CALIFORNIA CITRUS NURSERY BOARD

Progress Report for 2013
California Citrus Nursery Board
Agreement # 58-5302-3-392

Project Year: 2013-14

Progress Report: Final Report of a Two-Year Project

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Project Title: Development of a TaqMan® Array Plate for Multiplex Detection of Citrus Pathogens by Real-Time PCR from a Single Sample

General Objectives

Develop a usable Taqman® Array Card (TAC®) or plate system for simultaneous detection of multiple citrus pathogens from a single sample by real-time PCR (qPCR). The Taqman-probe-based qPCR can be used to detect multiple citrus pathogens and can be used with high throughput nucleic acid extraction to obtain citrus pathogen targets in propagative materials for certification.

Specific Objectives:

1. Develop singleplex qRT-PCR assays for RNA and DNA pathogens for use with TaqMan Array cards and plates.
2. Develop a custom Taqman Array card or plates as a standardized test for both RNA and DNA pathogens of regulatory importance for the California citrus nursery industry.

Final Report for Project 2013-2014 (2 year project)

Unique conserved sequences of citrus pathogens were selected from NCBI database and used to develop primer sets to identify each pathogen by quantitative PCR (qPCR) assay. Primers and TaqMan probes were selected from the pathogen database using Primer Express Version 3.0.1 (Life Technologies Corp, Carlsbad, CA). Assays were optimized with the same or highly similar annealing and melting profiles between pathogens or strains in a uniform set of PCR conditions (e.g. Supermix, template, primer, probe concentrations, thermocycling conditions, extension, etc.). Table 1 shows a list of the pathogens included.

Table 1.

Pathogen or strain	Type	ID
CTV	RNA	CTV P25
CTV T30	RNA	CTV T30
CTV T36	RNA	CTV P27 T36
CTV T36NS	RNA	CTV P27 T36NS
CTV VT3	RNA	CTV P27 VT3
Stubborn1	DNA	spirilin
Stubborn 2	DNA	citriP58
Internal Std 1	DNA	COX
Internal Std 2	RNA	NAD
Exocortis	RNA	CEVd
Hop stunt/Cahexia	RNA	HSVd/CVd-II
HLB	DNA	HLB
Viroid generic	RNA	CVd
Citrus bent leaf	RNA	CVd-I
Citrus viroid III	RNA	CVd-III
Citrus viroid IV	RNA	CVd-IV
Citrus viroid V	RNA	CVd-V
Citrus viroid VI	RNA	CVd-VI
Citrus tatterleaf	RNA	CTLV
Leprosis	RNA	CiLV
Citrus varigated chlorosis	DNA	CVC
Witches broom	DNA	Phytopl
Citrus leaf blotch-Dweet mottle	RNA	CLBV

High throughput extraction was developed with citrus samples from greenhouse and field infected with CTV, CEVd, HSVd, *Spiroplasma citri*, and HLB *C*LAS. Detection of *Citrus tristeza virus* (CTV), '*Candidatus* Liberibacter asiaticus' (*C*LAS) (Huanglongbing or HLB), *Spiroplasma citri,* The qPCR protocol successfully detected and differentiated severe CTV strains (VT and T3 genotypes) from mild CTV strains, *C*Las, CEVd or HSVd in single or mixed infections (Saponari et al. 2013). Success was also obtained to detect and differentiate Hopstunt viroid variants by real-time RT-PCR and high resolution melting temperature analysis (Loconsole et al. 2013).

A CTV RT-qPCR kit was designed to rapidly and economically differentiate between economic and non-economic CTV strains. The kit was tested and validated with 2013 field samples provided by the CCTEA and successfully detected a VT genotype of CTV in several locations in the Tulare County Pest Control District. To make the plate RT-qPCR assay user-friendly for the CCTEA, target acquisition was simplified by replacing high throughput nucleic acid extraction with immunocapture in PCR microtubes. The diagnostic thus uses the extract designed for ELISA for target RNA or virion capture. The immunocaptured target could then be tested by either a one-step of two-step qPCR assay. The immunocapture requires an effective antiserum. PCR inhibitors such as polyphenols are readily eliminated in the routine wash step after microtube antiserum coating.

