Final Report to the California Melon Research Board

Project title: **Monitoring an outbreak of *B. tabaci* whiteflies in melons in 2018 and continued development of vector-independent screening for whitefly-transmitted viruses infecting melons**

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There were two main goals for this project: 1) monitoring melon production in the Central Valley for outbreaks of whiteflies and use molecular methods to identify the whiteflies involved and 2) to continue to develop and test vector-independent methods for infecting melons with two economically important whitefly-transmitted RNA viruses: *Squash vein yellowing virus* (SqVYV) and *Cucurbit yellow stunting disorder virus* (CYSDV). However, in 2019, there were outbreaks of aphid-transmitted *Cucumber mosaic virus* (CMV) in Yuma in the spring and in the Central Valley in the fall. Furthermore, there was some evidence of mixed infection of CMV and potyviruses and association with an unusual internal discoloration and corky texture of cantaloupe fruit. Therefore, we carried some diagnostic and infectivity experiments to investigate the role of these viruses in these outbreaks.

Objective 1. Monitoring for whitefly outbreaks in the Central Valley. We conducted two surveys in Fresno County: an early season survey (June 18, 2019) and a later season survey (August 23, 2019). In the early season survey, a honeydew and cantaloupe field were surveyed at early green fruit stage and no adult whiteflies were observed at the four locations checked by the leaf-turn method. In the late season survey, three cantaloupe fields were surveyed, and none or trace populations of adult whiteflies were observed. These results revealed a very different situation from last year when late season whitefly outbreaks were observed in numerous melon fields. This likely reflects the cool wet spring conditions, which may have delayed or interfered with the presumed migration of adult whiteflies from southern locations, e.g., the Imperial Valley. This may indicate that a predictive temperature model (e.g., degree day) could be developed to predict the possibility of whitefly outbreaks in melon in the Central Valley.

Objective 2. Development of vector-independent inoculation and screening methods for SqVYV and CYSDV. Breeding for disease resistance is the most desirable management strategy for these viruses. Thus, efficient vector-independent inoculation systems can be very useful tools for a breeding program. These systems also allow for a wide array of basic research. The availability of assembly systems that accommodate large-sized DNA fragments made it more feasible to generate full-length infectious clones of plant-infecting RNA viruses with large genomes, i.e., >10,000 nucleotides. This process involves four steps: 1) amplification of the viral genetic material, 2) assembly into a vector that drives expression via the 35S promoter, 3) transforming the plasmid into *Agrobacterium* and 4) inoculating plants.

Following the introduction of SqYVV in 2014, we decided to attempt to generate an infectious clone and *Agrobacterium*-mediated inoculation system (agroclone for short) in anticipation of this

virus becoming an economic problem. A previously developed SqVYV agroclone was poorly infectious in squash and watermelon, and did not provide high rates of infection despite attempting many different methods of plant inoculation. Therefore, we decided to generate a new clone from the original sample collected in 2014 (stored at -80 C) and to use a new assembly approach (Hi-Fi, NEB) for the cloning. We were unable to amplify a full-length copy of the SqVYV genome from the frozen material. Therefore, pumpkin plants (cv. Big Max) were rub-inoculated with sap prepared from the original samples. After several attempts, one plant developed characteristic SqVYV symptoms (vein yellowing and mosaic) and was confirmed to be infected with SqVYV by RT-PCR with primers that targets the capsid protein (CP) gene.

From this SqVYV-infected plant, RNA was extracted and cDNA with specific primers for SqVYV was synthesized. The PCR amplification was optimized to a higher efficiency, and a putative full-length DNA fragment was obtained (~10,000 bp, Figure 1). The amplified fragment was excised from the gel and cleaned for improved assembly into the binary vector pJL89. The resulting assembled plasmids from two clones were partially sequenced and identity of 99% obtained when compared with the previous SqVYV clone. These two SqVYV clones were transformed into *Agrobacterium tumefaciens* C58 cells. The resulting *A. tumefaciens* colonies were screened for the presence of the SqVYV-pJL89 plasmids. Two-week-old pumpkin plants were agroinoculated with the newly developed SqVYV agroclone. However, plants inoculated with these agroclones failed to develop symptoms by 30 dpi. We then examined the nature of the plasmids in two different strains of *E. coli* (bacterium in which the plasmids are amplified before going into *Agrobacterium*) and found a wide range of different and mostly smaller-sized plasmids, suggesting that the constructs were not stable. Selected clones that had plasmids of the expected size were sequenced at the junctions of the cloned SqVYV insert and those with the correct sequences were selected and inoculated into giant pumpkin plants. However, no symptoms or infection was obtained. When we went back and examined these plasmids, we also found some different sizes and evidence of instability based upon restriction enzyme digestion analysis. We also considered that there could be toxicity of the viral sequence or possibly proteins in *E. coli* and tested a strain of *E. coli* that tolerates toxic proteins. Here we obtained more transformed colonies and a number of promising clones (out of 72 colonies screened). The ends of the promising clones were sequenced and most appeared to have the correct assemblies and 4 of these clones were agroinoculated into giant pumpkin plants. However, by 30 dpi, no symptoms were observed nor was SqVYV detected in newly emerged leaves of these plants by RT-PCR. We believe that the instability of the assembled plasmids in *E. coli* is the reason that we are unable to obtain infectious clones. To avoid this problem, we have been trying to directly transform *Agrobacterium* cells, but the efficiency of transformation has been very low, so this is ongoing.

