California Melon Research Board
2019 Annual Report

I. Project title:

Development of rapid detection methods for evaluation of germplasm for resistance and determination of the California host range of *Cucurbit chlorotic yellows virus* and *Squash vein yellowing virus*.

II. Principal investigator:

William M. Wintermantel, Ph.D., Research Plant Pathologist
U.S. Department of Agriculture, Agricultural Research Service
1636 East Alisal Street, Salinas, CA 93905
Phone: (831) 755-2824; FAX: (831) 755-2814
Email: bill.wintermantel@usda.gov

III. Co-PIs:

Robert L. Gilbertson, Ph.D., Professor, Dept. of Plant Pathology, University of California-Davis; Office: (530) 752-3163; Email: rlgilbertson@ucdavis.edu

James D. McCreight, Ph.D., Research Geneticist, USDA-ARS, 1636 East Alisal Street, Salinas, CA 93905; Office: (831) 755-2864; Email: jim.mccreight@ars.usda.gov

IV. Cooperating personnel

None

V. Location where work was performed

USDA-ARS, U.S. Agricultural Research Station, Salinas, California

VI. Objectives

1. Develop standard multiplexed RT-PCR primers for simultaneous detection of CCYV, CYSDV, CABYV, and SqVYV from plants and insect vectors.
2. Develop multiplexed real time (quantitative) RT-qPCR primers for simultaneous determination of titers of CCYV, CYSDV, CABYV, and SqVYV from plants that may be infected by one or more of these viruses simultaneously.
3. Use primers to begin to evaluate germplasm for presence and/or levels of each virus from field or greenhouse samples, and to determine the California host range of newly introduced virus, CCYV.

VII. Results and Analysis

Objective 1. *Develop standard multiplexed RT-PCR primers for simultaneous detection of CCYV, CYSDV, CABYV, and SqVYV from plants and insect vectors.*

DNA Primers were designed to portions of the genome sequence of each of the four RNA viruses targeted: Cucurbit chlorotic yellows virus (CCYV), Cucurbit yellow stunting disorder virus (CYSDV), Squash vein yellowing virus (SqVYV), and the aphid transmitted virus Cucurbit aphid-borne yellows virus (CABYV) which causes symptoms nearly identical to those of CYSDV and CCYV. Primers were developed such that each virus-specific primer set amplifies a different size fragment, making it easy to see which viruses are present in a sample and which are not when analyzed by electrophoresis on agarose gels (standard methods for evaluating results of RT-PCR). Primers for RT-PCR were designed to bind to and amplify portions of the RNA-dependent RNA polymerase genes of CCYV, CYSDV, and CABYV, whereas the primer for detection of SqVYV was designed to amplify a portion of the NIa gene of that virus. A method was developed in which primers for the RNA viruses (CCYV, CYSDV, SqVYV, CABYV) are combined into a single reaction mix and used to test for the presence of each of the viruses that might be present in a sample in a single test. The method has been validated and reliably detects each virus based on analysis of laboratory (**Fig. 1**) and field samples (data presented under Objective 3).

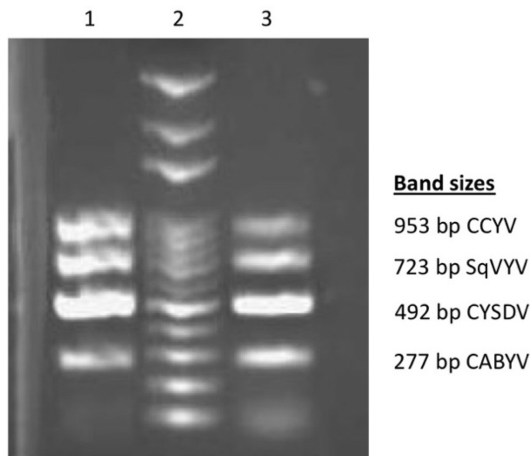

Figure 1. 1% Agarose gel showing how RT-PCR products differ in size to allow determination of which viruses are present during multiplex RT-PCR. Gel shows results of RT-PCR amplification of RNA extract of Lane 1) 100 ng RNA extract containing all 4 viruses; Lane 2) DNA size marker; Lane 3) 10 ng RNA extract containing all 4 viruses.