Improved sensitivity of *S. citri* was accomplished by developing qPCR primers to conserved multi-copy prophage gene sequences in the *S. citri* chromosome. In this case, detection by SYBR® Green was improved more the 10-fold (3 log units of cycle threshold (Ct)) Wang et al., 2015).

qPCR primers/probes included Table 1 detected target pathogens tested. Multiplexing in qPCR assays has limitations in the number of different targets that can be detected simultaneously. There can be cross reactions between primers causing primer-dimer complexes, competition for supermix reagents, availability of reporter dyes for probes and filters for the specific dye (ROX, CY5, Texas Red, FAM, etc.). Duplex detection was no problem and several triplex assays were successfully developed.

A cost-effective plate qPCR assay was developed to replace the more expensive TaqMan Array Assay. The plate assay involved adding the primer and probe mixture in each well on a PCR plate, air drying and covering the plate with a seal (See Appendix). The plates can be stored for days weeks or several months at room temperature or preferably in a desiccator at 4°C. Once the target extract had been prepared, they are simply added to duplicate wells along with a standard one-strep Supermix and qPCR performed in any qPCR instrument having the proper optic filters. qPCR plates were designed to be user-prepared and flexible. New pathogens or pathogen strains can be added, existing targets can be deleted by simply preparing a new custom plate. Cost of each plate would be approximately $130 and each plate test 44 samples. Thus, the cost per sample is $2.95 and includes test for two or three pathogens, excluding control A duplex test would cost $1.48 per pathogen. Cost per sample would increase by only $2.60 if for high throughput robotic extraction was used. Controls include citrus NADH (for RNA pathogens), COX (for DNA pathogens) and a non-template sample.

For the future, adaptation of the Droplet qPCR can overcome some of challenges of multiplexing target detection. Other techniques such as the Luminex assays utilizing microspheres each designed to capture a different target pathogen is expensive but has good promise.

Summary

- Multiplexing two to three pathogens in one reaction well was readily achieved. Adding more targets was limited by the need to incorporate controls for citrus nucleic acids to insure adequate extraction as well as pathogen controls per panel test.
- The qPCR in simplex and multiplex performed equally well when pathogen targets were obtained from infected citrus with commercial RNA and DNA Plant Extraction kits, high throughput robot utilizing magnetic beads, or CTAB extraction with chloroform:isoamyl alcohol and propanol.
- Commercial preparation of TaqMan Array Cards that requires a specific reader was deemed too expensive to be practical for the citrus industry. Even expanding annual testing 10 fold would not be cost-effective to get a favorable return on the cost of the custom array card.
- A custom qPCR plate format was developed in place of the array card system. The plate is durable, much cheaper than an array card and can be prepared by the end user, and has flexibility to change target as needed.

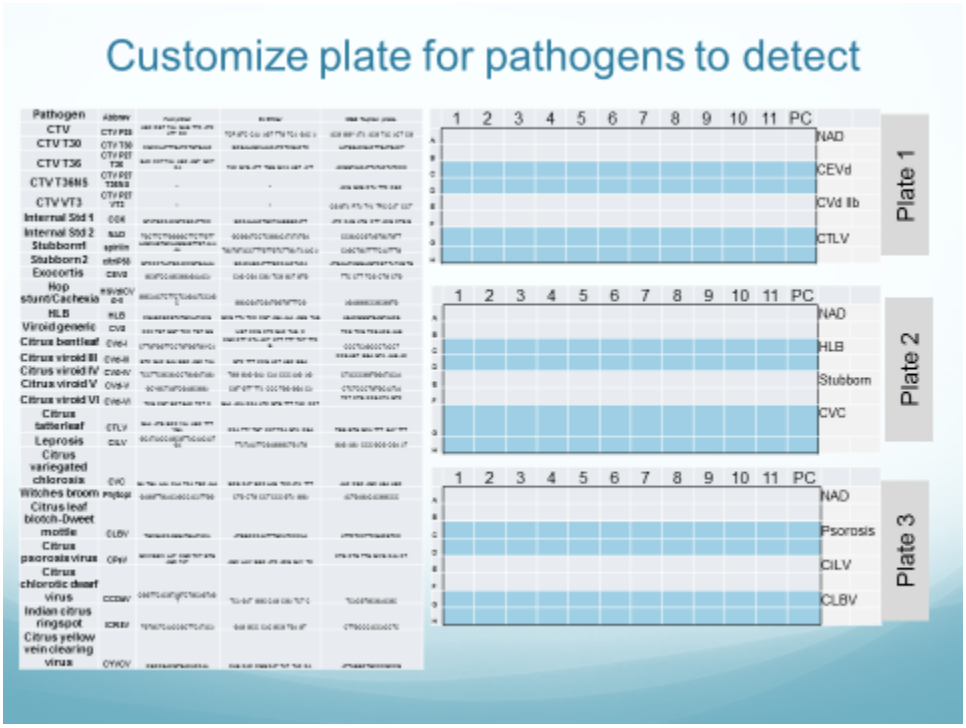
Publications.

