Cucurbit chlorotic yellows virus: Insights Into Its Natural Host Range, Genetic Variability, and Transmission Parameters

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Abstract

Cucurbit chlorotic yellows virus (CCYV) (genus *Crinivirus*, family *Closteroviridae*) is implicated in cucurbit yellows disease (CYV), causing typical interveinal yellowing symptoms in leaves, and is transmitted by *Bemisia tabaci* Mediterranean (MED) and Middle East-Asia Minor 1 (MEAM1). Due to its recent report in cucurbit crops in Greece, field surveys were conducted during 2011-2016 to determine the presence of the virus in symptomatic cucurbits and alternative hosts among arable weed species. Results indicated the restricted spread of the virus and

identified 13 weed species as CCYV hosts for the first time. Sequence analysis of the RNA-dependent RNA polymerase (RNA1) coat and minor coat proteins (RNA2) revealed very low genetic diversity (<0.1%) among the Greek isolates. Transmission experiments were also conducted using *B. tabaci* MED with retention determined at four days, whereas transmission efficiency was positively correlated with the number of adults used, features linked to the virus semipersistent mode of transmission.

Cucurbit chlorotic yellows virus (CCYV) (genus *Crinivirus*, family *Closteroviridae*) (Okuda et al. 2010) was initially reported in melon plants in Japan (Gyoutoku et al. 2009) in association with cucurbit yellows disease (CYD). The bipartite genome consists of 8607 nucleotides (nt) and 8041 nt for RNA1 and RNA2, respectively (Okuda et al. 2010). RNA1 encodes proteins involved in replication, while genes encoded by RNA2 are implicated in virus movement, encapsidation, and transmission by *Bemisia tabaci* (Okuda et al. 2010). CCYV is transmitted exclusively by the Mediterranean (MED, formerly biotype Q) and Middle East-Asia Minor 1 (MEAM1, formerly biotype B) cryptic species of *B. tabaci* (Gennadius) complex (Gyoutoku et al. 2009; Okuda et al. 2010) in a semipersistent manner (Li et al. 2016).

The virus has been reported in Middle East and Asian countries (Al-Saleh et al. 2015; Bananej et al. 2013; Gu et al. 2011; Hamed et al. 2011; Huang et al. 2010; Okuda et al. 2010), as well as in the Mediterranean region (Abrahamian et al. 2012; Amer 2015; Orfanidou et al. 2014a). Symptoms on cucurbits include brittleness, thickening, and interveinal yellowing of leaves that usually appear on basal leaves of the plant, but gradually expand to the new foliage and they are indistinguishable from those caused by nutrition deficiencies or by other criniviruses (Cucurbit yellow stunting disorder virus, CYSDV; Beet pseudoyellows virus, BPYV) (Abrahamian et al. 2013) or poleroviruses (Cucurbit aphid-borne yellows virus, CABYV; pepo aphid-borne yellows virus, PABYV).

In nature, CCYV has only been reported in cucurbits (melon, watermelon, cucumber, and zucchini). The experimental host range includes species of at least four botanical families (Asteraceae, Chenopodiaceae, Convolvulaceae, and Solanaceae) (Okuda et al. 2010), leaving an obvious knowledge gap on the true host range of the virus.

Bemisia tabaci is now recognized as a complex of at least 34 morphologically indistinguishable cryptic species (or genetic groups) (Boykin and De Barro 2014; De Barro et al. 2011), two of which are the most widespread: *B. tabaci* MEAM1 and MED. They differ in host range, behavior, insecticide resistance, and virus transmission efficiency (Brown 2010; De Barro et al. 2011). In Mediterranean basin countries such as Greece, *B. tabaci* MED is more widespread than

MEAM1 (Orfanidou et al. 2014b). A study on the transmission parameters of CCYV by *B. tabaci* MED has been recently conducted. This study involved the effect of acquisition access period (AAP), retention time, inoculation access period (IAP), and the number of whitefly adults on transmission efficiency of the virus. It was shown that the retention time of CCYV on *B. tabaci* MED is up to 12 days, whereas transmission reached 100% after 48 h AAP (Li et al. 2016). The biological validity of their data is limited as they evaluated retention time and AAP by SYBR Green real-time RT-PCR detection of the virus in the vector instead of detection in the host plants due to efficient transmission and multiplication of CCYV.

The purpose of this study was to improve knowledge about the epidemiology of CCYV, including characterization of Greek isolates and study of the natural host range and transmission efficiency by *B. tabaci* MED.