Objective 2. *Develop multiplexed real time (quantitative) RT-qPCR primers for simultaneous determination of titers of CCYV, CYSDV, CABYV, and SqVYV from plants that may be infected by one or more of these viruses simultaneously.*

DNA probes (modified primers for use in real-time/quantitative RT-PCR) for use in TaqMan assays were designed to quantify the amount of each of the four RNA viruses in melons and other host plants. As with the regular RT-PCR primers, probes for quantitative RT-PCR (usually abbreviated as RT-qPCR) were designed to bind to and amplify portions of the RNA-dependent RNA polymerase genes of CCYV, CYSDV, and CABYV, whereas the probe for detection of SqVYV was designed to amplify a portion of the NIa gene of that virus. Probes for each virus were validated against each target virus and against RNA samples containing RNA of one to four of the different viruses together. Initial testing confirmed optimized performance of probes for measuring differential levels of CCYV and CYSDV, but it was determined that probes for SqVYV and CABYV needed refinement. There was an issue with the fluorescent marker for SqVYV being too similar to that for another fluorescent marker, and therefore we had to reorder that probe with a different fluorophore. Probes for SqVYV and CABYV were redesigned and evaluated independently and in combination (**Fig. 2**) against samples of individual virus RNAs as well as samples in which all four virus RNAs were present (CCYV, CYSDV, SqVYV, CABYV).

