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ORIGINAL ARTICLE

Aphid-borne viruses infecting cultivated watermelon and squash in Spain: Characterization of a variant of cucurbit aphid-borne yellows virus (CABYV)

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Abstract

Aphid-borne viruses are responsible for major cucurbit diseases and hamper the sustainability of crop production. Systematic monitoring can reveal the occurrence and distribution of these viruses, in addition to unadvertised viruses, facilitating the control of diseases. For three consecutive (2018–2020) seasons, the presence of aphid-borne viruses was monitored from a total of 292 samples of watermelon and squash plants that showed yellowing symptoms in three major cucurbit-producing areas (Castilla La-Mancha, Alicante, and Murcia) in Spain. We observed that cucurbit aphid-borne yellows virus (CABYV) was the most common virus found (29%) in the plants from both crops. Likewise, except for squash samples from Castilla La-Mancha and Alicante, watermelon mosaic virus (WMV) was also found (23%) with a relatively high frequency. Furthermore, we observed the exacerbation of bright yellowing symptoms in watermelon plants that was often accompanied by considerable fruit abortion. CABYV was the only causative agent for this new yellowing disease, and two infectious cDNA clones (one from watermelon, CABYV-LP63, and another from melon, CABYV-MEC12.1) were constructed to further compare and characterize this CABYV disease. Based on the full-length genome, both isolates were grouped phylogenetically together within the Mediterranean clade. However, the Koch's postulates tests were only successfully completed for the LP63 isolate, which also showed several amino acid changes and two potential recombination events, as compared to MEC12.1. Remarkably, the LP63 isolate caused more severe symptoms and showed higher RNA accumulation than MEC12.1 in five cucurbit plant species. These results suggest that a novel CABYV variant that causes severe yellowing symptoms may be causing outbreaks in cucurbit crops.

KEYWORDS
aphid-borne plant virus, CABYV, cucurbits, WMV, yellowing diseases

1 | INTRODUCTION

Cucurbits are among the most important horticultural vegetables in the Mediterranean basin. However, the sustainability of their production is threatened by viral diseases that include at least 28 different viruses (Lecoq & Desbiez, 2012). Among them, the prevalence of aphid-borne viruses appears to be favoured not only because of the lack of effective countermeasures against plant viruses, but also by the increase of these crops in organic cultivation systems that may alter the vector population size, encouraging the incidence of these viral diseases. Thus, there is a need to perform systematic monitoring of the occurrence of viral diseases, as well as of their genetic structure and evolutionary epidemiology, which may facilitate the early detection of emerging diseases and control of viral diseases.

In particular, cucurbit aphid-borne yellows virus (CABYV, family *Luteoviridae*, genus *Polerovirus*) is one of the most prevalent viruses (Kassem et al., 2013; Mnari-Hattab et al., 2009). CABYV was initially described in France in 1992 (Lecoq et al., 1992), and subsequently it has been reported in cucurbit crops from Algeria, Greece, Italy, Iran, Lebanon, Spain, Tunisia, and Turkey (Juárez et al., 2004; Mnari-Hattab et al., 2009; Tomassoli & Meneghini, 2007). Most recently, it has also been reported in Germany (Menzel et al., 2020), Slovenia (Mehle et al., 2019), and Poland (Minicka et al., 2020). CABYV is especially spreading through the northern hemisphere where it is known to cause the most harmful epidemic viral diseases in agriculture (Lecoq et al., 1992). CABYV is transmitted primarily by two aphids, *Aphis gossypii* and *Myzus persicae*, and the transmission is considered to be circulative, persistent, and nonpropagative (Dogimont et al., 1996). In Spain, CABYV was first identified in 2004 in the Murcia region (southeastern Spain; Juárez et al., 2004). From that point on, it has spread to cucurbit crops, becoming highly prevalent in melon and zucchini crops in southeastern Spain (Juárez et al., 2013; Kassem et al., 2007).