Materials and Methods

Plant material and molecular detection. Samples were collected from 17 localities between 2011 and 2016. A total of 615 melon, watermelon, cucumber, and zucchini plants exhibiting typical CYD symptoms (Fig. 1) and 342 plants of arable weed species (Table 1), i.e., the naturally occurring wild species found in the cropped area, from the island of Rhodes were included in the study. The weeds were collected within or surrounding the affected fields irrespective of the presence of symptoms in May, during the growing season, and in November, when cucurbits are not grown.

Total RNA was extracted from all samples according to the extraction protocol A developed by Chatzinasiou et al. (2010). Primers 'CC-RdRp-up' (5'-CCTAATATTGGAGCTTATGAGTACA-3')/'CC-RdRp-do' (5'-CATACACTTTAAACACAACCCC-3') that amplify a 709-bp region of the RNA-dependent RNA polymerase (RdRp) gene were used in RT-PCR detection of CCYV (Orfanidou et al. 2014a). In addition, primers 'BPYV I'/'BPYV II', 'CYSDV1'/'CYSDV2' (Boubourakas et al. 2006), and 'CABYVUp'/'CABYVDo' were used in RT-PCR assays for detection of BPYV, CYSDV, and CABYV, respectively (Lotos et al. 2014).

Sequencing of the RNA-dependent-RNA-polymerase (RdRp), the coat protein (CP), and the minor coat protein (CPm) encoding genes. The RdRp encoding gene from the RNA1 and coat protein (CP) and coat protein minor (CPm) encoding genes from the RNA2 of CCYV were selected for sequence analysis. Isolates from cucurbits and weeds from two different geographical regions were used in the study (Supplementary Table 1). In order to amplify the complete coding region, primers targeting flanking regions of the three genes were designed (Supplementary Table 2) based on available sequences in EMBL-EBI.

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In order to amplify the complete nucleotide sequence of the RdRp gene, two overlapping RT-PCR assays were performed using primers CC5938F/CC6895R and CC6638F/CC7698R. Reverse transcription (RT) was carried out in a final volume of 20 µl containing: 2 µl of RNA template, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM each dNTP, 0.4 µM reverse primer, 15 Units ribonuclease inhibitor (HT Biotechnology), 50 Units Moloney murine leukemia virus reverse transcription (Invitrogen). The reaction was carried out for 1 h at 45°C followed by 15 min at 70°C. For the PCR, 2 µl of the cDNA were used and the reaction mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.2 µM of each primer, 1 Unit Dynazyme II DNA polymerase (Finnzymes), and DEPC treated water to a final volume of 20 µl. The thermal profile was: 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a final step of 72°C for 5 min. A two-step RT-PCR assay was applied to amplify the CP gene using the primers CC4701F/CC5760R, whereas for CPm, two overlapping RT-PCR assays were also performed using primers CC5418F/CC6277R and CC6168F/CC7191R. RT-PCR took place in a similar two-step protocol with the same cycling conditions described for the RdRp gene.

Amplified PCR products were purified from the agarose gel using the NucleoTrap purification kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and sequenced on both directions at Eurofins Genomics (Vienna, Austria).

Sequence and phylogenetic analysis. Sequences obtained in the present study as well as those available in EMBL-EBI were included in the analysis. The sequences were aligned with CLUSTALW available in MEGA7 (Kumar et al. 2016). Appropriate nucleotide substitution models were determined using the same program. The estimation of the phylogenetic relationships was carried out with the Maximum Likelihood (ML) method incorporated in MEGA and the reliability

of the phylogenetic hypothesis was evaluated using the nonparametric bootstrap analysis (NPB).

Transmission studies. A *Bemisia tabaci* MED colony was maintained on tobacco (*Nicotiana tabacum* var. Virginia) in isolated, insect-proof cages at temperatures ranging from 22 to 25°C. The identity of the *B. tabaci* species was confirmed by real-time PCR (Papayiannis et al. 2009) and sequencing of the cytochrome oxidase I mitochondrial gene (Tsagkarakou et al. 2007). No attempts were made to balance the sex ratio of whiteflies used. Isolate CC75 (EMBL-EBI accession number LT716000) from a naturally infected cucumber plant (hybrid Litoral) collected in 2013 in Tympaki (Crete) was maintained in an insect-proof cage by serial passages onto cucumber plants (*C. sativus*, hybrid Galeon) using *B. tabaci* MED.