Because the phloem-limited CYSDV is currently the most economically important melon-infecting virus and it is not sap (mechanically) transmissible, we also decided to generate a CYSDV *Agrobacterium*-mediated infection system. To this end, CYSDV-infected melon leaves were obtained from the laboratory of Dr. Willam Wintermantel (USDA) and total RNA was extracted and used as a template for cDNA production with specific primers for each of the two RNAs (RNA-1 and RNA-2) that compose the genome of CYSDV (Table 1). Putative full-length fragments of both genomic RNAs were obtained (Figure 1), and assembled into the binary vector pJL89. The presence of RNA-1 and RNA-2 clones in the constructs was confirmed by sequencing

the 5’ and 3’ ends. Watermelon plants were co-agroinoculated with the RNA-1 and RNA-2, but no symptoms were observed nor was CYSDV detected in newly emerged leaves of these plants by RT-PCR. We next co-infiltrated a viral anti-defense protein (the potyvirus HC-Pro) in an attempt to enhance infectivity, but this did not result in infection.

Table 1. Primers used for amplification of the complete genomes of SqVYV and CYSDV.

	Forward (5’-3’)	**Reverse (5’-3’)**
CYSDV RNA-1	GTTCATTTCATTTGGAGAGGGAAATATTT TCCTTGGTATAAG	TGGAGATGCCATGCCGACCCTAAAGT CATCGTTAATTTTGAG
CYSDV RNA-2	GTTCATTTCATTTGGAGAGGGAAATACT ACGGTAAATCCATTG	TGGAGATGCCATGCCGACCCCGACCT AGTTTATTTATAAC
SqVYV	CCTCTCCAAATGAAATGAACATGTTAGA TAGCATTCAACCAC	TGGAGATGCCATGCCGACCCGTGGTT GAATGCTATCTAA

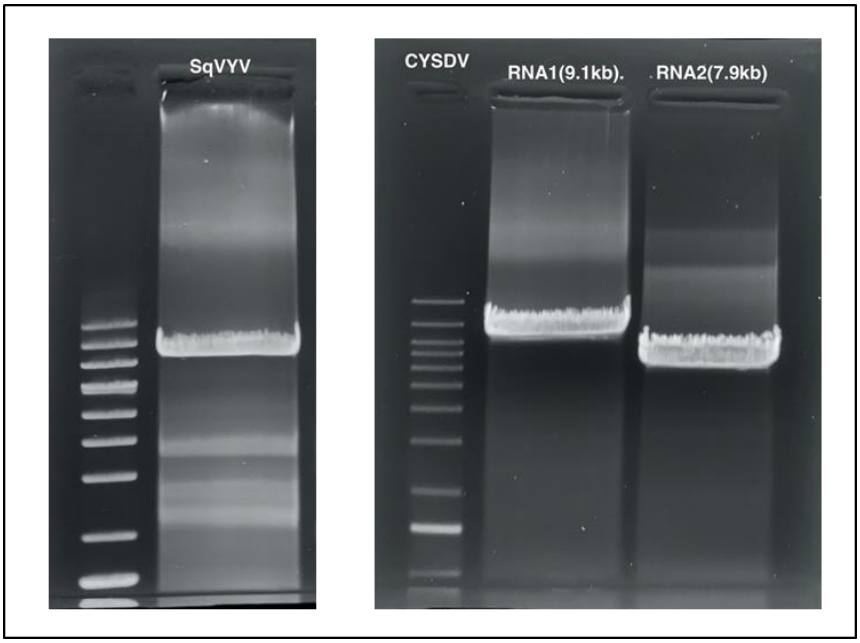

Fig.1. RT-PCR amplification of the RNA genomes of SqVYV and CYSDV

Objective 3. Studies with aphid-transmitted viruses. It is not uncommon that the most important or prevalent virus in a crop can vary from year to year. Thus, in 2019, we received or collected a number of samples of cantaloupe or honeydew melons with mosaic symptoms and, for the late planted fields in the Central Valley, fruit symptoms of discoloration and corky areas from some fields.

