Creation of a Transient Expression Vector for Citrus: An Effective Defense against Citrus Greening

Case Study 16

Research goal	Manipulation of the *Citrus tristeza virus* (CTV) genome for transient expression of foreign genes in citrus trees.
Beneficiaries	Florida citrus growers, US citrus processing industry.
Activities conducted in order to achieve the objectives	Creation of a manipulatable genetic system with the full-length cDNA copy of the CTV genome. Examination of strategies to develop a highly stable CTV expression vector resulted in a suite of stable vectors that express foreign genes from different genomic positions. Use of the CTV expression vector to test efficacy of antimicrobial peptides against citrus greening.
Funding	4 BARD awards: US-2346-94, IS-2711-97C, US-3171-00C and US-3483-03C; $1,242,443. Additional funds: $3,750,000.
Publications	66 journal publications, 25 of them in the top impact factor quartile, 5 with more than 100 citations.
Students involved	4 graduate students and 7 post-doctoral researchers were involved in the BARD supported research for CTV manipulation as a transient-expression vector. Current positions: 8 in academia in Israel, the US, Spain and Uruguay, 1 established a biotech industry, 1 is in the biological industry in Russia and 1 is in government service in Israel.
Stakeholders' collaboration	The CTV expression vector is a base for 7 USDA research projects.
Environmental impact	Reduced use of neonicotinoid insecticides.
Social impact	Rise in citrus industry jobs (revival of juice processors in Florida).
Commercial engagement	Southern Citrus Gardens.
Patents	2 patents
Practical agricultural applications	Inoculation of citrus trees *via* grafting with CTV containing one or more spinach defensin genes.
Economic benefit	The USDA APHIS multisite permit for use of the CTV vector with the defensin gene is still pending. Therefore, we did not calculate benefits for this project.

Green- Academic information; Yellow - Social and environmental information; Blue - Economic information

1 Objective: A Transient Expression Vector for Citrus Used to Protect against Citrus Greening

Citrus tristeza virus (CTV) is distributed worldwide and is the causal agent of what was once one of the most economically important diseases of citrus, having caused the decline and death of millions of orange trees where the sour orange (*Citrus aurantium*) was used as rootstock. The initial research objective was to determine the biological activity of CTV isolates and to characterize the CTV genome. At a later stage, based on the initial findings, the research goal evolved into using a harmless strain of CTV to develop a highly expressing and stable transient vector for citrus.

2 Research Activities

Between 1988-1994 5 BARD awards were granted to Moshe Bar-Joseph (ARO) and Richard F. Lee (U. Florida) for biological research on citrus tristeza virus (CTV) isolates. In a subsequent BARD award in 1994 (US-2346-94), M. Bar-Joseph collaborated with William Dawson (U. Florida), a research that led to successful cloning of CTV and in which libraries of cDNA were prepared against several Israeli and Florida CTV strains. The full-length sequence clone of the Florida CTV strain provided the research basis of 3 further BARD awards between 1997 -2006 (IS-2711-97C, US-3171-00C and US-3483-03C). In these research projects M. Bar-Joseph and W. Dawson aimed to "manipulate" the CTV virus and utilize its recombinant properties to facilitate CTV as a tool for the transfer of informational molecules into citrus plants. The Dawson laboratory focused on manipulation of the viral genome and the Bar-Joseph laboratory focused on the virus-host biology. The potential for application of the molecular engineering tool was directed at this stage for construction of a new set of tools for protecting citrus against severe CTV isolates. The research activities in these latter 3 BARD studies included:

1) Development of a transient-expression vector

The researchers examined several strategies to develop a CTV based vector for transient expression of foreign genes in citrus trees using a green fluorescent protein (GFP) as a reporter. Engineered vector constructs were examined for replication, encapsidation, GFP expression during multiple passages in protoplasts, and for their ability to infect, move, express GFP, and be maintained in citrus plants. The most successful CTV vectors were shown to be unusually stable, continuing to produce GFP fluorescence for up to 10 years in citrus trees.

2) Use of CTV as a delivery vehicle to screen for potential gene products that could provide resistance to citrus canker and greening.

The CTV expression vector was used to test efficacy of antimicrobial peptides against citrus canker and greening. In follow up studies, the researchers showed that CTV is tolerant to manipulation at several positions within the genome, resulted in a suite of stable

transient expression vectors that express foreign genes from different genomic positions, each with characteristics that are optimal for specific conditions.

3 Academic Impact

3.1 Publications

66 peer-reviewed journal publications were published based on research from the 4 BARD awards. Of these, 25 publications were in the top impact factor quartile and 5 have been cited more than 100 times.

3.2 Capacity Building

4 graduate students and 7 post-doctoral researchers were involved in the BARD supported research for CTV manipulation as a transient-expression vector. Of them, 8 currently hold positions in academia in Israel, the US, Spain and Uruguay, 1 established a biotech industry, 1 is in the biological industry in Russia and 1 is in government service in Israel.