CCYV-infected cucumber plants (hybrid Galeon) were used as virus source three weeks post inoculation (wpi). Noninfected cucumber plants of the same hybrid were used as negative controls. In all transmission experiments plants were used at the second true leaf stage. Groups of 5, 10, 15, 25, 40, and 60 adult whiteflies were given a 24-h AAP, whereas IAP was 72 h. Then, clip cages were removed and one wpi the leaf on which the whiteflies fed was removed in order to prevent possible maturing of whitefly nymphs. Plants were transferred to different chambers, where they were grown for 4 weeks. For retention evaluation, ten batches of 60 adult whiteflies were given a 24-h AAP on infected cucumber (hybrid Galeon). Whiteflies were then allowed to feed on healthy cucumber (hybrid Galeon) plants for a 24-h IAP: by the end of each IAP, each batch of whiteflies was transferred every day for a total of a week onto healthy cucumber plants. Three days after each IAP, clip cages were removed and plants treated with insecticide (imidacloprid) before being transferred to the greenhouse (22 to 25°C) and observed for symptom development. In all transmission experiments, there were five replicates of 10 cucumber plants each. In the case of 40 and 60 adults, 2 clip cages were used per group in order to avoid

Fig. 1. Map of Greece: Black triangles indicate regions from which cucurbit samples showing yellowing symptoms were collected between 2011 and 2016.

overcrowding. At 3 wpi, detection was performed using as template RNA extracted from approximately 0.3 g of pooled material collected from the base of the 3rd to 8th leaves as described above.

Statistical analysis of the results was conducted by one-way analysis of variance and Tukey's honestly significant difference was employed to compare the mean values at $P \leq 0.01$ using the SPSS statistical package (version 23.0; IBM, Armonk, NY).

Results

Natural host range of CCYV. CCYV was present in cucumber, melon, watermelon, and zucchini crops in Crete (Heraklion) and Rhodes (Fig. 1) at a rate of 22.4%, whereas the virus was not detected in other regions of Greece. The majority of the symptomatic samples were found infected by other criniviruses (BPYV, CYSDV) or poleroviruses (CABYV) (*unpublished data*). It is worth mentioning that mixed infections of CCYV and CYSDV were also observed in both Crete and Rhodes in 30, or 12%, of all 4 cucurbits (*unpublished data*).

Arable weeds were collected from several fields in Rhodes. CCYV was detected in 13 species belonging to 11 families in a percentage of 13.2% of the collected material and the majority of infected weeds were collected in November in the absence of cucurbits (Table 1).

Sequence and phylogenetic analysis. The complete RdRp, CP, and CPM sequences were obtained from seven CCYV isolates from cucumber, melon, and weeds. Comparative analysis revealed 99 to 100% identity between the Greek isolates.

Phylogenetic analysis of CP (Fig. 2A) clustered the Greek isolates in one group, "group I," together with isolates from Lebanon (KC990504), China (JQ904629), Japan (AB523789), Taiwan (JF502222), Sudan (JF807053), and Saudi Arabia (KT946809), whereas "group II" consisted of two isolates from Iran. Specifically for "group I," a further subdivision was observed, as the Saudi Arabian isolate (KT946809) formed a different clade (subgroup Ib) from the other sequences (subgroup Ia).

The low genetic diversity and bootstrap support of CCYV isolates as well as the lack of sequences from more divergent isolates such as Iranian or Saudi Arabian resulted in inability of discrimination of any group from complete RdRp (*data not shown*) and CPM (Fig. 2B) sequences. Nevertheless, when two partial sequences from Saudi Arabia (LM653109, LM653110) were included in the phylogenetic analysis of the RdRp gene, they formed a different clade from all CCYV sequences (Fig. 2C).

Transmission efficiency and retention of CCYV by MED. Transmission parameters of the CCYV isolate CC75 by *B. tabaci*

Table 1. Detection of Cucurbit chlorotic yellows virus (CCYV) in annual (An), biennial (Bi), and perennial (Pe) weed species in the island of Rhodes. Newly identified hosts for CCYV are indicated in bold.