The early season samples were received from Yuma on May 9. These samples showed crumpling and mosaic, and were strongly positive with the CMV immunostrip, but negative with the potyvirus immunostrip. This outbreak remained limited and did not cause economic losses.

We received or collected samples later in the growing season (September-October) from the Central Valley. Here, the mosaic symptoms were mostly in the leaves of the young shoots and, in some fields, there were symptoms that ranged from off colored or mottled to discoloration of the flesh, including corky areas. We tested these samples by 1) immunostrips for CMV and potyvirus and 2) by inoculation of the bioindicator plant *Nicotiana benthamiana*. The results are presented in Table 2. Overall, we found widespread infection with CMV in many of the leaf and fruit samples, with less consistent detection of potyvirus imfection.

One of the challenges with the potyvirus immunostrip is that the signal can be weak, especially for older plants, making it difficult to know if it is a weak positive or false positive. To try to increase the amount of virus, we inoculated *N. benthamiana* plants with sap prepared from these samples. Plants inoculated with sap from all the samples induced symptoms in these plants and, in most cases, the symptoms were severe leaf crumpling and curling. Tests of symptomatic leaves indicator plants revealed that all samples were infected with CMV and that some were also infected with a potyvirus (most likely *Watermelon mosaic virus*). In some cases, the original samples were negative with immunostrips, but then was positive when the bioindicator test was performed.

Table 2. Detection of *Cucumber mosaic virus* (CMV) and potyviruses (POTY) in melon samples received on 2019.

Sample ID	Original samples				Sap transmission to *N. benthamiana*		
	Plant part	Symptoms	Immunostrip[1]		Symptoms[2]	Immunostrip[1]	
			CMV	POTY		CMV	POTY
19-304	Branch	Yellowing on leaves	+	+	++	+	+
19-305	Branch	Blistering and crumping on leaves	-	++	+	+	-
19-306	Branch	Blistering and crumping on leaves	-	-	++	+	+
19-307	Branch	Leaves with yellowing and necrosis at leaves' border	-	-	++	+	+
19-308	Fruit	No obvious symptoms	+	+	+++	+	-
19-309	Fruit	Necrosis inside	++	++	++	++	++
19-317	Fruit (Cantaloupe)	Necrosis inside	++	++	+++	++	-
19-318	Leaves (Cantaloupe)	Blister and crumpling on leaves	++	++	+++	++	-

[1] - = negative, + = weak positive and ++ : medium positive

[2] epinasty, crumpling and blister on leaves: + = mild, ++ = moderate and +++ = severe

Therefore, CMV was clearly associated with the symptoms in leaves and fruits, with likely mixed infections with a potyvirus in some samples. It appeared that these infections were relatively late, possibly from aphids moving through the fields, which is why the symptoms were strongest in the young leaves and fruits (strong sinks). It also appears that immunostrips alone may not be suitable for diagnosis because 1) low titer infections may not be detected and 2) the challenges with interpreting the 'weak' potyvirus immunostrip.

It is not possible to conclude that CMV infection alone or a mixed infection of CMV and a potyvirus was responsible for the fruit symptoms. One reason is that there were fields with mosaic symptoms in the leaves that did not have symptoms in the fruits. Also, a search of the literature failed to reveal this type of fruit symptom associated with CMV infection. One possibility is that the CMV infection, together with additional stress, resulted in the fruit symptoms.

Conclusions

In 2019, whitefly populations were extremely low in melon fields in Central California, and no outbreaks such as those observed in 2018 were observed. This was likely due to the cool rainy spring weather in 2019. Despite trying a number of different approaches, we were not able to develop agroclone systems for SqVYV or CYSDV. We have identified the problem to be stability of these large constructs in *E. coli* strains used to increase the assembled plasmids. We are now looking into other approaches to deal with this problem. Finally, aphid-transmitted viruses were more important in 2019, and we showed that CMV was the predominant virus involved in the fields we received samples from. The nature of an unusual fruit symptom associated with CMV infections is still not clear.