CALIFORNIA CITRUS NURSERY BOARD
2011-02-06 Final Report (year 1 of 4)

The Future of the Cooperative Registration Program of Nursery Owned Citrus Source Trees

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Objectives

1. Molecular characterization of the psorosis isolates in the CCPP disease bank, for the development of efficient and efficacious laboratory PCR based diagnostic tools.

Twelve out of the 14 isolates in the CCPP collection have been processed and the sequence of the coat protein gene has been identified.

Table 1. CCPP disease bank psorosis isolates.

	Psorosis Isolate	Shock	Young Leaf	Coat Protein Gene Sequence
1.	P 200	Sometimes	Moderate	Yes
2.	P 201	Sometimes	Moderate	Yes
3.	P 202	Severe	Severe	Yes
4.	P 203 M	Severe	Severe	Yes
5.	P 205	Severe	Severe	Yes
6.	P 208	Moderate to Severe	Moderate to Severe	Yes
7.	P 209	Severe	Moderate	Yes
8.	P 212 M	Mild	Moderate	Yes
9.	P 213	Mild	Moderate	No
10.	P 214	No	Moderate	No
11.	P 215 M	Severe	Severe	Yes
12.	P 216 M	Severe	Severe	Yes
13.	P 250 LB	Severe	Mild	Yes
14.	P 251A LB	Severe	Severe	Yes

Based on our sequence results and different sequences from various geographic areas we designed and currently testing two different sets of degenerate primers and Taqman probes for reverse transcription quantitative polymerase chain reaction (RT-qPCR).

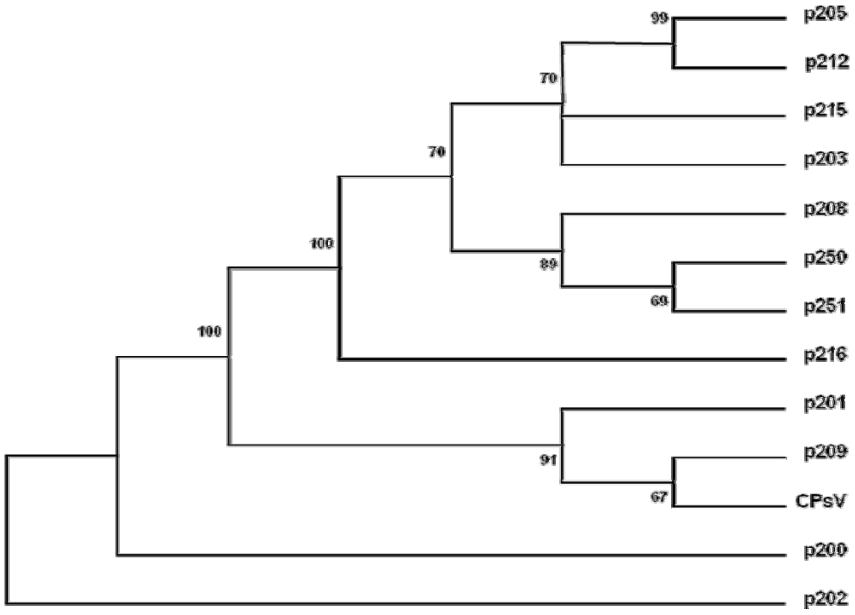

Fig.1. Phylogenetic analysis of the psorosis coat protein gene.

2. Development, employment, and validation of PCR and Real Time PCR protocols for: Spiroplasma citri (stubborn), Citrus viroids (CVds), Citrus tristeza virus (CTV), Candidatus Liberibacter sp., Citrus leaf blotch virus.

This past year we focused our efforts on citrus viroids and CTV since their testing is crucial for the implementation of the new mandatory registration regulations.

Both CTV and CVds are RNA pathogens so we tested different RNA extraction methods. We tested the Buffard buffer method and kits from Sigma and Qiagen. All methods provided adequate quantities of RNA for RT-qPCR.

a. CTV: We designed CTV degenerate primers based on the sequences of the different CTV genotypic groups. To test the performance of those primers we extracted RNA from approximately 50 CTV isolates from the CCPP collection with positive results. In addition, we received and processed approximately 60 (out of the aprx 300) CTV isolates from the collection in the Central California Tristeza Eradication Agency (CCTEA) and RT-qPCR testing will be performed in the following year. RNA material from the CCPP and CCTEA CTV isolates is currently under genetic characterization based on the CTV genotypic markers T30, T36, T3, T68, and VT. We also made arrangements with the International Collection of Exotic Diseases in Beltsville, Maryland, to receive and test our diagnostic protocol against additional CTV isolates from around the world.

b. Citrus viroids: We designed RT-qPCR primers and probes specific for the, *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (cachexia, HSVd), *Citrus dwarfing viroid* (CDVd), *Citrus bark cracking viroid* (CBCVd), and *Citrus exocortis viroid* (CEVd). We fine-tuned the reaction conditions (denaturation, cDNA synthesis, qPCR reaction etc), type of enzyme and reaction kit (tested Invitrogen, Biorad, and Quanta) for all CVds (Fig. 2) while we acquired positive controls for the newly characterized CVd-V and –VI.

