°C and 200V for 3h, as described previously (D'Urso *et al*., 2000).

Sequence analysis. Twenty nested RT-PCR products of each particular SSCP pattern were inserted into the vector PMD-18T (TaKaRa) and cloned into *E. coli* JM-109. Five clones of each RT-PCR products were sequenced (Shenggong).

Standard curves. The cDNA used as template for *in vitro* transcription was obtained by RT-PCR with primer PM198R and PM261F that includes T7 promote sequence at its 5′ terminal (Ruiz-Ruiz *et al*., 2007). RT-PCR products were transcribed in vitro with T7 RNA polymerase (Promega), and then the transcripts were purified with Transcript RNA Clean Up Kit (TaKaRa). The concentrations of transcripts were detected by NanoDrop ND-1000 UV Spectrophotometer (Thermo Scientific) twice. Ten 10-fold serial dilutions were prepared using RNA extracts (10 ng/μl) from healthy citrus, and stored at -80℃. Dilutions from 109 to 102 were employed to generate the standard curve.

Detection of CTV by real-time RT-PCR. Real-time RT-PCR with and without reverse transcriptase were run in parallel, to ensure the absence of DNA template in transcript preparations. The RNA extracts from *T. citricida* and CTV donor plants were tested in the iCycler iQ platform (BIO-RAD) with primers PM261F/PM198R targeting conserved sequence in CTV ORFs 1b (Ruiz-Ruiz *et al*., 2007).

Statistic analysis. The percentages of viruliferous *T. citricida* that acquired different CTV isolates were statistically analyzed using the generalized linear model. Differences among quantitative levels of CTV obtained in single *T. citricida* were calculated with the one-way ANOVA method of the SPSS13.0 package (Bertolini *et al*., 2008).

RESULTS

Single aphid transmission. The single aphid transmission rate of different CTV isolates ranged from 1.1% to 47.7% (Table 1). The average transmission rate of severe and mild CTV isolates was 25.3% and 18.9%, respectively. No significant difference was observed between the transmission rate of mild and severe CTV isolates ($P=0.892$). Based on the transmissibility by *T. citricida*, CTV isolates were grouped into three groups: low: 0%-5%, intermediate: 6%-15% and high: over 16% (Yokomi *et al*., 2010). Most of the CTV isolates were grouped in high transmissibility.

Table 1 Detection of CTV in the receptor plants after single aphid transmission.

Isolates	Pathogenicity	Transmissibility	Means of Transmissibility (%)	Isolates	Pathogenicity	Transmissibility	Means of Transmissibility (%)
SS7	Severe	7/30[a]	22.2	CT11	Mild	11/30	37.8
		8/30				11/30	
		5/30				12/30	
HH12		15/30	47.8	ST9		2/30	8.89
		15/30				3/30	
		13/30				3/30	
HB1		9/30	28.9	HH3		7/30	27.8
		10/30				9/30	
		7/30				9/30	
GS14		0/30	2.22	LJ1		0/30	1.11
		1/30				0/30	
		1/30				1/30	

[a]Numerator = number of plants infected; denominator = number of test plants used.

Detection of CTV in aphids by nested RT-PCR analysis. After 24h acquisition period, CTV targets were detected in 533 out of 720 aphids (74.0%). No significant difference was found between the transmissibility of *T. citricida* and the percentage of viruliferous *T. citricida* that acquired different isolates ($P=0.127$). The highest percentage of viruliferous aphids was 91.1%, when GS14 was acquired by *T. citricida*, and the lowest detection rate was 63.3%, when HH12 was obtained.

Detection and quantification of CTV in donor plants and single aphid. To estimate the amount of CTV in donor plants, the plants were analyzed by real-time RT-PCR, and the results showed the titre of CTV targets in donor plants ranged from 474226829 to 682576873.

The detection rates obtained by real-time RT-PCR ranged from 71.1% to 91.1% (Table 2). When CTV isolates with high, intermediate and low transmissibility were acquired by *T. citricida*, the mean values were 2984953993593 and 1207566 copies of CTV-targets in single viruliferous aphid, respectively (Table 2). According to the mean number of CTV-targets quantified, significant differences were observed when CTV isolates with high transmissibility was compared to those with intermediate transmissibility ($P=0.0257$), and those with low transmissibility ($P=0.0412$). However, when low and intermediate transmissibility groups were compared, no significant difference was observed in the number of acquired CTV-targets ($P=0.783$).