Family	Weed species	Life span/spring (S)/winter (W) weeds	No. of collected plants	No. of plants positive to CCYV
Amaranthaceae	<i>Amaranthus blitoides</i> ^y	An (S)	15	2
	<i>Amaranthus retroflexus</i> ^{xy}	An (S)	32	3
Asteraceae	<i>Chamomila recutita</i> ^x	An (S)	1	0
	<i>Chrysanthemum</i> sp. ^x	Pe	7	0
	<i>Cirsium arvense</i> ^x	Pe	1	0
	<i>Conyza</i> sp. ^x	An (W)	6	0
	<i>Cynara scolymus</i> ^y	Pe	7	0
	<i>Dittrichia viscosa</i> ^x	Pe	1	0
	<i>Sonchus asper</i> ^y	An (W)	1	0
	<i>Sonchus oleraceus</i> ^{xy}	An (W)	42	2
Boraginaceae	<i>Heliotropium europaeum</i> ^y	An (S)	10	2
Brassicaceae	<i>Capsella bursa-pastoris</i> ^{xy}	An (W)	7	2
	<i>Myagrum perfoliatum</i> ^x	An (S)	1	0
	<i>Sinapis arvensis</i> ^{xy}	An (W)	2	0
	<i>Sisymbrium</i> sp. ^x	An (W)	3	3
Caparaceae	<i>Capparis spinosa</i> ^{xy}	Pe	6	1
Caryophyllaceae	<i>Stellaria media</i> ^x	An (W)	2	0
Chenopodiaceae	<i>Chenopodium album</i> ^{xy}	An (S)	33	4
Convolvulaceae	<i>Convolvulus arvensis</i> ^{xy}	Pe	3	2
Cucurbitaceae	<i>Ecballium elaterium</i> ^{xyz}	Pe	39	14
Euphorbiaceae	<i>Chrozophora</i> sp. ^x	An (S)	1	0
Fabaceae ^z	<i>Medicago sativa</i> ^{xz}	Pe	8	3
	<i>Trifolium</i> sp. ^x	An (Pe)	8	0
Lamiaceae	<i>Lamium amplexicaule</i> ^x	An (W)	2	0
Malvaceae	<i>Malva sylvestris</i> ^{xy}	Bi (Pe)	25	2
Papaveraceae	<i>Papaver rhoeas</i> ^x	An (W)	2	0
Plantaginaceae	<i>Plantago major</i> ^x	Pe	2	0
	<i>Veronica hederifolia</i> ^x	An (W)	3	0
	<i>Veronica pericaria</i> ^x	An (W)	1	0
Polygonaceae	<i>Polygonum aviculare</i> ^x	An (S)	2	0
Portulacaceae	<i>Portulaca oleracea</i> ^y	An (S)	5	0
Primulaceae	<i>Anagallis foemina</i> ^x	An (W)	9	0
Rubiaceae	<i>Galium aparine</i> ^x	An (W)	5	0
Solanaceae	<i>Solanum eleagnifolium</i>	Pe	2	0
	<i>Solanum melongena</i> ^x	An (S)	1	0
	<i>Solanum nigrum</i> ^x	An (S)	22	0
Urticaeae	<i>Parietaria officinalis</i>	Pe	13	0
	<i>Urtica urens</i> ^x	Pe	3	0
Zygophyllaceae	<i>Tribulus terrestris</i> ^y	Pe	8	5
SUM	...	...	341	15

^x Species collected in spring.

^y Species collected in winter.

^z Species that showed interveinal yellowing of leaves.

MED were also evaluated. Groups of 5, 10, 15, 25, 40, and 60 whiteflies transmitted CCYV with efficiencies of 16%, 22%, 22%, 34%, 66%, and 78%, respectively (Fig. 3). Results showed that transmission efficiency of the virus was positively correlated with the whitefly density ($F = 138.877$, $df = 5$, $P \leq 0.01$), but it did not reach 100% (Fig. 3).

Virus retention in *B. tabaci* MED was at least 4 days (Fig. 4). Transmission efficiency ranged from 66 to 45% the first 3 days post acquisition, but decreased rapidly by the 4th day ($F = 271.224$, $df = 6$, $P \leq 0.01$) (Fig. 4). No transmission was observed from days 5 to 7.

Discussion

CYD has been observed in Greece for the past 20 years (Boubourakas et al. 2006; Livieratos et al. 1998) with initial studies showing that BPYV, CYSDV, and CABYV are associated with the disease (Boubourakas et al. 2006). The consistent detection of the already reported viruses in symptomatic cucurbit samples led to the assumption that no other viruses were implicated in CYD in Greece. CCYV was recently reported in Greece infecting cucumber in Heraklion (Crete), and watermelon and melon exhibiting yellowing symptoms in Rhodes both in single and mixed infections with CYSDV (Orfanidou et al. 2014a). Under such circumstances, the study of the epidemiological parameters of this virus in Greek fields has been the target of this research.

Since its first report in Greece, CCYV has been consistently detected every year in cucurbit fields in Rhodes and Crete (Fig. 1). Indeed, CCYV seems to be established in all cucurbit species (cucumber, melon, watermelon, and zucchini). It was detected in at least nine

different fields, mainly in the Southwest part of Rhodes, whereas in Crete the virus appears to be restricted in Heraklion, as it has not been detected in the rest of the island or the mainland. The restricted spread of the virus may be due to its introduction into the country being recent, and therefore its spread is an ongoing process. However, in Rhodes, CCYV has adapted to arable weeds (Table 1). Also, CCYV has been found in mixed infections with CYSDV (*unpublished data*) with which they share the same vectors that are native in Greece (Roditakis et al. 2009; Orfanidou et al. 2014b). Both criniviruses were also detected often in mixed infections in the majority of the reported countries (Abrahamian and Abou-Jawdah 2014; Al-Saleh et al. 2015; Amer 2015; Bananej et al. 2013); as such, it would be worthwhile to determine if there is a synergistic/antagonistic interaction between these viruses that may affect the spread and predominance of CCYV in Greek fields.