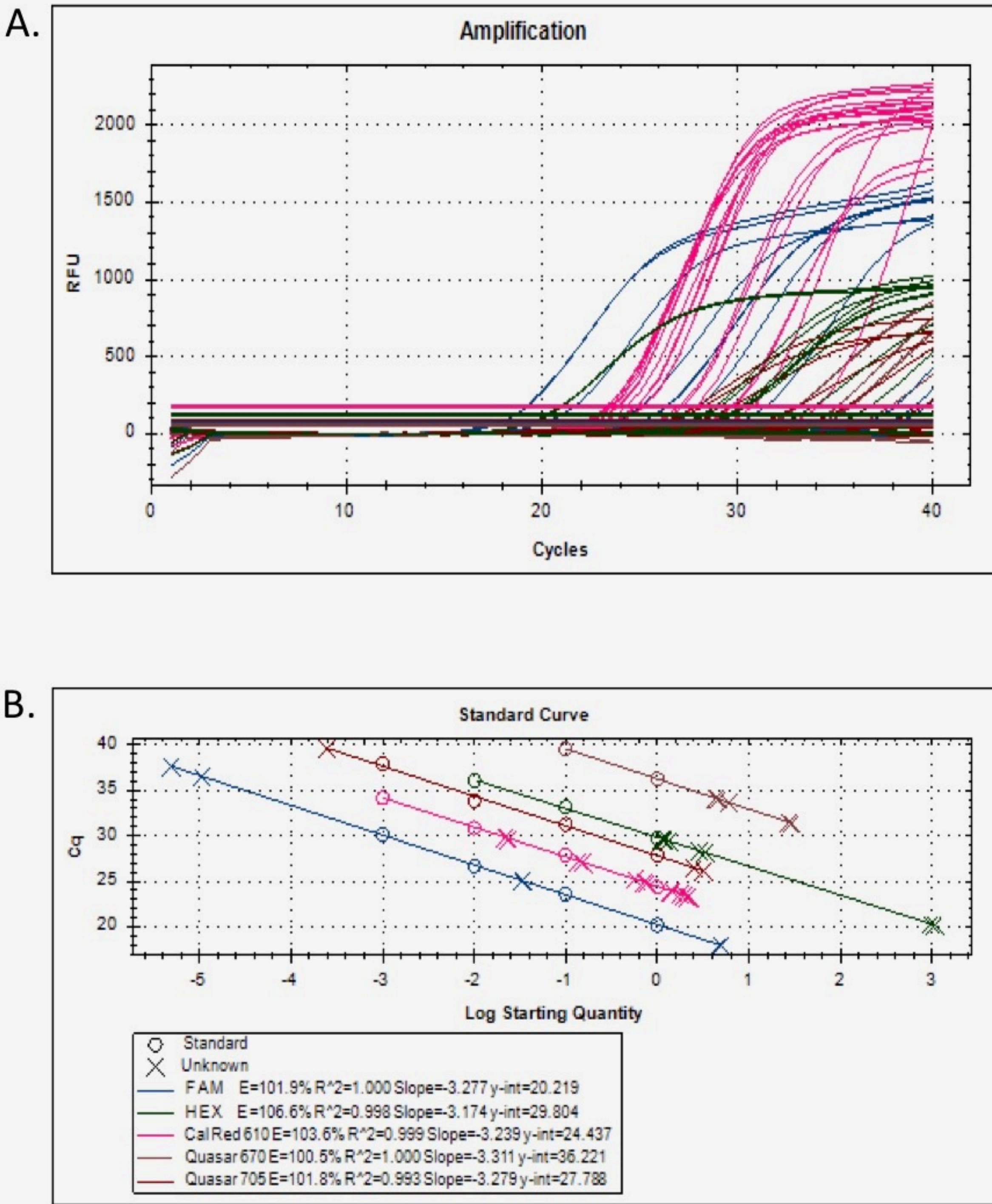

Figure 2. (A) Quantitative PCR assay showing amplification of viruses CCYV, CYSDV, SqVYV, CABYV, plus melon reference gene ADP (internal control). **(B)** Standard curves for the 4-virus multiplex for quantification of virus titer. Description of colors associated with fluorescent markers: FAM = CCYV; HEX = CYSDV; Quasar670 = SqVYV; Quasar705 = CABYV; CalRed610 = Melon ADP (internal control reference gene).

Objective 3. *Use primers to begin to evaluate germplasm for presence and/or levels of each virus from field or greenhouse samples, and to determine the California host range of newly introduced virus, CCYV.*

Spring Season Results:
Primers for standard multiplex RT-PCR were used in combination with primers for CuLCrV on samples at the end of the spring season (June) from melon breeding plots at the University of California, Desert Research Extension Center (DREC) near Holtville, CA. Fewer numbers of yellowed melons are identified in the spring season than during the fall season, and only a limited sampling of these spring sampes were evaluated for viruses in order to determine which viruses were present in the plot. This was the first field-test of the new multiplex RT-PCR system. All primers performed well and determined the presence of both CCYV and CYSDV in the breeding plots from the spring trial (**Table 1**). CCYV was by far the most prevalent virus in the spring trial, and CYSDV was found only in co-infections with CCYV. The quantitative detection system (designed in Objective 2) was not fully operational in June, so it was only possible to estimate ratios of each virus using intensity of bands on gels. Interestingly, only CCYV produced strong amplifications by RT-PCR, and only two plants showed coinfection of CYSDV with CCYV. Those two plants had faint bands for CYSDV compared to the intensity of CCYV bands (not shown). CuLCrV and CABYV were not detected from the spring field samples from DREC. SqVYV was also not detected in the spring trial (*correction from mid-season report*).

Table 1. Mid-season sampling of yellowed melon plants from resistance trial at the Desert Research and Extension Center, Holtville, CA

SAMPLE #	SAMPLE ID	SYMPTOMS (AS NOTED ON BAG)	RT-PCR RESULT			
			CCYV	CYSDV	SQVYV	CABYV
1	2018-1	YELLOW CROWN	POS	NEG	NEG	NEG
2	2087-2	CROWN YELLOWING	POS	NEG	NEG	NEG
3	1039-2	CYSDV? YELLOWING	POS	NEG	NEG	NEG
4A	1116-2	CRUMBLING ON YOUNGER	POS	NEG	NEG	NEG
4B	1116-2	YELLOW CROWN	POS	NEG	NEG	NEG
5A	1140-2	MID BRANCH CHLOROTIC	POS	POS (F)	NEG	NEG
5B	1140-2	CRUMPLE	POS	NEG	NEG	NEG
6	1120-2	UNUSUAL VEIN BANDING, CRUMPLE, SPECKLES, SQVYV?	POS (F)	NEG	NEG	NEG
7	2039-1	MID BRANCH CHLOROTIC	POS (F)	NEG	NEG	NEG
8	1160-2	MID BRANCH CHLOROTIC	POS (F)	POS (F)	NEG	NEG

(F) = FAINT BAND

Fall Season Results:
Fall breeding plots at DREC were sampled the week of Sept. 23, 2019, along with several commercial and research fields in California and Arizona. These samples were evaluated with the standard multiplex RT-PCR method developed in Objective 1 (**Table 2**).

Table 2. Virus incidence among commercial fields and research plots[1] in low desert production regions of California and Arizona sampled in September 2019.

