Suppression of *Cucurbit chlorotic yellows virus* Accumulation in Melon Breeding Line MR-1 under Natural Infection in Imperial Valley, California

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Introduction

Cucurbit chlorotic yellows virus (CCYV) infection of melon was observed for the first time in the New World in Imperial Valley, California in 2018 (Wintermantel et al., 2019). CCYV was first reported in Japan (Okuda et al., 2010) followed by reports of its presence in China (Gu et al., 2011; Zeng et al., 2011), Taiwan (Huang et al., 2011), Sudan (Hamed et al., 2011), Lebanon (Abrahamian et al., 2012), Iran (Bananej et al., 2013), and Greece (Orfanidou et al., 2014).

CCYV-susceptible melons react to infection by the virus with foliar symptoms and disease development nearly identical those induced by *Cucurbit yellow stunting disorder virus* (CYSDV) (Kuo et al., 2007; Wintermantel et al., 2017). Resistance breeding for control of CYSDV has been ongoing in Imperial Valley, California since 2006 (McCreight and Wintermantel, 2008) with resistance to CYSDV conditioned by a single recessive gene in PI 313970 (McCreight and Wintermantel, 2011) and TGR 1551 (= PI 482420) (McCreight et al., 2017), but selection under naturally-infected conditions was confounded unbeknownst to us by CCYV beginning in Fall 2014. CCYV and CYSDV have been co-infecting melons in Imperial Valley since 2014; however, due to similarity in symptoms on melon plants infected by these viruses, the presence of CCYV was not determined until 2018 (Wintermantel et al., 2019).

Five potential sources of resistance to CCYV in melon were found among 51 lines in controlled inoculation tests: JP 138332, JP 215154, JP 21515, JP 216751, and JP 91204, a.k.a. MR-1 (Okuda et al., 2013). These five lines exhibited no or only faint symptoms in response to CCYV inoculation. JP 138332 was, however, the only one of the five putative CCYV-resistant lines with a detectable virus titer significantly lower than that of 'Earl's Seine'. Okuda et al. (2013) also noted susceptibility of TGR 1551 (= PI 482420) to CCYV including CCYV-induced yellowing symptom expression in a preliminary study. We observed CCYV-induced yellowing of PI 313970 and TGR 1551, lines exhibiting resistance to CYSDV, before we detected CCYV in Imperial Valley in 2018 (Wintermantel et al., 2019). Our stocks of MR-1 exhibited CYSDV-induced yellowing symptoms in numerous field tests in Imperial Valley beginning in Fall 2006 (McCreight and Wintermantel, 2008) and every year thereafter (McCreight and Wintermantel, unpublished). Here we report the reaction of MR-1 to CCYV in a field test naturally co-infected with CYSDV and CCYV as measured by virus copy number and foliar yellowing.

Materials and Methods

Four melon lines were planted at the University of California Desert Research and Extension Center, Holtville: 'Ananas Yoqne'am' (AY), 'Top Mark' (TM) and PI 313970 (PI), and MR-1. AY is an Israeli melon and a member of the Ameri Group (Burger et al., 2010). TM is a western U.S. shipper type cantaloupe susceptible to CYSDV and CCYV (unpublished data). PI, a land race from India, is resistant to CYSDV (McCreight and Wintermantel, 2011) and susceptible to CCYV (unpublished data). MR-1 was derived from PI 124111 for uniform reaction to downy mildew caused by two races of *Pseudoperonopsora cubensis* and races 1, 2 and 3 of powdery mildew caused by *Podosphaera xanthii* (Thomas, 1986). The test was planted and watered with subsurface drip irrigation on 15 August 2019. Seeds were sown in standard western U.S. melon beds, on 2 m centers and two hills spaced 1.5 m apart within 3 m-long plots. Plants were sampled for virus content and rated for virus yellowing ca. 40 and 70 days post-planting (dpp). Imidacloprid was applied after emergence in order to prevent the plants from being damaged by whitefly feeding (Wisler et al., 1998).