It is very well established that arable weeds play an important role in plant virus epidemiology (Bos 1981; Duffus 1971; Sacristán et al. 2004; Thresh 1981; Webster et al. 2007). Their role is crucial in virus spread and overwintering, especially for viruses that are not seed transmitted, such as criniviruses. Therefore, weed species that play an important role, especially when the main crop is not present, have been recognized as crinivirus hosts (Boubourakas et al. 2006; Kil et al. 2015; Orfanidou et al. 2014b; Tugume et al. 2016). The constant detection of CCYV in Rhodes for the last five years led to the hypothesis that it has been established in alternative hosts. In doing so, two surveys in 2015 were conducted on the island—the first in May when cucurbit crops were cultivated and exhibited yellowing symptoms, and the second in

Fig. 2. Maximum likelihood phylogenetic trees inferred from the entire **A**, CP and **B**, CPm, and from **C**, partial RdRp gene sequences of CCYV determined in this study and published in the databases. Greek isolates are reported with their name, accession number, and regions (black triangles). Already published isolate sequences from other countries are reported with their accession numbers and country of origin. CYSDV (accession number NC_004810) is used as outgroup. The numbers at the nodes are the nonparametric bootstrap (NPB) probabilities given as percentages of 1000. Bootstrap values >70 are indicated on the branches. The bar represents substitutions per site.

November, when cucurbits are not present in the field—in order to shed light on the natural host range of CCYV. Results revealed the presence of CCYV in 13 wild species for the first time (Table 1), seven annuals and six perennials. Both winter and perennial weeds possibly play a crucial role in virus epidemiology forming a “green” bridge when cucurbits are not present. Reinforcing this notion is the fact that over 80% (37/45) of the infected weeds was collected in November (Table 1) with most infected species being perennials in the island.

Moreover, CCYV was detected in *Ecballium elaterium* (wild cucumber) in a high frequency (14/39) (Table 1), with almost all the plants showing yellowing symptoms. The virus was also detected in three *M. sativa* (alfalfa) plants that also exhibited mild yellowing symptoms and a symptomless *Capparis spinosa* (caper) plant (Table 1). Alfalfa is perennial and it is considered an important forage crop in Greece and worldwide, whereas caper is a common species in the Mediterranean flora. As these three hosts are actually perennials in Rhodes, their role in the epidemiology of CCYV needs to be further examined. The expansion of cucurbit and tomato criniviruses in other crops has been recently reported. Wintermantel and colleagues (2016) highlighted the importance of CYSDV in five non-cucurbit crops, including alfalfa, by evaluating these species as efficient sources of the virus. Conversely, tomato chlorosis virus (ToCV), a tomato crinivirus, was reported to infect pumpkin in China (Sun et al. 2016). The shift of criniviruses from their main hosts to other botanical family species seems to occur in the fields and the mechanisms involved remain to be elucidated.

Criniviruses, in general, show limited genetic diversity as several studies on *Tomato infectious chlorosis virus* (TICV), ToCV, CYSDV, and BPYV from different hosts revealed high nucleotide sequence similarities even when virus isolates originated from geographically distant locations (Akhter et al. 2016; Orfanidou et al. 2014b; Orlio and Navas-Castillo, 2009; Rubio et al. 2001). Also, phylogenetic analysis of the CP gene of CCYV isolates resulted in clustering the Iranian sequences in “group II,” while “group I” consisted of five isolates from China, Lebanon, Japan, Sudan, and Taiwan (Keshavarz et al. 2013). In the present study, determination of the RdRp, CP, and CPm sequences of Greek CCYV isolates was conducted in order to unveil the evolutionary relationships of the virus isolates present in the country with the published isolates in EMBL-EBI database. Sequencing analysis of the RdRp, CP, and CPm genes of seven Greek isolates originated from cultivated and weed species revealed high identity both in nucleotide and amino acid levels. The phylogenetic analysis of RdRp and CP gene sequences resulted in classification of all Greek isolates into “group I,” which was further divided into 2 subgroups, as “subgroup Ib” includes isolates from Saudi Arabia (Fig. 3A, 3C). The formation of groups was not possible when analyzing the CPm gene, owing to the lack of sequencing

information on the most diverse isolates (i.e., Iranian and Saudi Arabian) that supported the branches in the CP tree.