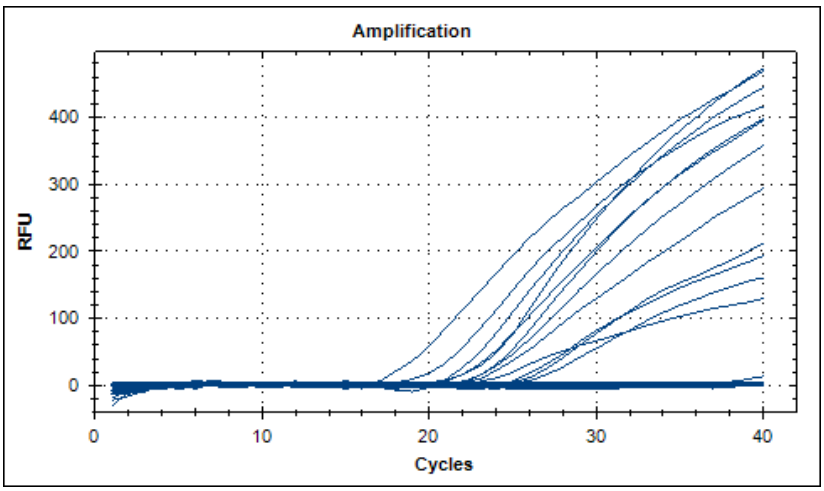

Fig. 2. Detection of CBLVd, HSVd, and CDVd, in mixed infections of three 11 years old clementine trees.

In order to minimize the number of necessary reactions for the detection of the seven (7) known CVds we designed degenerate primers for the three different genera of the CVds (Table 2).

Table 2. Known Citrus Viroids used for the design of degenerate primers by genus

Family	Genus	Species
Pospiviroidea	Pospiviroid*	CEVd & CBCVd*
	Hostuviroid	CVd-II (-IIa, -IIb, -IIc, & -909)
	Apscaviroid	CBLVd, CDVd, CVd-V, CVd-VI

*Including the CBCVd calssified as Cocadviroid

CEVd: *Citrus exocortis viroid*, CBCVd: *Citrus bark cracking viroid*, CVd-II: Citrus viroid II (variants of *Hop stunt viroid*), CBLVd: *Citrus bent leaf viroid*, CDVd: *Citrus dwarfing viroid*, CVd-V: *Citrus viroid V*, CVd-IV: *Citrus viroid IV*.

We tested field samples inoculated with various mixtures of citrus viroids, green- and screen-house samples inoculated with different viroid species, as well as samples with unknown viroid content with very encouraging results. There was no cross reactivity among different viroid species, and the PCR products of different viroid species had distinct properties (i.e. melting temperatures) (Fig. 3). The upcoming year we will proceed with the validation of our protocols using the cooperative registration samples of 2010-11 program.