Sample #	CYSDV	CCYV	SqVYV	CABYV
1	+[2]	–	–	–
2	+	–	–	–
3	+	–	+	–
4	+	–	+	–
5	+	–	–	–
6	+	–	–	–
7	+	+	+	–
8	+	+	–	–
9	+	+	–	–
10	+	–	+	–
11	+	+	–	–
12	+	+	+	–
13	–	–	–	–
14	+	–	+	–
15	+	+	–	–
16	–	–	–	–
17	+	–	+	–
18	+	–	+	–
19	–	–	–	–
20	–	–	–	–
21	–	–	–	+
22	–	–	–	+
23	–	–	–	+
24	+	–	–	
25	+	–	–	+
26	–	–	–	–
27	+	+	–	–
28	+	–	–	–
29	+	+	–	–
30	+	+	–	–
31	+	+	–	–
32	+	+	–	–
33	+	+	–	–
34	+	+	–	–
CYSDV positive	+	–	–	–
CCYV positive	–	+	–	–
SqVYV positive	–	–	+	–
CABYV positive	–	–	–	+

[1] Not including results at DREC.; [2] + = positive, – = negative

Results demonstrated an abundance of CYSDV among the fall melon samples from throughout the region with 76 percent of plants sampled (26/34 plants) testing positive for CYSDV. CCYV was also detected in 38 percent of plants tested, and in 50 percent (13/26) of the CYSDV infected plants. No single infections of CCYV were identified during the fall season. Some melon fields had nearly universal co-infection of both CYSDV and CCYV. SqVYV was detected in 24 percent of plants tested (8/34), and in 31 percent of the CYSDV infected plants. Like CCYV, all SqVYV infected plants were also co-infected with CYSDV. Interestingly, all SqVYV infected plants were from Arizona, no SqVYV was detected in samples collected from California in the fall of 2019. CABYV is aphid-transmitted and symptoms resemble those of CYSDV or CCYV, which is why it was included in the multiplex detection system. CABYV is more prevalent in the Central Valley of California than in the Low Desert region, but we did identify four melon samples with CABYV infections, all from Arizona. Three of these melon plants were singly infected with CABYV alone, whereas one was co-infected with CYSDV. These results strongly suggest CYSDV remains the dominant virus in the region during the fall season, and contrasted with the results of the spring sampling in which CCYV was by far the most prevalent of the viruses, but in a far more limited sampling focused on the Desert Research and Extension Center in Holtville.

Relative quantification of virus for QTL mapping against CYSDV and CCYV in melon using the multiplex detection system.

The quantitative detection multiplex system (designed in Objective 2) was used to evaluate a mapping population for titers of CYSDV and CCYV during the fall season. Results are summarized below.

Two mapping populations:

1. $F_{2:3}$ populations PI 313970 x Top Mark (TM) for CYSDV resistance
2. RIL population (F_{10} generation) of MR-1 x Ananas Yokne'am (AY) for CCYV resistance.

PI 313970 is CYSDV resistant and is susceptible to CCYV. Conversely, MR-1 is resistant to CCYV and susceptible to CYSDV. 'Top Mark' is susceptible to both viruses. Both populations were planted and phentoyped in a naturally infected field test at DREC, Holtville, CA during the Fall of 2019. The PI 313970 x TM $F_{2:3}$ population was genotyped with genotyping-by-sequencing (GBS) technology to develop a linkage map and subsequent QTL mapping analysis.

Previous studies conducted in 2018 after the discovery of CCYV in Imperial County, and during the spring of 2019 (**Table 1**) demonstrated both CCYV and CYSDV can occur together in the same leaf of the same melon plant as described above, and both produce the same yellowing symptom on melon leaves. Therefore, we consider the phenotypic data (symptom severity) invalid for mapping QTL for resistance against these viruses in this situation of co-infection by the two viruses. The relative quantification of both viruses in each line of our population was calculated using RT-qPCR data and will be used for mapping a resistance QTL against these two viruses.

At least three leaf samples were collected for each line from at least one replication to extract viral RNA. Approximately 100 mg of leaf tissue was harvested from each line. A MagMax Plant RNA Isolation kit was used to extract RNA. The cDNA was synthesized using the iScript RT Supermix Kit. RT-qPCR was conducted with virus-specific probes for each virus and the multiplex protocol developed in Objective 2. Standard dilution was included for each virus in each run to generate a standard curve with a 10-fold serial dilution and the initial amount of virus was calculated.

Results show that the RT-qPCR system is very efficient in distinguishing CYSDV and CCYV from one another. Top Mark and PI 313970 are both susceptible to CCYV, but PI 313970 carries a source of resitance to CYSDV. The CCYV titer was higher than CYSDV in most of the $F_{2:3}$ lines as shown below in **Figure 3**.