A significant aspect in virus epidemiology is the transmission efficiency by vectors. Especially in the case of criniviruses, which are not mechanically or seed transmitted (Tzanetakis et al. 2013), whitefly-vectors play a unique role in spread and predominance. In order to frame our understanding on the transmission mode of CCYV, two basic parameters were studied: transmission efficiency and retention time. Transmission efficiency using AAP and IAP of 24 h and 72 h, respectively, was positively correlated with the number of *B. tabaci* MED adults, but it did not reach 100%. On the other hand, 24-h AAP of *B. tabaci* MED on CCYV cucumber plants resulted in 95% positive whiteflies (Li et al. 2016). This variability might be explained by the different methodological approaches. In our study, the effect of the 24-h AAP on CCYV transmission was evaluated by testing the virus presence in the recipient plants instead of detecting the virus in the whitefly vector itself. In any case, research on other plant viruses provide some circumstantial evidence that efficient transmission of a specific virus in a given agroecosystem is affected by AAP and IAP (Irwin and Ruesink 1986). Nevertheless, in order to have a complete image of the effect of AAP in transmission efficiency, alternative feeding times must be tested. Enhanced acquisition on the infected plant was shown to favor

Fig. 4. Persistence of Cucurbit chlorotic yellows virus (CCYV) in *Bemisia tabaci* MED. Percent transmission is compared by the number of days post acquisition during seven serial passages. Error bars indicate the standard deviation. Means followed by the same letter indicate not statistically significant differences ($P \leq 0.01$) according to the HSD test.

Fig. 3. Transmission efficiency of Cucurbit chlorotic yellows virus (CCYV) by *Bemisia tabaci* MED using groups of whiteflies (5, 10, 15, 25, 40, and 60 individuals per plant). AAP was of 24 h and IAP was of 72 h. Error bars indicate the standard deviation. Means followed by the same letter indicate not statistically significant differences ($P \leq 0.01$) according to the HSD test.

transmission efficiency of semipersistently transmitted viruses, such as ToCV (Orfanidou et al. 2016) or CYSDV (Celix et al. 1996), so this might be the case for CCYV as well.

An important factor in insect transmission is retention time within the vector. Li et al. (2016) successfully detected CCYV in *B. tabaci* MED 12 days post acquisition by RT-qPCR; however, based on studies on other criniviruses, they presume that *B. tabaci* MED may not be able to transmit the virus after 3 to 5 days due to the rapid reduction of positive whiteflies by the sixth day. Indeed, our study showed that retention period of CCYV in *B. tabaci* MED is 4 days (Fig. 4).

To conclude, this study explored important epidemiological parameters of CCYV in Greece, such as host range, genetic diversity, and transmissibility by *B. tabaci* MED. The emergence of criniviruses as an important agricultural threat over the past 20 years holds potential for a rich area of research, and future studies are needed to unveil the fundamental mechanisms underlying this ongoing process.