Yellowing leaves were collected from two plants of each of the four lines and stored in a cold room until sampled for RNA extraction. Approximately 100 mg of leaf tissue from each plant was collected, lyophilized, and ground to a fine powder. Total RNA was extracted from each sample using the MagMax Plant RNA extraction kit (Thermo Fisher Scientific) in a KingFisher Flex Magnetic particle processor, and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. The CYSDV and CCYV-

specific primers and probes were designed targeting the RNA dependent RNA polymerase (RdRp) gene on RNA1 of each virus (Table 1). The CYSDV probe was labeled at the 5' end with HEX, CCYV with FAM and ADP with CALFluor610. The melon ADP gene was used as an internal control for RT-qPCR. All primers were subjected to NCBI BLASTn to verify specificity and were validated against both viruses and healthy melon prior to use in these studies.

RT-qPCR was conducted in a CFX96 Real-time system thermal cycler (Bio-Rad) with cycling parameters: denaturation at 95° for 3 min followed by 40 cycles of 95° for 10 s and 60° for 1 min. Each single RT-qPCR reaction contained 5 µl of 5x PerfecTa Multiplex qPCR supermix (Quantbio), 0.6 µl forward primers, reverse primers and the respective probes for CYSDV, CCYV and ADP, 13.6 µl nuclease free water and 1 µl cDNA for a total reaction of 25 µl in a 96-well PCR plate. Two technical replications for each sample were included in RT-qPCR to minimize the error. A non-template control using nuclease-free water was used for each run. The data were analyzed using Bio-Rad CFX manager software V3.1. The quantification cycle value (Ct) was determined at the default settings. A 5-fold dilution series of CYSDV and CCYV was generated with known cDNA concentration and run in each plate to construct standard curves by plotting log value of cDNA against Ct number. The amplification efficiency (*e*) and the coefficient of determination (R^2) of the primers used in qPCR were calculated automatically by Bio-Rad CFX manager software according to the Ct value generated for the serial dilutions against the corresponding $\log_{10}$ amount of the cDNA template. Mean relative virus copy number in the sample was calculated as follows. Virus copy number/ µl= [cDNA concentration (g/µl)/ (PCR product in bp x 660)] x 6.022 x 10^{23}.

Results and Discussion

MR-1 and TM exhibited extensive virus yellowing symptoms 41 dpp compared with intermediate symptoms on AY and PI, but by 70 dpp all four lines exhibited extensive yellowing (Table 2). PI is resistant, not immune, to CYSDV (McCreight and Wintermantel, 2011). The titer of CYSDV in PI was 0.023 % of the level in TM, whereas the CYSDV titer in MR-1 was 527 % higher than in TM. Extensive yellowing exhibited by PI was, therefore, likely due to CCYV infection. The CCYV titer in PI was 136 % of that in TM (Table 2), whereas the CCYV titers in MR-1 and AY were 1 % and 2 % that of TM, respectively. The extensive yellowing of MR-1 and AY may have been largely due to CYSDV infection, if their CCYV titers are indicative of resistance. Resistance to CYSDV in PI resulted in much greater reduction of CYSDV titer relative to TM than the putative resistance to CCYV did in MR-1 and, possible resistance in AY relative to TM. Interestingly, the lower titer of CCYV in MR-1 contradicts the results of Okuda et al. (2013) who found that although MR-1 exhibited mild symptoms in response to inoculation with CCYV, virus titer was comparable to that in the CCYV-susceptible 'Earl's Seine'.