SSCP analysis. The sequence assay of five clones from each nested RT-PCR production showed that no more than four nucleotide differences were found among the same SSCP pattern. Therefore, the haplotype of CTV can be indicated by the same SSCP patterns in this study.

CTV in the receptor plants showed less haplotype diversity than that in the aphids, most of the receptor plants and single viruliferous aphid contained one haplotype of CTV. Com-

pared with the severe CTV isolates, mild isolates ST9 and CT111 were more easily separated. When the CTV isolates with high transmissibility were transmitted by *T. citricida*, the major haplotypes of CTV in the aphids were more easily remained in the receptor plants than other haplotypes (Table 3).

Table 2 Detection and quantitation of CTV in T. citricida by nested RT-PCR and real-time RT-PCR

Transmissibility	Isolate	Detected by nested RT-PCR		Detected by real-time RT-PCR		
		Number of aphids positive[a]	Detection rate (Mean ± SE[b])	Number of aphids positive[a]	Ct ± S.D[c]	Number of CTV targets (Mean± SE[d])
High	HH12	18/30	0.7471± 0.1415a	20/30	25.4±1.1	2984953± 1019170a
		17/30		21/30		
		22/30		23/30		
	CT11	27/30		27/30	25.5±0.7	
		15/30		22/30		
		25/30		25/30		
	HB1	26/30		26/30	26.3±0.3	
		22/30		23/30		
		25/30		25/30		
	HH3	25/30		24/30	25.7±0.8	
		18/30		22/30		
		22/30		23/30		
	SS7	17/30		23/30	26.8±0.5	
		22/30		25/30		
		26/30		24/30		
Intermediate	ST9	11/30	0.6567± 0.2483a	22/30	28.5±1.1	993593± 278637b
		24/30		21/30		
		24/30		25/30		
Low	GS14	28/30	0.8283± 0.0998a	28/30	27.9±0.9	1207566± 468849b
		27/30		27/30		
		27/30		27/30		
	LJ1	24/30		22/30	28.5±0.7	
		20/30		25/30		
		23/30		24/30		

[a] Numerator: number of viruliferous aphids; denominator: number of test aphids used.

[b] Means followed by different letters are significantly different using the generalized linear mode statistical analysis.

[c] Average threshold cycle and standard deviation

[d] Means followed by different letters are significantly different using a one-way ANOVA after transforming the response variable by the natural logarithm statistical analysis.

Table 3 Summary on the CTV isolates by SSCP analysis

Haplotypes of CTV isolates	ST9		GS14		HH12		CT11	
	Aphids[a]	Plants[b]	Aphids	Plants	Aphids	Plants	Aphids	Plants
ST9Ⅰ	20	8						
ST9Ⅱ	3	0						
ST9Ⅲ	29	0						
ST9Ⅰ+Ⅲ	7	0						
GS14Ⅰ			11	1				
GS14Ⅱ			14	0				
GS14Ⅲ			13	0				
GS14Ⅰ+Ⅱ			21	1				
GS14Ⅰ+Ⅲ			23	0				
HH12Ⅰ					34	30		
HH12Ⅱ					5	3		
HH12Ⅲ					3	0		
HH12Ⅰ+Ⅱ					10	8		
HH12Ⅰ+Ⅲ					5	2		
CT11Ⅰ							45	34
CT11Ⅱ							22	0

[a] The number of CTV haplotype in the single viruliferous aphid.

[b] The number of CTV haplotype in the receptor plants after single aphid transmission.

DISCUSSION

In this study, although no significant differences were observed between the transmission rates corresponding to severe and mild CTV isolates, most of severe CTV isolates have high transmissibility. It might relate to that severe CTV isolates could spread quickly in the fields (Sharma. 1989), but some severe components of CTV might not be transmitted whereas the mild components could be. So the sub-isolates of aphid transmission should be characterized by biological indexing in the future study.

