CITRUS RESEARCH BOARD

PROJECT PLAN - RESEARCH GRANT PROPOSAL FOR FY2010-2011

Fiscal Year: __2011__________ **Anticipated Duration of Project:** Nov. 1, 2009 – October 31, 2011

This Project is: ______New or __X____ **Ongoing (Year** __3___ **of** __3___**)**

Project Leader: __Raymond K. Yokomi
Name

Location: _USDA, ARS, 9611 S. Riverbend Ave., Parlier, CA 93648
Address (University department, if applicable)

Mailing Address (if different): ____

Phone: __(559) 596-2990___**FAX:**__(559) 596-2992___ **E-Mail:**__ray.yokomi@ars.usda.gov______

Cooperating Personnel: __Maria Saponari and Pasquale Saldarelli (Istituto di Virologia Vegetale del CNR, Bari, Italy), Harshavardhan Doddapaneni (Roy J. Carver Center Comparative Genomics, Dept. of Biology University of Iowa, Iowa City, IA).

Project Title: Investigation of seedling yellows cross protection by mild components of the Dekopon *Citrus tristeza virus* (CTV) strain and use of California CTV strains to protect against virulent isolates

Keywords: Mild strain cross protection, gene silencing, stem pitting, real time reverse transcription polymerase chain reaction, deep sequencing.

Abstract *(limit 200 words):(clearly and succinctly state what your project is about why you are doing it and expected out come and how the industry will use these outcomes)*
This is the 3rd year of a project examining cross protection of CTV. Year 1 research documented amelioration of severe CTV SY symptoms in sour orange (SO) and grapefruit (DGF) trees infected with a mixture of VT, T3 and non-standard (NS) genotypes. Year 2 focused on strain accumulation and small RNA (siRNA) profiles using real-time RT-PCR (qRT-PCR) and high-throughput sequencing. Experiments used the field mixture in Dekopon (DK) source plants or a mixture reconstituted after the strains were separated by aphid transmission. BLASTN analysis of short reads obtained by using the Illumina Genome Analyzer II, showed for the three CTV strains an accumulation of CTV-derived siRNA in the 3'end region of the virus genome encompassing the three gene silencing suppressors. We also identified, for the first time, an accumulation of homologues of siRNA in the sub-genomic loci on the *Poncirus trifoliata* Ctv resistance locus. For Year 3, we propose to continue evaluation of DK for cross-protection against T3 isolates recently found in central California which induce severe SY; extend deep sequencing of siRNA for mRNA and degradome analysis ; validate deep sequencing results by quantitative analysis. These studies should elucidate the genes involved in the silencing pathways in CTV cross-protected plants

Problem and its Significance*: *(include literature review)*

Cross-protection of plant viruses is when a mild isolate of the virus protects the host plant against disease development caused by a severe strain of the same virus. Because selective removal of CTV-infected trees have been adopted and field surveys show presence of T3 genotype strains capable of inducing strong seedling yellows (SY) in Tulare Co., aggressive isolates will likely spread. This will eventually lead to stem pitting (SP) CTV strains which affect citrus regardless of rootstock and decreases fruit quality and production. Cross protection is a proven strategy to control CTV SP and this may be a useful future strategy for CTV mitigation. Although a recent study showed cross-protection occurs between isolates of the same genotype (Folimonov et al. (9), its mechanism remains unknown. This lack of knowledge is a major limitation in the use of cross protection. Pre-infection of trees with cross protective CTV strains on

*Use as much space as necessary; attach additional pages as needed; budget info and signatures will appear on last page.

Not for publication without the express written consent of the project leader*. Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*

tolerant or resistant rootstocks has permitted continuation of citrus production in Brazil (7); Australia (4,8), South Africa (14,16) and Peru (5) where severe CTV isolates and the brown citrus aphid are abundant. In these cases, cross protection prolonged the economic life of sweet orange and grapefruit but its performance and durability are not equivalent to resistance.