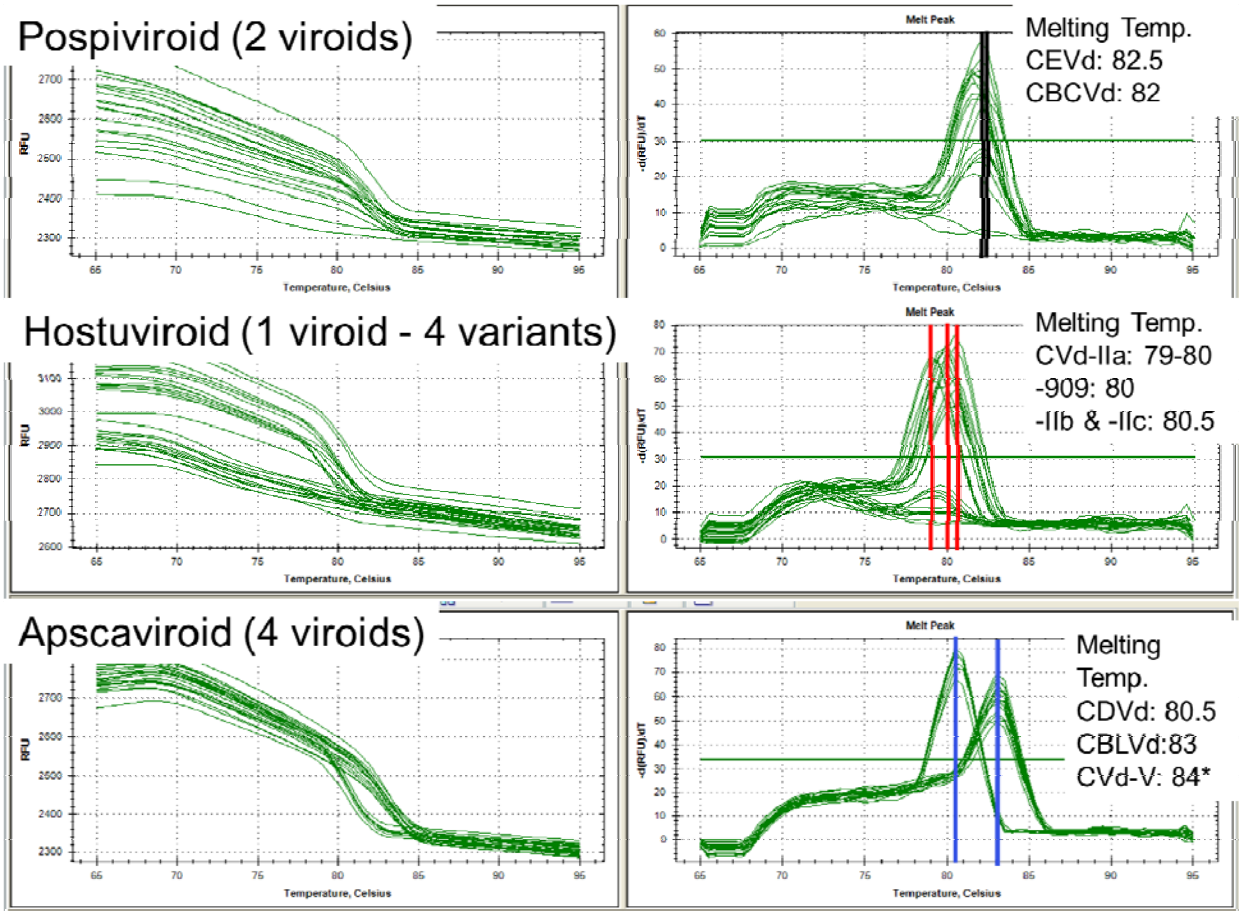

Fig. 3. Detection of CVds in mixed infected citrus via RT-qPCR with degenerate primers per viroid genus.

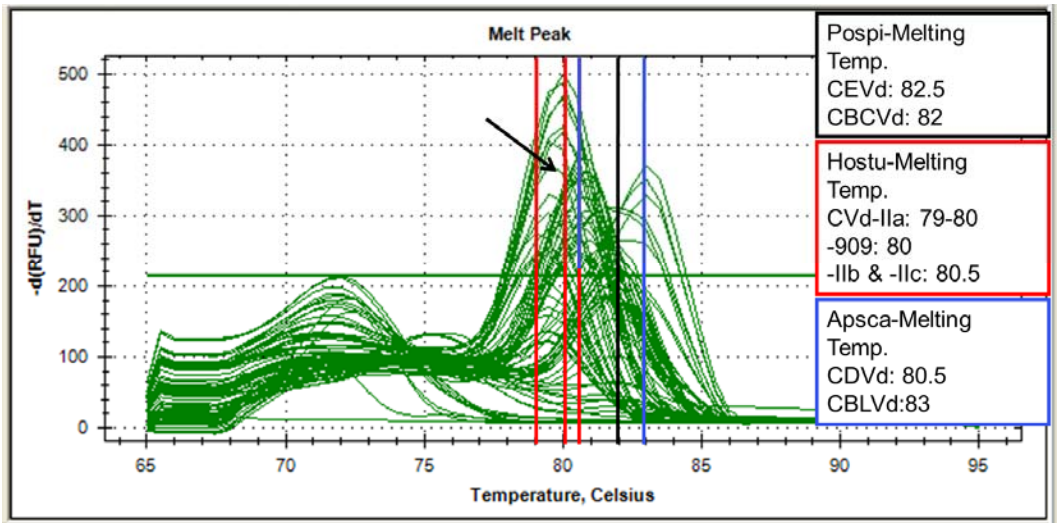

Fig. 4. Detection of CVds in mixed infected Navel, Clementine and Oroblanco and foreign introduction via RT-qPCR with degenerate primers per viroid genus.