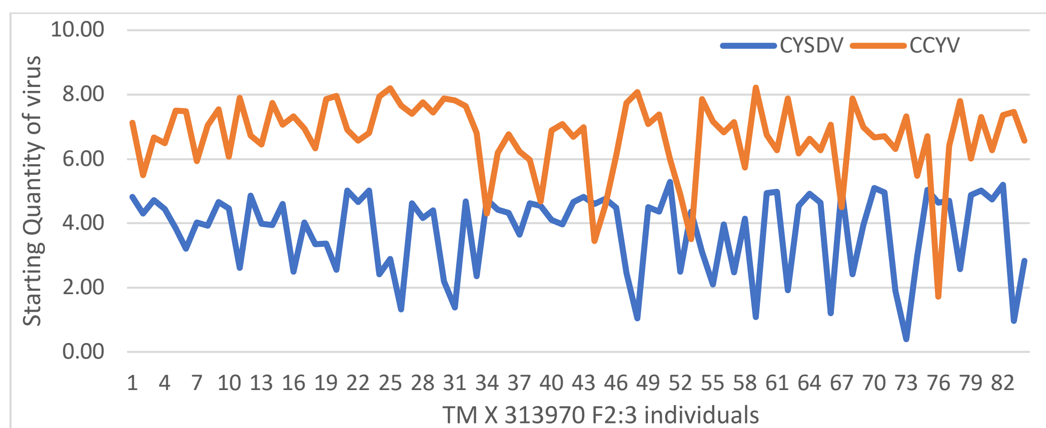

Figure 3. CYSDV and CCYV assay on $F_{2:3}$ PI 313970 x Top Mark family. The Y-axis represents the starting quantity of CYSDV and CCYV and the X-axis represents the $F_{2:3}$ PI 313970 x Top Mark lines (individual plants).

Interestingly, samples from Topmark melon in border rows adjacent to the research plot were found to have an abundance of CYSDV, and only two plants (numbers 2 & 3) had detectable levels of CCYV. Neither SqVYV nor CABYV were detected at DREC during September. This result is important because it suggests that CYSDV may have been the dominant virus in

comparison with CCYV during the fall season in the absence of a CYSDV resistance source at the DREC where the resistance trial was conducted. RT-qPCR analysis of sample number 2, which was positive for CYSDV and CCYV found approximately twice the amount of CYSDV in this plant (Sample 3 was not tested by RT-qPCR).

Table 3. Virus prevalence in Topmark melon (susceptible to all four viruses) at DREC, September 2019.

Sample #	CYSDV	CCYV	SqVYV	CABYV
1	+	–	–	–
2	+	+	–	–
3	+	+	–	–
4	+	–	–	–
5	+	–	–	–
6	+	–	–	–
7	+	–	–	–
8	+	–	–	–
9	+	–	–	–
10	+	–	–	–
CYSDV positive	+	–	–	–
CCYV positive	–	+	–	–
SqVYV positive	–	–	+	–
CABYV positive	–	–	–	+

Conclusions

- Highly effective and robust methods for multiplex RT-PCR based detection and differentiation of viruses associated with the yellowing complex have been developed that allow detection of CYSDV, CCYV, SqVYV and CABYV in a single reaction. The method has been validated with field sampling.

- Reliable methods for determining differential accumulation of the same four yellowing complex associated viruses have been developed, lab-tested, and validated in a research trial at DREC.

- CCYV was far more prevalent than CYSDV during limited sampling in the 2019 spring production season at the Desert Research and Extension Center in Holtville, CA.

- CYSDV was far more prevalent than CCYV throughout the entire AZ/CA production region during the 2019 fall season based on extensive sampling of grower fields and research trials. Some SqVYV co-infections with CYSDV were detected in Arizona. Three melon plants were identified with CABYV infection alone, and one with co-infection with CYSDV.

- Further studies will be needed to determine if the pattern of CCYV as the dominant virus in the spring season and CYSDV as the dominant virus in the fall season will be needed, as current results are based on a single sampling year and limited spring data.

- Research to clarify the host range of CCYV and SqVYV in the Sonoran Desert using these detection methods is in progress.

- Breeding studies demonstrate the effectiveness of CYSDV resistant lines in suppressing levels of CYSDV in comparison to susceptible Topmark controls during the fall season when whitefly populations and virus incidence is at its peak. This shows that the dominant or most prevalent virus in these putative CYSDV resistant plants was CCYV during the fall season, and demonstrates a need to continue development of CCYV resistance in addition to advancement of resistance to CYSDV.