We have been characterizing the molecular and biological properties of CTV isolates from California for a number of years. The majority of the isolates cluster with the mild T30 genotype, however, severe genotypes or strains (VT, T36, T3) have been found but their spread has been limited by the eradication program. During Year 1 of this project, we determined that the mild non-standard (NS) genotype component of the virulent Dekopon (DK) CTV strain obtained by aphid transmission was associated with reduced seedling yellows (SY) reaction SO and DGF. Specific probes to target and determine the accumulation of each strain in the mixture were successfully developed. In Year 2, accumulation of the three strains in both natural and artificial infected seedlings were evaluated and components correlated with symptom expression. XP with the artificial co-infected SO seedlings was reproduced, thus, confirming that SY cross-protection was due to the interaction of the three strains previously identified. However, the SO plants artificially infected by the mixture, were slightly stunted and a mild SY reaction observed on the apical leaves compared to plants infected with the original parental isolate containing the mixture of isolates (Fig.1). However, in time-course graft-inoculations (i.e. NS component 1 month before the VT and T3 components and vice versa), we were not able to reproduce XP, even if single strand conformational polymorphism analysis and strain specific qRT-PCR revealed that the mild NS component was replicating in the mixture. Thus, the presence of the mild strain was not sufficient to protect the plants from SY symptom development in the presence with a mixture of T3 and VT strains.

Small interfering (si) RNAs fractions (21-24nt in size) were characterized from the XP and non-XP SO seedlings using deep sequencing by the Illumina Genome Analyzer II. From this we determined the profile distribution of CTV viral siRNAs, identifying the highest siRNA accumulation in the 3' portion of the genome, thus, strongly suggesting that this region is highly targeted by the plant defense mechanism (silencing). This result could be related to the presence of several subgenomic RNA encompassing this region or to the association of the SY determinant to the P23 and 3'UTR gene region (1).

Deep sequencing provided new insights on host response to CTV infection through the RNA silencing pathway. Although only a limited amount of *Citrus* reference genome sequences are available in public databases, the largest fraction of host-derived siRNA in SO mapped to the previously sequenced 282 Kb *Poncirus trifoliata* Ctv resistance locus (18). Using our small RNA sequencing approach, for the first time, we were able to distinctly identify two localized differentially expressed loci in this region. The first is centered in the intergenic and overlapping UTR regions between CTV.11 and CTV. 12; the second one is between CTV.19 and CTV.20 (overlapping) genes. This is interesting because CTV.11 is a known R-gene and CTV.12 is a zinc ion binding protein. Also, a Gipsy-like retrotransposone C is identifiable in this region. Retrotransposons elements (RE) are heterogeneous sequences, widespread in eukaryotic genomes, which refer to the so-called mobile DNA. Copia- and Gypsy-like retrotransposons are the two main classes of retroelements now known to be ubiquitous in plant genomes. In the past two decades, some examples have correlated the emerging of RE activity with a stress mediated reaction and it has been proposed that RE plays a role in regulating gene expression (6, 11). Therefore, our further studies will include understanding the dynamics of this influence though transcriptome sequencing.

The data acquired in the 2nd year using the deep sequencing are preliminary and further investigations through mRNA (transcriptome) and degradome analysis are critical to examine CTV and host-genes regulation during infection development. The transcriptome is the complete set of transcripts in a cell under different conditions. Understanding the transcriptome is essential for: interpreting the functional elements of the genome; understanding the changes occurring in the expression levels of each transcript and their role in development; abiotic stress and/or pathogen responses. The development of novel high-throughput DNA sequencing methods has provided a new method for mapping and quantifying transcriptomes. This method, termed RNA-Seq (RNA sequencing), has clear advantages over existing approaches and is expected to revolutionize how eukaryotic transcriptomes are analyzed (13). Since RNA-Seq is quantitative, it can be used to determine RNA expression levels and provide a comprehensive view of the genes involved in host disease response. Further, unlike a DNA resequencing project, a complete genome sequence is not a prerequisite for RNA-Seq. This makes it advantageous for Citrus research with limited genome sequence in the public domain.