4 Stakeholder's Collaboration

- The early BARD studies led in 1998 to the taxonomic classification of the virus family *Closteroviridae,* of which CTV is a species. Closteroviruses explore the upper size limit for the RNA-based genomes and their genetic plasticity makes them attractive vehicles for the delivery and expression of recombinant genes engineered into viral genomes
- Since detection in 2005 of the bacterial disease Huanglongbing (also called citrus greening) in Florida, the use of the CTV vector has evolved and it has been used as a platform for many targeted solutions for treatment and prevention of citrus greening. The CTV vector is a component of 7 USDA Speciality Grants Program for Citrus Greening, four of which the U. Florida group have received funding for and three for which the U. Florida group are building CTV constructs for with no additional funding.
- The CTV strain T-36 is native to Florida. Generally, citrus growers prevent the import of viruses that are exotic to their industry. The U. Florida group has collaborated with researchers in Brazil, California, and South Africa to build CTV vectors from their isolates of CTV to be used in defense against citrus greening.
- Additional expression *Closteroviruses* viruses were developed by other groups based on this research (e.g. - the use of beet Yellows Virus[1] and Grapevine leafroll-associated virus[2]). CTV was the first use of a viral vector in woody trees (following

[1] J. of Virology, 2002, 76 (21) 11003-11011; DOI**:** 10.1128/JVI.76.21.11003-11011.2002
[2] J. of Virology 2012, 86 (11) 6002-6009; DOI: 10.1128/JVI.00436-12

upon the earlier research of W. Dawson on tobacco mosaic virus (TMV)-based expression vectors used with herbaceous annual plants). The BARD research provided a platform for following developments for viral expression vectors in other woody trees[2].

5 Commercial Engagement

The U. Florida patent for the transient expression vector for citrus has been licensed to Southern Gardens Citrus Inc of Clewiston, FL. Initially, Southern Gardens used the CTV vector as a tool to identify antimicrobial genes with activity against the HLB bacterium (*Candidatus* Liberibacter) targeted for transformation into citrus. However, HLB has spread rapidly in Florida and as transgenic plants will not be available in time to save the industry, alternative solutions based on CTV use as an expression vector are being pursued.

5.1 Patents

1) *Citrus tristeza virus-based vectors for foreign gene/s expression*, William O. Dawson, Svetlana Y. Foliminova and Choaa El-Mohtar, US20130125254A1, Granted 9-10-2018 to University of Florida.

2) *Viral-based transient-expression vector system for trees*, William O. Dawson, Svetlana y. Foliminova and Alexey S. Folimonov, US20100017911A1, Granted 14-01-2004 to University of Florida.

6 Practical Agricultural Applications

1) Southern Gardens has used the CTV vector to express spinach defensin proteins[3], shown to induce disease resistance of trees infected with the Liberibacter bacterium that causes citrus greening. The genetically engineered CTV expressing the proteins (CTV-SoD) have been field tested since 2010 under confined conditions. Southern Gardens has approached the USDA, EPA, and FDA for permission to commercially use the vector to provide resistance to HLB. The CTV treatment is considered a microbial pesticide by the EPA and is under review by the USDA for an application permit throughout Florida. The USDA 2-year process began in April 2017, and if approved would be the first transient expression vector approved for commercial use.

Southern Gardens proposes to plant and grow trees of various citrus varieties that have been inoculated *via* grafting with one or more strains of CTV-SoD[4], which is the CTV vector carrying a defensin protein derived from spinach. After the genetically engineered

[3] Licensed by Southern Gardens from Texas A&M
[4] https://www.aphis.usda.gov/biotechnology/downloads/CTV_Q&A.pdf

CTV is introduced into the tree, the tree will produce the defensin protein which leads to inducement of the citrus own resistance system against the bacteria.

The environmental impact statement of USDA - Animal and Plant Health Inspection Service (APHIS)[4] states that the process shows promise both in protecting trees from contracting citrus greening disease, and in treating trees that are already infected.

2) A second approach based on the use of CTV as a transient expression vector has focused on control of its spreading vector, the Asian citrus psyllid (ACP). Coincident cohabitation in phloem tissue by the *Candidatus* Liberibacter bacterium, ACP and CTV has been exploited by Southern Gardens to successfully inhibit the reproductive system of the psyllids using RNA interference (RNAi) carried by the CTV transient expression vector[5].

7 Economic Impact

7.1 Investment Cost

BARD contributed $1,242,443 in research funds between 1994-2006. Additional funds contributed approximately $3,750,000. These funds were from a USDA cooperative agreement with the University of Florida (1993 -1998), the US Citrus Research and Development Foundation and its predecessor, the Citrus Production Research Advisory Board (199 -2010), and the USDA Specialty Crops Grant Program for Citrus Greening (2011-to date).

7.2 The Benefits

We assess the potential benefits of the inoculation of citrus trees with CTV-SoD by first assessing the costs of the citrus greening disease.

Cost of Disease:

Citrus greening disease has been identified in in all counties with commercial citrus throughout Florida, as well as in other US states such as Alabama, California, Georgia, Louisiana, South Carolina, and Texas. Citrus greening disease puts at risk America's entire citrus crop[6].