The different reactions of MR-1 could have been due to CCYV strain differences, although this is not likely, since the genomes of CCYV isolates from Japan and California are 99% identical to each other (Wintermantel unpublished). Another possibility would be differences in the MR-1 sources between the two sets of experiments. Different test conditions may have played an important role, too. Okuda et al. (2013) exposed the test plants to 4-days of feeding by 20-30 viruliferous *B. tabaci* Q biotype whiteflies per plant. The plants in the Imperial Valley test were subjected to much higher numbers (often > 100 adults per leaf) of *B. tabaci* MED biotype whiteflies feeding continuously from time of emergence through the second leaf sample collection, with mean daily maximum temperatures of 42, 37 and 31°C in August, September and October, respectively. A more likely explanation may be that co-infection of CCYV with CYSDV in the Imperial Valley field test influenced CCYV titer in test plants. Co-infection of CCYV and CYSDV often resulted in reduced titers of both viruses, especially CCYV in cucumber plants (Abrahamian et al., 2013; Orfanidou et al., 2020). In this regard it is interesting to note the CCYV:CYSDV ratio within each line. By this measure, the CCYV titer was greater than the CYSDV titer in all four lines, ranging from 7x for MR-1 to 1.8E+07x for PI in this test (Table 1). CYSDV infection may have resulted in extensive virus yellows symptoms and perhaps suppression of CCYV multiplication in MR-1 and the other lines relative to TM, although CCYV titers still exceeded those of CYSDV within each entry.

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Literature Cited

1. Abrahamian, P.E., H. Sobh, and Y. Abou-Jawdah. 2012. First Report of *Cucurbit chlorotic yellows virus* on cucumber in Lebanon. Plant Dis. 96:1704.
2. Abrahamian, P.E., R. Seblani, H. Sobh, and Y. Abou-Jawdah. 2013. Detection and quantitation of two cucurbit criniviruses in mixed infection by real-time RT-PCR. Journal of Virology Methods 193:320–326.
3. Bananej, K., W. Menzel, N. Kianfar, A. Vahdat, and S. Winter. 2013. First report of *Cucurbit chlorotic yellows*

virus infecting cucumber, melon and squash in Iran. Plant Dis. 97:1005.

4. Burger, Y., H.S. Paris, R. Cohen, N. Katzir, Y. Tadmor, E. Lewinsohn, and A.A. Schaffer. 2010. Genetic diversity of *Cucumis melo*, p. 165–198. In: J. Janick (ed.). Hort. Rev. Wiley-Blackwell, New York.
5. Gu, Q.S., Y.H. Liu, Y.H. Wang, and W.G. Huangfu. 2011. First report of *Cucurbit chlorotic yellows virus* in cucumber, melon, and watermelon in China. Plant Dis. 95:73.
6. Hamed, K., W. Menzel, G. Dafalla, A.M.A. Gadelseed, and S. Winter. 2011. First report of *Cucurbit chlorotic yellows virus* infecting muskmelon and cucumber in Sudan. Plant Dis. 95:1321.
7. Huang, L.-H., H.-H. Tseng, J.-T. Li, and T.-C. Chen. 2011. First report of *Cucurbit chlorotic yellows virus infecting* cucurbits in Taiwan. Plant Dis. 94:1168.
8. Kuo, Y.-W., M.R. Rojas, R.L. Gilbertson, and W.M. Wintermantel. 2007. First report of *Cucurbit yellow stunting disorder virus* in California and Arizona, in association with *Cucurbit leaf crumple virus* and *Squash leaf curl virus*. Plant Dis. 91:330.
9. McCreight, J.D. and W.M. Wintermantel. 2008. Potential new sources of genetic resistance in melon to *Cucurbit yellow stunting disorder virus*, p. 173–179. In: M. Pitrat (ed.). Cucurbitaceae 2008, Proceedings of the IXth EUCARPIA meeting on genetics and breeding of Cucurbitaceae, May 21-24th, 2008. INRA, Avignon (France).
10. McCreight, J.D. and W.M. Wintermantel. 2011. Genetic resistance in melon PI 313970 to *Cucurbit yellow stunting disorder virus*. HortScience 46:1582–1587.
11. McCreight, J.D., W.M. Wintermantel, E.T. Natwick, J.W. Sinclair, K.M. Crosby, and M.L. Gómez-Guillamón. 2017. Recessive resistance to *Cucurbit yellow stunting disorder virus* in melon TGR 1551. Acta Hort. 1151:101–107.
12. Okuda, M., S. Okazaki, S. Yamasaki, S. Okuda, and M. Sugiyama. 2010. Host range and complete genome sequence of *Cucurbit chlorotic yellows virus*, a new member of the genus Crinivirus. Phytopathology:560–566.
13. Okuda, S., M. Okuda, M. Sugiyama, Y. Sakata, M. Takeshita, and H. Iwai. 2013. Resistance in melon to *Cucurbit chlorotic yellows virus*, a whitefly-transmitted crinivirus. European J. Plant Pathol. 135:313–321.
14. Orfanidou, C., A. Katsiani, L. Papayiannis, Katis Nikolaos I., and V.I. Maliogka. 2020. Interplay of *Cucurbit yellow stunting disorder virus* with cucurbit chlorotic yellows virus and transmission dynamics by *Bemisia tabaci* MED. Plant Dis. https://doi.org/10.1094/PDIS-03-20-0621-RE
15. Orfanidou, C.G., V.I. Maliogka, and N.I. Katis. 2014. First report of Cucurbit chlorotic yellows virsu in cucumber, melon, and watermelon in Greece. Plant Disease 98:1446.
16. Thomas, C.E. 1986. Downy and powdery mildew resistant muskmelon breeding line MR-1. HortScience 21:329.
17. Wintermantel, W.M., R.L. Gilbertson, E.T. Natwick, and J.D. McCreight. 2017. Emergence and epidemiology of *Cucurbit yellow stunting disorder virus* in the American desert southwest, an development of host pant resistance in melon. Virus Research 241:213–219.
18. Wintermantel, W.M., L.L.J. Hladky, P. Fashing, K. Ando, and J.D. McCreight. 2019. First report of *Cucurbit chlorotic yellows virus* infecting melon in the New World. Plant Dis. 103:778. https://doi.org/10.1094/PDIS-08-18-1390-PDN
19. Wisler, G.C., J.E. Duffus, H.Y. Liu, and R.H. Li. 1998. Ecology and epidemiology of whitefly transmitted closteroviruses. Plant Dis. 82:270–280.
20. Zeng, R., F.M. Dai, W.J. Chen, and J.P. Lu. 2011. First Report of *Cucurbit chlorotic yellows virus* infecting melon in China. Plant Dis. 95:354.