However, our work will greatly benefit if a complete Citrus genome sequence is available so we can reliably identify UTRs and novel transcriptionally active regions of the genome. Different citrus genome sequencing projects are

Not for publication without the express written consent of the project leader. *Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*

underway for different *Citrus* spp. and hybrids and are expected to provide overlapping whole genome sequencing by the end of 2010. In the mean time, we will continue efforts to organize current available database and make the best use of it. Through such an effort, our preliminary comparative analysis between our siRNA sequences of SO siRNA vs. Carrizo genome assemblies (generated by us using the 454 reads from http://citrus.pw.usda.gov) identified homologous regions in the more than 100,000 contigs (total length 41 Mb) and 2K predicted proteins of Carrizo genome.

Further experiments will contribute to the identification of specific genetic signatures associated with XP that can allow expanding cross protection to include SP using other CTV genotypes in California. With our studies, it is possible to identify the sub-genomic loci such as described above that could be linked to CTV resistance and lead to deeper understanding of resistance gene regulation. If durable, broad spectrum cross protection can be developed, it can be used in both established citrus and new plantings using current propagation techniques. This strategy will also be helpful if the brown citrus aphid and more virulent CTV strains are introduced into California. It should be noted here that experimental transgenic citrus with CTV resistance using viral sequences have, so far, not been successful (3).

In conclusion, we propose to continue using deep sequencing to complete the siRNA profiles on the selected panel of indicators displaying XP or severe SY and integrate this approach with qRT-PCR quantitation, TILLING (Targeting Induced Local Lesions IN Genomes) (2), transcriptome and degradome analysis to determine which genes are involved in the host defense responses.

Objectives*: *(succinctly state each objective and milestone, ie the time expected to successfully complete an achieve each objective)*

The overall objective of this 3-year proposal is to gain information about the host-CTV interactions occurring in seedling yellows cross-protection. We focused our study on the DK strain of CTV, a mixture of VT, T3 and NS strains showing XP in SO and DGF. In year 1&2 we investigated if the replication/accumulation of each strain in single and mixed infections could affect the symptom expression. In the 2nd year a deep sequence approach was used to characterize the siRNA fractions in 2 of the SO indicator plants included in the biological tests. These analyses need to be extended to more indicator plants (end of year 2) as well as validated by the quantification of the predominant siRNA found.

Thus the objectives we propose for Year 3 are:

1) Quantification of the CTV- and host-predominant siRNA by development of qRT-PCR assays and application for the identification of siRNA markers which correlate with a specific phenotype;
2) Analysis of the siRNA profile associated to the Ctv resistance gene locus in different Citrus spp. and their hybrids following CTV infection;
3) Perform high throughput sequencing for "degradome analysis" to provide information on the mRNAs decay profiles produced during infection and comparison of these profiles among the single/mixed infections will elucidate which genes are involved in gene-silencing pathways of cross-protected plants and how different CTV isolates interacts with the host.
4) Validate the SY cross-protection against the severe T3 isolates recently recovered in San Joaquin Valley (SJV).

Project's Benefit to the Industry*: *(How will the industry utilize your research results or product)*

Mild strain cross protection is a proven control strategy for stem pitting CTV. The protective strain could be inoculated into existing plantings or grafted into budwood source trees and would be present in all trees propagated from the "protected" source tree. The latter has been done for many years in Brazil and So. Africa. This strategy could be applied to all cultivars immediately once it is proven to be stable, mild and effective against local severe isolates. Cross protection can be considered a form of biological control. No recombinant DNA or transgenic citrus is involved in the delivery of cross protection; hence, no lengthy regulatory delays or environmental issues are expected. In addition, the knowledge gained in this project in evaluating disease expression markers should provide a valuable background for the rapid extension of this technology to the study other important diseases such as Huanglongbing (greening disease).

The project will develop an immense amount of data on siRNA occurring in different Citrus spp. as well as those produced in presence of genetically and biologically diverse CTV strains. Since the first description of RNA interference (RNAi) less than a decade ago, there has been rapid progress towards its use as a therapeutic modality against human and plant diseases. RNAi-based therapies might soon provide powerful tools against pathogens and diseases. A first application of RNAi mediated resistance for CTV has been reported by Lopez et al. (12). Although, the accumulation of a transgene-derived siRNAs was not sufficient for protection against CTV in Mexican lime, other siRNAs need to be tested

Not for publication without the express written consent of the project leader. *Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*

for this purpose. The data acquired in this project will greatly help to screen for more effective siRNA.