Florida's citrus bearing grove area declined from over 750,000 acres in the year 2000 to around 435,000 acres in 2016, a reduction of 42 percent, while production volume utilized declined by 68 percent, primarily due to losses from citrus greening disease (HLB), which entered the state in 2005**Error! Bookmark not defined.**.

A study conducted by the Food and Resource Economics Department in the University of Florida on the impact of citrus greening (HLB) confirmed the continued increasing

[5] Journal of Biotechnology 176 (2014) 42–49
[6] https://www.aphis.usda.gov/biotechnology/downloads/CTV_Q&A.pdf

economic impact of HLB disease on the Florida citrus industry. Using a model for the world orange juice industry, orange and grapefruit grower revenues for processed utilization were estimated under a hypothetical scenario without HLB,(compared to the actual historic production values for processing, with HLB). It was concluded that HLB has caused a cumulative loss of $1.672 billion in grower revenues over the 2012-13 to 2015-16 period, or an average of $418 million annually. This revenue loss resulted in average annual economic impacts to the Florida economy of -7,945 jobs, -$658 million in value added, and $1.098 billion in industry output. The analysis was conducted to assist citrus industry stakeholders, government regulators, policy makers, and researchers to better understand the economic importance of finding solutions to citrus greening.[7,8]

Currently, tree removal, nutrient applications and intensive insecticide applications as soon as ACP is detected are the only available management options for HLB.

Potential of CTV-SoD Implementation

Having determined the cost of the disease we assess the potential use of CTV-SoD.

The CTV vector delivery tool is based on the parent wild-type strains, T30 and T36, that are native to Florida. To implement the strategy in a different global region, cloning of the local CTV strain would need to be executed and further work conducted to elucidate the appropriate genomic position of the foreign gene/protein. The large size and the complexity of the CTV virus entails this as a non-trivial project. Therefore, all benefits should currently be assessed for Florida alone.

Additionally, The USDA permit application from Southern Gardens describes the release sites as within the 67 counties in Florida, also limiting the future economic benefits of CTV-SoD use in the next years for Florida alone.

To assess the potential impact of CTV-SoD we need the following assumptions, which are not all available for assessment to-date:

- Following USDA, EPA and FDA approval, the biological control of greening using CTV-SoD is expected to enter the market by 2020 (based on the release of the draft Environmental Impact Statement and Pest Risk Assessment mid-2018).
- CTV-SoD would be part of an Integrated Pest Management (IPM) strategy.
- Although CTV-SoD efficacy is demonstrated also as a treatment for infected trees, the release of CTV-SoD under the permit would likely be for inoculation of young nursery trees, i.e. for use as replacement trees and for new plantations in the abandoned areas. As such, the impact would not be immediate.

[7] https://fred.ifas.ufl.edu/economicimpactanalysis/publications/2015-citrus-industry/

[8] National Academies of Sciences, 2018. *A Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting a Ravaging Disease*. https://doi.org/10.17226/25026.

- The rate at which orange production and the citrus industry in Florida could recover depends on the effectiveness of CTV-SoD against HLB. The efficacy of the field trials is not available until the regulatory process is completed.

8 Economic Results

The potential benefits of the integration of CTV transfer techniques to lessen the impacts of the disease are immense, as detailed in Section 7.2. However, as the USDA APHIS multisite permit for use of the CTV vector with the defensin gene is still pending, it is too early to evaluate its full success. Therefore, we did not calculate any benefit to this project yet.

9 Appendix A: BARD Awards

Table 1: Details of the 4 BARD awards between 1994 - 2003

Project No	Full Title				
	Investigators	Institutes	Budget	Duration	Start Year
US-2346-94	Citrus Tristeza Virus: Molecular Approaches to Cross Protection				
	Dawson, W.O. Bar-Joseph, M. Niblett, C.L. Gafny, R. Lee, R.F. Mawassi, M.	U. Florida ARO, Min. Ag U. Florida ARO, Min. Ag U. Florida ARO, Min. Ag	$300,000	3 years	1994
IS-2711-97C	The Role of Defective RNAs in Citrus Tristeza Virus Diseases				
	Bar-Joseph, M. Dawson, W.O.	ARO, Min. Ag U. Florida	$342,000	3 years	1997
US-3171-00C	Creating an Ally from an Adversary: Genetic Manipulation of Citrus Tristeza				
	Bar-Joseph, M. Dawson, W.O.	ARO, Min. Ag U. Florida	$310,355	3 year	2000
US-3483-03C	Creating an ally from an adversary: genetic manipulation of Citrus tristeza virus				
	Bar-Joseph, M. Dawson, W.O.	ARO, Min. Ag U. Florida	$290,088	3 year	2003

10 Appendix B: Information providers: Personal communication

William Dawson – Plant Pathology, Citrus Research and Education Center, U. Florida

Moshe Bar-Joseph - Plant Pathology, ARO, Min. of Agr.