Table 1. CYSDV and CCYV-specific primers and probes for multiples qPCR assay.

Virus	Forward primer	Reverse Primers	Probes
CYSDV	TGATGACGGGAAGGTTAGAGT	CTTCGGATCGGGTTGGACA	HEX-TGCCAGATGCACAGAGGATGTTCG-BHQ1
CCYV	ACGGTGGGAGAGTTAGAGTGA	CTCTTCGTCTGATTGGTGTGGATA	FAM-CACCAGACGCGCAGAGGATGTTC-BHQ1
ADP	GTGGTGGATAGCAATGACAGAGA	CCTCAGCTCGTCCTCATTCAAC	CALFluor610-TCGAAGCTAGGGATGAGCTGCAC-BHQ2

Table 2. Mean titer (copies/µl) of CYSDV and CCYV and virus yellows symptoms in a naturally-infected field test in Imperial Valley, California, Fall, 2019.

	CYSDV			CCYV		Yellowing (dpp)[z]	
Line	copies/µl	R_{TM}[y]	CCYV: CYSDV	copies/µl	R_{TM}	41	70
Ananas Yoqne'am	8.1E+10	2.12264	3.0E+01	2.4E+12	0.02	4.5	8.0
MR-1	2.0E+11	5.26730	7.1E+00	1.4E+12	0.01	7.0	8.5
PI 313970	8.8E+06	0.00023	1.8E+07	1. 6E+14	1.36	4.0	7.0
Top Mark	3.8E+10	1.00000	3.0E+03	1.1E+14	1.00	7.5	6.5

[z]Virus yellowing rated on a 1 (asymptomatic) to 10 (100 % yellowed leaves) visual scale; dpp = days post planting.
[y]Virus copies/µl relative to 'Top Mark'