Research Collaboration* (*be specific*):
Dr. Yokomi will supervise the research and provide to the collaborators all the plant tissues necessary. The ARS-Parlier team will conduct inoculations and biological evaluation of the cross protection against the SY symptoms induced by the severe CTV T3 strain recovered in SJV in 2009. Dr. Saponari (CNR, Bari, Italy) will be responsible for conducting experiments towards objective 1. Dr. Saldarelli (CNR, Bari, Italy) has much experience in post transcriptional gene silencing (PTGS) for grape viruses and will contribute valuable insight and direction to insure appropriate hallmarks of gene silencing are examined. Deep sequencing will be performed in CNR lab in Bari, Italy and in University of Iowa, Iowa City, IA. Dr. Doddapaneni, (University of Iowa) will be responsible for conducting data bioinformatics. Communication will be done through email correspondence and annual site visits by Yokomi to CNR, Bari, Italy funded through other sources.

Plans and Procedures* *(use this section to describe your experimental design site location(s)and elaborate on objectives and milestones)*
The experiments will be continued on the parental DK isolate of CTV identified as P108A and its aphid transmitted progeny maintained in different host/indicator plants: Carrizo, Madame vinous, SO and DGF.

Objective 1. *Quantification of the CTV- and siRNA.* Small RNA profiles obtained through deep sequencing will reveal those that are more represented and thus potentially involved in the CTV-host interactions. To confirm the association of specific phenotypes to CTV-siRNA and host-miRNA, dedicated qRT-PCR protocols will be developed by designing of specific stem-loop primers and universal probes (15). Trizol-based total RNA from 200 individual indicators (MV, SO and DGF) inoculated in year 2 and displaying differential SY symptoms will be used in the test.

Objective 2. *Analysis of siRNA profiles associated to the Ctv resistance gene.* A remarkable accumulation of Ctv resistance locus – derived siRNA was found in the cDNA libraries sequenced in Year 2 from SO. No data are available yet on the expression or the role of all Ctv genes associated to this locus (fully sequenced in *Poncirus trifoliata*). We will use 2 different approaches to investigate involvement of this locus which contains several different REs with regard to virus accumulation and host response. The first approach includes qRT-PCR to determine the accumulation of the mRNA of Gipsy-like retrotransposon C and Ctv12 gene located in this CTV-R region producing siRNAs. A second approach will include the TILLING technology to identify polymorphisms in target Ctv genes among the different Citrus spp. for comparison with the resistant *P. trifoliata* genotype. This will be accomplished by designing specific primers and performing assays on the Licor platform.

Objective 3. *Perform high throughput sequencing for "degradome analysis".* Transcriptome analysis will be carried out on total RNA extracted from the indicators showing various levels of SY; poly(A) mRNA will be used to isolate and prepare a non-directional Illumina RNA-Seq library. Conventional 5' RACE protocols will be also used to determine the mRNA targeted by specific miRNA identified by the deep sequencing analysis of the libraries. Degradome analysis will be carried out on selected virus combinations according to the protocol of German et al. (10). Healthy controls for each host species will be included to determine the reads that hits host-derived sequences and to allow transcriptome comparison between healthy and infected plants. Degradome libraries will be constructed by ligation of polyA-enriched RNA samples to a custom RNA adapter containing a 3' MmeI site, followed by reverse transcription (RT), second-strand synthesis, MmeI digestion, ligation of a 3' dsDNA adapter, gel-purification, and PCR amplification. Amplified degradome tag libraries will then be sequenced using the Illumina genome analyzer. Raw reads will be processed in a pipeline to remove 5' and 3' adapter sequences; tags with sizes of 20 or 21nts (the sizes expected from MmeI cleavage) will be retained and tags that did not correspond to structural RNAs (rRNA, tRNA, snRNA, snoRNA) will be mapped to reference genomes.