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Literature Cited

- Abrahamian, P. E., and Abou-Jawdah, Y. 2014. Whitefly-transmitted criniviruses of cucurbits: Current status and future prospects. *Virus Dis.* 25:26-38.
- Abrahamian, P. E., Sobh, H., and Abou-Jawdah, Y. 2012. First report of *Cucurbit chlorotic yellows virus* on cucumber in Lebanon. *Plant Dis.* 96:1704.
- Abrahamian, P. E., Seblani, R., Sobh, H., and Abou-Jawdah, Y. 2013. Detection and quantitation of two cucurbit criniviruses in mixed infection by real-time RT-PCR. *J. Virol. Methods* 193:320-326.
- Akhter, M. S., Bhor, S. A., Hlaele, N., Nao, M., Sekine, K.-T., Yaeno, T., Yamaoka, N., Nishiguchi, M., Gubba, A., and Kobayashi, K. 2016. Review of *Beet pseudoyellows virus* genome structure built the consensus genome organization of cucumber strains and highlighted the unique feature of strawberry strain. *Virus Genes* 52:828-834.
- Al-Saleh, M. A., Al-Shahwan, I., Amer, M. A., Shakeel, M. T., Abdalla, A. O., Orfanidou, C., and Katis, N. I. 2015. First report of *Cucurbit chlorotic yellows virus* in cucumber in Saudi Arabia. *Plant Dis.* 99:734.
- Amer, M. A. 2015. Serological and molecular characterization of *Cucurbit chlorotic yellows virus* affecting cucumber plants in Egypt. *Int. J. Virol.* 11:1-11.
- Bananej, K., Menzel, W., Vahdat, A., and Winter, S. 2013. First report of *Cucurbit chlorotic yellows virus* infecting cucumber, melon, and squash in Iran. *Plant Dis.* 97:1005.
- Bos, L. 1981. Wild plants in the ecology of virus diseases. Pages 1-34 in: *Plant Diseases and Vectors: Ecology and Epidemiology*. K. Maramorosch and K. F. Harris, eds. Academic Press, London, UK.
- Boubourakas, I. N., Avgelis, A. D., Kyriakopoulou, P. E., and Katis, N. I. 2006. Occurrence of yellowing viruses (*Beet pseudo-yellows virus*, *Cucurbit yellow stunting disorder virus* and *Cucurbit aphid-borne yellows virus*) affecting cucurbits in Greece. *Plant Pathol.* 55:276-283.
- Boykin, L. M., and De Barro, P. J. 2014. A practical guide to identifying members of the *Bemisia tabaci* species complex and other morphologically identical species. *Front. Ecol. Evol.* 2:45.
- Brown, J. K. 2010. Phylogenetic biology of the *Bemisia tabaci* sibling species group. Pages 31-67 in: *Bemisia: Bionomics and Management of a Global Pest*. P. A. Stansly, and S. E. Naranjo eds. Springer, New York.
- Celix, A., Lopez-Sese, A., Almaraz, N., Gomez-Guillamon, M. L., and Rodriguez-Cerezo, E. 1996. Characterization of Cucurbit yellow stunting disorder virus, a *Bemisia tabaci*-transmitted closterovirus. *Phytopathology* 86:1370-1376.
- Chatzinasiou, E., Dovas, C. I., Papanastassopoulou, M., Georgiadis, M., Psychas, V., Bouzalas, I., Koumbati, M., Koptopoulos, G., and Papadopoulos, O. 2010. Assessment of bluetongue viraemia in sheep by real-time PCR and correlation with viral infectivity. *J. Virol. Methods* 169:305-315.
- De Barro, P. J., Liu, S.-S., Boykin, L. M., and Dinsdale, A. B. 2011. *Bemisia tabaci*: A statement of species status. *Annu. Rev. Entomol.* 56:1-19.
- Duffus, J. E. 1971. Role of weeds in the incidence of virus diseases. *Annu. Rev. Phytopathol.* 9:319-340.
- Gu, Q. S., Liu, Y. H., Wang, Y. H., Huangfu, W. G., Gu, H. F., Xu, L., Song, F. M., and Brown, J. K. 2011. First report of Cucurbit chlorotic yellows virus in cucumber, melon, and watermelon in China. *Plant Dis.* 95:73.
- Gyoutoku, Y., Okazaki, S., Furuta, A., Etoh, T., Mizobe, M., Kuno, K., Hayashida, S., and Okuda, M. 2009. Chlorotic yellows disease of melon caused by Cucurbit chlorotic yellows virus, a new crinivirus. *Jpn. J. Phytopathol.* 75:109-111.
- Hamed, K., Menzel, W., Dafalla, G., Gadelseed, A. M. A., and Winter, S. 2011. First report of Cucurbit chlorotic yellows virus infecting muskmelon and cucumber in Sudan. *Plant Dis.* 95:1321.
- Huang, L.-H., Tseng, H.-H., Li, J.-T., and Chen, T.-C. 2010. First report of Cucurbit chlorotic yellows virus infecting cucurbits in Taiwan. *Plant Dis.* 94:1168.
- Irwin, M. E., and Ruesink, W. G. 1986. Vector intensity: A product of propensity and activity. Pages 13-33 in: *Plant Virus Epidemics: Monitoring Modelling and Predicting Outbreaks*. G. D. McLean, R. G. Garrett, and W. G. Ruesink, eds. Academic Press, Sydney.
- Keshavarz, T., Shams-Bakhsh, M., Izadpanah, K., and Malboobi, M. A. 2013. Occurrence and genome analysis of *Cucurbit chlorotic yellows virus* in Iran. *J. Phytopathol.* 162:523-526.