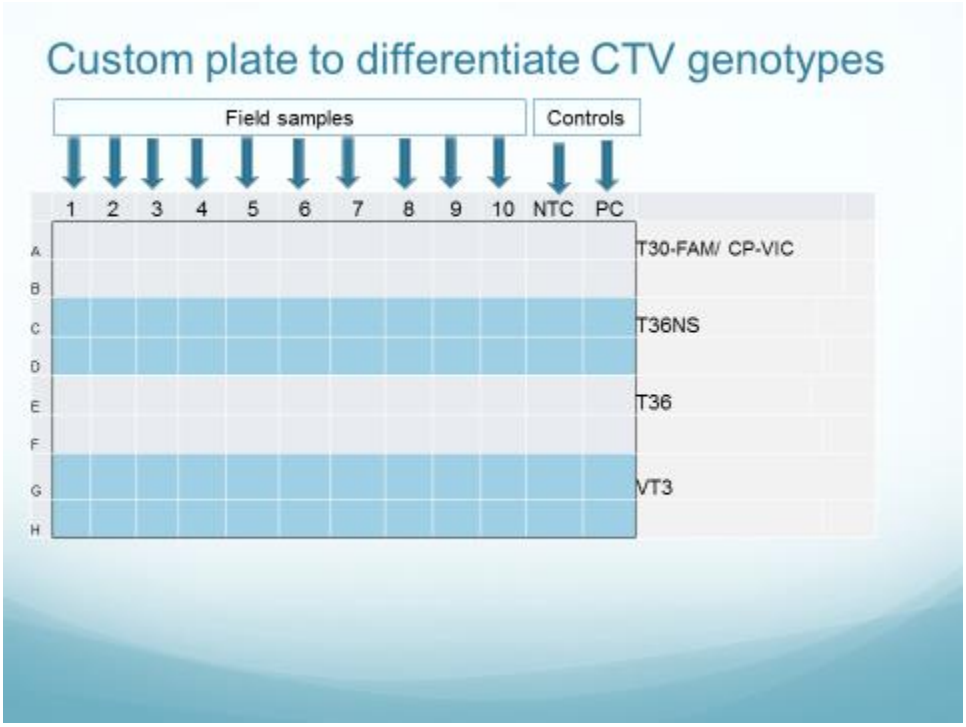
Loconsole, G., Onelge, N., Yokomi; R. K., Abou Kubaa, R., Savino, V., and Saponari, M. 2013. Rapid differentiation of citrus Hop stunt viroid variants by real-time RT-PCR and high resolution melting analysis. Molecular and Cellular Probes. 27: 221-229.

Saponari, M., Loconsole, G., Liao, H.-H., Jiang, B., Savino, V., and Yokomi, R. K. 2013. Validation of high-throughput real time polymerase chain reaction assays for simultaneous detection of invasive citrus pathogens. Journal of Virological Methods 193:478-486.

Wang, X., Doddapaneni, H., Chen, J., and Yokomi, R. K. (2015). Improved real-time PCR diagnosis of citrus stubborn disease by targeting prophage genes of *Spiroplasma citri*. Plant Disease 99:149-154.

Appendix


Customize plate for pathogens to detect
Pathogen
CTV
CTV T30
CTV T36
CTV T36NS
CTV VT3
Internal Std 1
Internal Std 2
Stubborn
Stubborn2
Exocortis
Hop stunt/Cachexia
HLB
Viroid generic
Citrus bentleaf
Citrus viroid III
Citrus viroid IV
Citrus viroid V
Citrus viroid VI
Citrus tatterleaf
Leprosis
Citrus variegated chlorosis
Witches broom
Citrus leaf blotch-Dweet mottle
Citrus psorosis virus
Citrus chlorotic dwarf virus
Indian citrus ringspot
Citrus yellow vein clearing virus
1 2 3 4 5 6 7 8 9 10 11 PC
NAD
CEVd
CVd IIb
CTLV
Plate 1
NAD
HLB
Stubborn
CVC
Plate 2
NAD
Psorosis
CiLV
CLBV
Plate 3


Custom plate to differentiate CTV genotypes
Field samples
Controls
1 2 3 4 5 6 7 8 9 10 NTC PC
A
B
C
D
E
F
G
H
T30-FAM/ CP-VIC
T36NS
T36
VT3