Objective 4. *Validate the SY cross-protection against the severe T3 isolates* The data acquired in the 2nd year proved that the SY occurs when all DK subisolates are inoculated simultaneously. This mixture will be used to challenge other SY-inducing isolates recovered in SJV including the T3 genotype strain found in the Exeter, CA area. For these biological experiments SO, DGF and MV (as control) seedlings will be challenged simultaneously by graft-transmission with the DK isolate and severe T3 isolates. Symptoms and strains replication will be monitored in a time

---

Not for publication without the express written consent of the project leader. *Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*

Project Title: Investigation of seedling yellows cross protection by mild components of the Dekopon Citrus tristeza virus (CTV) strain

course experiments.

Other Funding Sources for this Project *(current, pending, potential; can this project be used as matching funds for other funding sources)*

Some fundamental and program support come from the PI's in-house CRIS from ARS. The PI has a proposal pending in the CDFA Specialty Crops Block Grant for some partial technician support for greenhouse/laboratory assistance. The PI has also submitted a proposal to the ARS Postdoc Program on a portion of this research relating to the *Poncirus trifoliata* CTVr gene(s).

Technology Transfer* *(include any potential intellectual property issues; steps necessary for grower utilization extension/communication component):*

Progress reports will be provided as required to the CRB. Research results will be presented as oral presentations and posters at professional society meetings (local, national, international), workshops, and invitational reports or updates at CRB and Extension meetings. Manuscripts will be written and submitted for publication in professional journals as well as local trade magazines

Budget Justification:

0.55 FTE of a Biological technician (GS5) is requested ($23,040 including benefits) for ARS-Parlier; $17,000 for each for SCA to CNR-Bari and Univ. Iowa for deep sequencing service (Total $34,000); $3,000 each for ARS-Parlier, CNR- Bari, and Iowa State (Total $9,000) for genomic library kits, reagents and plasticware and $3,500 for meeting travel.

References Cited

1. Albiach Marti M., Robertson C., Godwa S., Tatineni S., Belliure B., Garnsey S., Folimonova S., Moreno P., Dawson W., 2010. The pathogenicity determinant of Citrus tristeza virus causing the seedling yellows syndrome maps at the 3′-terminal region of the viral genome. Molecular Plant Pathology 11(1): 55 – 67.
2. Barkley N.A, Wang M.L., 2008. Application of TILLING and EcoTILLING as Reverse Genetic Approaches to Elucidate the Function of Genes in Plants and Animals. Curr. Genomics 9(4): 212–226.
3. Batuman, O, Mawassi M, Bar-Joseph M (2006) Transgenes consisting of a dsRNA of an RNAi suppressor plus the 3′ UTR provide resistance to Citrus tristeza virus sequences in Nicotiana benthamiana but not in citrus. Virus Genes 33:319–327.
4. Broadbent, P., Bevington, K.B., Coote, B.G. 1991. Control of stem pitting of grapefruit in Australia by mild strain protection. In Proc. 11th Conf. IOCV, 64-70 IOCV, Riverside, CA.
5. Bederski, K., Roistacher, C.N., Muller, G.W. 2005. Cross Protection against the severe Citrus tristeza virus stem pitting in Peru. In Proc. 16th Conf. IOCV, 117-126, IOCV, Riverside, CA.
6. Clark RM, Wagler, TN, Quijada, P, Doebley, J. 2006. A distant upstream enhancer at the maize domestication gene tb1 has pleiotropic effects on plant and inflorescence architecture. Nat Genet. 38: 594–597.
7. Costa, A.S., and Muller, G. 1980. Tristeza control by cross protection: a US-Brazil cooperative success. Plant Disease 64: 538-541.
8. Cox, J.E., Frazer, L.R., and Broadbent, P. 1976. Stem pitting of grapefruit: Field protection by use of mild strains, and evaluation of trials in two climatic districts. In: Proc. 7th Conf. IOCV, p. 68-70. IOCV, Riverside, CA.
9. Folimonov, A., Folimonova, S., Robertson, C., Garnsey, S., Hilf, M.E., Dawson, W. 2010. Infection with Strains of Citrus Tristeza Virus Does Not Exclude Superinfection by Other Strains of the Virus. Journal of Virology, 84, 3, 1314-1325.
10. German MA, Pillay, M, Jeong, DH, Hetawal, A, Luo, S, Janardhanan, P, Kannan, V, Rymarquis, LA, Nobuta K, German R, De Paoli E, Lu C, Schroth, G, Meyers BC, Green PJ. 2008. Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. Nat. Biotechnology. 26(8): 941-6.
11. Kobayashi S, Goto,-Yamamoto N, Hirochika, H. 2004. Retrotransposon-induced mutations in grape skin color. Science 304: 982.
12. Lopez C., Cervera, M., Fagoaga, C., Moreno P., Navarro L., Flores R., Pena L., 2010. Accumulation of transgene-