- Kil, E.-J., Lee, J.-J., Cho, S., Auh, C.-K., Kim, D., Lee, K.-Y., Kim, M.-K., Choi, H.-S., Kim, C.-S., and Lee, S. 2015. Identification of natural weed hosts of *Tomato chlorosis virus* in Korea by RT-PCR with root tissues. *Eur. J. Plant Pathol.* 142: 419-426.
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870-1874.
- Li, J., Liang, X., Wang, X., Shi, Y., Gu, Q., Kuo, Y.-W., Falk, B. W., and Yan, F. 2016. Direct evidence for the semipersistent transmission of *Cucurbit chlorotic yellows virus* by a whitefly vector. *Sci. Rep.* 6:36604.
- Livieratos, I. C., Coutts, R. H. A., and Katis, N. 1998. Differentiation between *Cucurbit yellow stunting disorder virus* and *Beet pseudo-yellows virus* by a reverse transcription-polymerase chain reaction assay. *Plant Pathol.* 47: 362-369.
- Lotos, L., Efthimiou, K. E., Maliogka, V. I., and Katis, N. I. 2014. Generic detection of poleroviruses using an RT-PCR assay targeting the RdRp coding sequence. *J. Virol. Methods* 198:1-11.
- Okuda, M., Okazaki, S., Yamasaki, S., Okuda, S., and Sugiyama, M. 2010. Host range and complete genome sequence of *Cucurbit chlorotic yellows virus*, a new member of the genus *Crinivirus*. *Phytopathology* 100:560-566.
- Orfanidou, C., Maliogka, V. I., and Katis, N. I. 2014a. First report of *Cucurbit chlorotic yellows virus* in cucumber, melon, and watermelon in Greece. *Plant Dis.* 98:1446.
- Orfanidou, C. G., Dimitriou, C., Papayiannis, L. C., Maliogka, V. I., and Katis, N. I. 2014b. Epidemiology and genetic diversity of criniviruses associated with tomato yellows disease in Greece. *Virus Res.* 186:120-129.
- Orfanidou, C. G., Pappi, P. G., Efthimiou, K. E., Katis, N. I., and Maliogka, V. I. 2016. Transmission of *Tomato chlorosis virus* (ToCV) by *Bemisia tabaci* biotype Q and evaluation of four weeds as viral sources. *Plant Dis.* 100: 2043-2049.
- Orfilio, A., and Navas-Castillo, J. 2009. The complete nucleotide sequence of the RNA2 of the crinivirus *Tomato infectious chlorosis virus*: Isolates from North America and Europe are essentially identical. *Arch. Virol.* 154:683-687.
- Papayiannis, L. C., Brown, J. K., Seraphides, N. A., Hadjistyli, M., Ioannou, N., and Katis, N. I. 2009. A real-time PCR assay to differentiate the B and Q biotypes of the *Bemisia tabaci* complex in Cyprus. *Bull. Entomol. Res.* 99: 573-582.
- Roditakis, E., Grispou, M., Morou, E., Kristoffersen, J. B., Roditakis, N., Nauen, R., Vontas, J., and Tsagkarakou, A. 2009. Current status of insecticide resistance in Q biotype *Bemisia tabaci* populations from Crete. *Pest Manag. Sci.* 65:313-322.
- Rubio, L., Abou-Jawdah, Y., Lin, H.-X., and Falk, B. W. 2001. Geographically distant isolates of the crinivirus *Cucurbit yellow stunting disorder virus* show very low genetic diversity in the coat protein gene. *J. Gen. Virol.* 82:929-933.
- Sacristán, S., Fraile, A., and García-Arenal, F. 2004. Population dynamics of *Cucumber mosaic virus* in melon crops and in weeds in central Spain. *Phytopathology* 94:992-998.
- Sun, X. H., Gao, L. L., Wang, C. L., Wang, S. L., Zhao, J., Qiao, N., and Zhu, X. P. 2016. Natural occurrence of *Tomato chlorosis virus* in pumpkin in China. *Plant Dis.* 100:2543.
- Thresh, J. M. 1981. The role of weeds and wild plants in the epidemiology of plantvirus diseases. Pages 53-70 in: *Pests Pathogens and Vegetation*. J. M. Thresh, ed. Pitman Sci. Pub., London, UK.
- Tsagkarakou, A., Tsigenopoulos, C. S., Gorman, K., Lagnel, J., and Bedford, I. D. 2007. Biotype status and genetic polymorphism of the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) in Greece: Mitochondrial DNA and microsatellites. *Bull. Entomol. Res.* 97:29-40.
- Tugume, A. K., Mukasa, S. B., and Valkonen, J. P. T. 2016. Mixed infections of four viruses, the incidence and phylogenetic relationships of *Sweet potato chlorotic fleck virus* (Betaflexiviridae) isolates in wild species and sweetpotatoes in Uganda and evidence of distinct isolates in East Africa. *PLoS One* 11:e0167769.
- Tzanetakis, I. E., Martin, R. R., and Wintermantel, W. M. 2013. Epidemiology of criniviruses: An emerging problem in world agriculture. *Front. Microbiol.* 119: 1-15.
- Webster, C. G., Coutts, B. A., Jones, R. A. C., Jones, M. G. K., and Wylie, S. J. 2007. Virus impact at the interface of an ancient ecosystem and a recent agroecosystem: Studies on three legume-infecting potyviruses in the southwest Australian floristic region. *Plant Pathol.* 56:729-742.
- Wintermantel, W. M., Gilbertson, R. L., McCreight, J. D., and Natwick, E. T. 2016. Host-specific relationship between virus titer and whitefly transmission of *Cucurbit yellow stunting disorder virus*. *Plant Dis.* 100:92-98.