In the previous studies on CTV, *Potato leaf roll virus* (PLRV) and *Barley yellow dwarf virus* (BYDV) hinted that the percentage of viruliferous migrant aphids is one of the major factors for the epidemiology of virus (Singh *et al*., 1995; Cambra *et al*., 2000; Fabre *et al*., 2003). Due to high sensitivity, nested RT-PCR and real-time RT-PCR could detect the virus in the samples that tested CTV negative by other techniques (Olmos *et al*., 1999; Bertolini *et al*., 2008). In this study, all of the aphid samples were tested by nested RT-PCR and real-time RT-PCR. The detections showed that more than 71.1% of the *T. citricida* were viruliferous after 24h acquisition access period. No significant differences were found between the transmissibility of *T. citricida* and the percentage of viruliferous *T. citricida* that acquired different CTV isolates. Cambra *et al*. (2000) once found that although there was a correlation between PCR detection of CTV in individuals of *A. gossypii* and the transmission to Mexican lime seedling, CTV isolates were still detected consistently in *T. citricida*, *A. spiraecola*,

T. aurantii, *A. nerii* and *Hyalopterus pruni*, regardless of their transmissibilities. Furthermore, Moreno *et al*. (2009) showed 88.5% of the tested aphids inoculated PPV into the receptor plants, whereas only 20% plants were infected PPV. These results indicated that the amount of virions, which involved in aphid transmission rather than the percentage of viruliferous aphid might relate with the aphid transmissibilities.

Escriu *et al*. (2000) reported that the efficiency of transmission of *Cauliflower mosaic virus* (CMV) depended on the accumulation of the virus in plants, and a correlation between virus accumulation and transmissibility with a plateau at virus concentrations. Previous studies indicated that aphid might obtain higher transmissibility when it was fed on CTV-infected Mexican lime, because the titre of CTV in Mexican lime was higher than that in other citrus plants (Marroquin *et al*., 2004). In this study, interference from the most abundant sgRNAs and dRNAs was avoided by targeting the ORFs 1b (Ruiz-Ruiz *et al*., 2007). However, no obvious relationship between transmissibility and CTV accumulation in the donor plant has been found in this study, and the amount of CTV in different donor plants were similar.

Moreno *et al*. (2009) found that the amount of PPV which inoculated by a single aphid in the plant could influence the frequency of infected plants. Now CTV isolates were analyzed in the different aphid species by ELISA or real-time RT-PCR, but few reports are available on the number of CTV acquired by the single aphid for its transmissibility (Cambra *et al*., 1981; Saponari *et al*., 2008). In this study, CTV isolates with high transmissibility had much more copies in the single *T. citricida* than the others. It is suggested that a threshold of the quantity for CTV in *T. citricida* might play a role on the transmission. This finding may have potential value for epidemiological studies of CTV in the regions where annual epidemics are caused by migrant viruliferous aphids. Furthermore, in other studies, the number of virions that required for effective infection and the aphid behavior also involved in the virus acquisition and inoculation process (Soosaar *et al*., 2005; Moreno *et al*., 2009).

Nolasco *et al*. (2008) found that although 54% of aphids carried the similar haplotype with very low nucleotide diversity, most of the variation of CTV could be found among individual *T. citricida*, and some aphids contained more than one haplotypes. Considering the conservation of 3′ UTR region, the region of CTV genome is acceptable for assessing changes in CTV populations. So in this study, the nested RT-PCR productions of 3′ UTR regions were used for further analysis on the genetic structures of CTV populations during aphid transmission by SSCP and sequencing. These results also indicated that some aphids carried a viral content distinct from the others, and most of aphids that acquired one haplotype of CTV. This phenomenon might explain the reason of separation by single aphid. In this study, some CTV haplotypes can not be detected in the receptor plants. It presumed that CTV isolate may contain some non-transmissible components, or the transmission rates of some components are too low to be detected with the number of replications used in the study (Bar-Joseph *et al*., 1989; Rocha-Pena *et al*., 1995). A further step toward improving real-time RT-PCR with specific primers or probes may be used to detect the different haplotypes of CTV, so as to provide a better understanding about the relationship between genotype and its transmission.

ACKNOWLEDGMENTS

This work was supported by Chongqing Research Program of Foundation Research and Frontier Technology (cstc2015jcyjBX0043).