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

Not for publication without the express written consent of the project leader. *Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*

derived siRNAs is not sufficient for RNAi-mediated protection against Citrus tristeza virus in transgenic Mexican lime. Molecular Plant Pathology, 11, 1, 33-41.

13. Mortazavi, A., Williams B. A., McCue K., Schaeffer L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods, 5, 621 – 628.
14. van Vuuren, S.P., Collins, R.P., and da Graca, J.V. 1993. Evaluation of Citrus tristeza virus for cross protection of grapefruit in South Africa. Plant Dis. 77, 24-28.
15. Varkonyi-Gasic, E., Wu R., Wood M., Walton E. F., Hellens ,R. P., 2007. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods 2007, 3:12.
16. Von Bromembsen, L., and A.T.C. Lee. 1988. South Africa's Citrus Improvement Programme. In: Proc. 10th IOCV, p 407-416, IOCV, Riverside, CA.
17. Wang Z., Gerstein M., Snyde, M. 2009. Innovation: RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews Genetics 10, 57-63.
18. Yang ZN, Ye XR, Molina J, Roose ML, Mirkov TE. 2003. Sequence analysis of a 282-kilobase region surrounding the citrus Tristeza virus resistance gene (Ctv) locus in Poncirus trifoliata L. Raf. Plant Physiol. 131: 482-92.

Fig.1. Sour orange plants 1-year after inoculation with the 3 DK aphid-transmitted isolates (A) or with the original field infected source (B).

Not for publication without the express written consent of the project leader. *Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*

CRB Project Plan – Research Grant Proposal for FY2010-2011 **CRB Project No. 5300-138**

Project Title: Investigation of seedling yellows cross protection by mild components of the Dekopon Citrus tristeza virus (CTV) strain

Department Account Number: *(if applicable)* 093-5302-186

	Year: **2010-2011**	Year: **2011-2012**	Year: **2012-2013**
Salaries and Benefits:			
Postdocs/Research Assistants	$		
SRA's			
Lab/Field Assistance	23.040		
Benefits			
Supplies and Expenses:	9,000[1]		
Equipment:			
Operating Expenses and Travel:	3,500[2]		
SCA for Univ. Iowa[3] :	17,000		
SCA for CNR[4]:	17,000		
Other:			
ANNUAL TOTAL:	69,540		

Specifics regarding contract (i.e., "split" funding to more than one PI):

[1]Libraries kits, reagents, plasticwares

[2]Travel to CRB and APS annual meeting

[3]SCA for Univ. Iowa is for Harshavardhan Doddapaneni to perform deep sequencing at a rate of 75-cycle paired end cluster gen.and seq. per lane @$2,154 (8 lanes per plate).

[4]SCA for CNR (Maria Saponari and Pasquale Saldarelli) for adapter–ligation kits, bioinformatic services and deep sequencing with a similar fee schedule from CNR-Udine.

Signatures

Project Leader:____________________ **Date:**________

____________________ **Date:**________

____________________ **Date:**________

____________________ **Date:**________

Dept. Chair: ____________________ **Date:**________

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

Not for publication without the express written consent of the project leader. *Before quoting or reproducing any information in whole or extracted in any form, contact the project leader responsible.*