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Chongqing Lvkang Fruits Co., Ltd

Chongqing Lvkang Fruits Co., Ltd was established in Xiema, Beibei, Chongqing on June, 2000. It is now one of the leading agricultural enterprises in Chongqing, also one of the important national companies to produce virus-free citrus nursery trees and budwoods, The main activities of the company are commercialization, propagation and demonstration of fruit cultivars such as citrus, pear, plum, cherry, loquat and waxberry. The agricultural products wholesale and delivery, fruit production, as well as design, construction and management of leisure fruit picking orchard are some other businesses of the company.

The company has achieved over 20 scientific technology and practical technology achievements, and registered 8 trademarks such as "Yuzhou", "Three Gorges" and "Early Crystal Pear". The fruit products have been certified as the national green food. The company has the capability to produce 4 million container nursery trees for over 100 elite fruit varieties annually, and supply to more than 30 counties, cities and regions.

Chongqing Lvkang Fruits Co., Ltd devotes itself to the improvement of fruit industry. We are keen to cooperate with the partners in China and all over the world.

Jiangxi Yang's Fruits Co., Ltd

Jiangxi Yang's Fruits Co., LTD. (Yang's) is a modern agricultural enterprise engaged in planting, purchasing, processing, warehousing, sorting, pre-cooling, trading with own brand. Mr. Yang is the Yang's founder who is rich with more than 30 years' experience in this industry, witnessing overall development process from none to now, small to great.

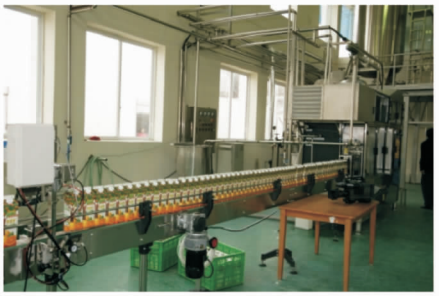
Yang's adheres to operation Philosophy of branding, specialization, standization, large-scale, industrialization and networking, and provides consumers with the freshest, safe & good tasting fruits as a duty. Orange & mandarin are star products of Yang's, which is the reason why Yang's become the best valuable chain supplier. Yang's has not only implemented & certificated its management system with ISO22000(HACCP), ISO9001 & GLOBAL G.A.P, but also won the title of leading enterprises , famous brand of Jiangxi Province, Top 10 citrus brand 2015, Top 100 citrus brand enterprise & Top 10 brand of potential investment .

"Possession of origin, expansion of terminal, reduction intermediate link" is the overall strategic concept of Yang's. In order to implement this strategy, Yang's insists to improving industrial layout, large plants have been established individually in Jiangxi, Guangdong, Guangxi, Hunan province, to ensure produce quality and safety fruits from favorable geographical position of growing areas. In addition, there are five wholesale companies crossing Southern, Eastern, Northern market in China which realizes Differential management of selling network.

Besides, Yang's is also developing import and export business. On the one hand, Yang's purchases overseas fruits selling to domestic market; on the other hand , exports own products to many countries, such as Canada, UAE, Russia, Singapore, Malaysia, Thailand, Philippines, Sri Lanka, Vietnam, Hong Kong, Macao, etc.

Chongqing Pai Sen Bai Orange Juice Co., Ltd

As the first NFC orange juice processing plant in China, Chongqing Pai Sen Bai orange juice CO., Ltd has engaged in citrus industry for over 20 years, established 14 thousands hectares of exclusive raw material bases for orange juice processing. Its main products include NFC orange juice, diced orange peel, organic fertilizer made from orange peel and orange jam. As both national key leading enterprise of agricultural industrialization and poverty alleviation enterprise, the company took on over 20 science and technology development projects at both national and Chongqing level. A complete citrus industry chain has formed, including "selecting of superior varieties for processing"-"propagating virus-free container budlings"-"establishing standard orchards"-"processing NFC orange juice"-"integrated management of scrap peel".



The company has helped more than 200 thousands farmers including 50 immigrants to increase their income by citrus cultivation in the Three Gorges Region. As Chongqing top brand and famous trademark, Pai Sen Bai orange juice has been authorized as certificates of organic food, HACCP and ISO9001.