Additionally, potyviruses, such as watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), and papaya ringspot virus (PRSV), as well as cucumber mosaic virus (CMV, genus *Cucumovirus*), among others, have been reported to affect cucurbit crops (Bertin et al., 2020; Desbiez et al., 2020; Juárez et al., 2013), with a relatively high importance in Spain (De Moya-Ruiz et al., 2021; Juárez et al., 2013). Furthermore, the number of multiple viral infections is comparatively high in cucurbit plant species in Spain, and CABYV and WMV are often found in mixed virus combinations (Juárez et al., 2013; Kassem et al., 2007, 2013). The sequencing of different CABYV isolates has identified four major groups: Asian or Chinese (C), Mediterranean (N), and Taiwanese (TW), with the phylogenetic analysis further distinguishing four clusters, including recombinant (R) groups (Knierim et al., 2010). Thus, CABYV populations are considered to be genetically structured. Further analysis of the open reading frames (ORF3; coat protein, CP) has shown that CABYV has a high nucleotide substitution rate (0.01 substitutions/site/year) (Pagán & Holmes, 2010). This could indicate a relatively high mutation rate as the source of genetic diversity. Additionally, it has been reported that the genetic diversity of *Polerovirus* populations can be prone to recombination amongst themselves or viruses belonging to other families (Knierim et al., 2010). These factors provide genetic plasticity that may allow for the rapid evolution and adaptation of viruses to new agricultural conditions and may therefore be contributing to the creation of new CABYV variants that could challenge the prevailing control strategies.

Heterogeneous host genotype populations (i.e., different host plants and varieties of cucurbits along with particular agroecological practices) may alter the epidemiological patterns of viral diseases (De Moya-Ruiz et al., 2021; Juárez et al., 2019; Valverde et al., 2020). Hence, the systematic monitoring of the occurrence of viral diseases and their causative agents, combined with ecological and quantitative epidemiological approaches, is essential to understand and mitigate their consequences on crops and natural ecosystems (Jeger, 2020; McLeish et al., 2020). In addition, the genetic and biological characterization of the emerging viruses increases our understanding of the ecological epidemiology of the diseases and could facilitate their early detection and prompt action for their control. Thus, the aim of this study was to increase our understanding of the occurrence and distribution of aphid-borne viruses that cause yellowing diseases in watermelon and squash crops. We monitored the occurrence of cucurbit aphid-borne viruses in these cultivated plants for three consecutive seasons in three major crop-producing areas. Additionally, we observed and further characterized an emerging CABYV variant that causes bright yellowing disease in watermelon plants. The construction of two CABYV infectious cDNA clones allowed us to assess the symptom expression and viral RNA accumulation in five cucurbit plant species in order to understand the impact of this novel CABYV variant on the cultivated cucurbits.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 292 apical leaf samples were collected from yellowing watermelon (*Citrullus lanatus*) and squash (*Cucurbita moschata*) plants grown in 73 different open fields: 32 plots in Alicante (38°03′26″N, 0°51′17″W), 27 in Murcia (37°43′20″N, 0°57′59″W), and 14 in Castilla La-Mancha (39°09′34″N, 3°18′48″W) during field inspections in the 2018, 2019, and 2020 seasons. In each plot, a single plant variety is normally grown; the watermelon plots included cultivars Boston, Fashion, Kasmira, Leonor, Rosalinda, and Style, and the squash plots included cv. Butternut. Four samples were collected from each plot and processed for total RNA extraction. All plant samples were stored frozen at −80 °C for further use.

2.2 | Cucurbit virus detection

The total RNA from the plant samples was extracted using TRI reagent (Sigma-Aldrich), and 1.5 μl was used for virus detection either by dot-blot hybridization or reverse transcription (RT)-PCR. Because

no differences were found between both techniques for the viral detection, we provide data from molecular hybridization. RNA from each sample was placed twice on five positively charged nylon membranes, and the RNA was fixed with a UV light crosslinker. A dot-blot molecular hybridization was carried out using specific RNA probes for CABYV, CMV, PRSV, WMV, and ZYMV detection (Kassem et al., 2007). The membranes were incubated overnight at 68 °C with the specific digoxygenin (DIG)-labelled probes. After the hybridization, a series of washes were carried out, followed by an incubation with the anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) and the chemiluminescent substrate CDP-Star (GE Healthcare) (Gómez-Aix et al., 2019). The membrane analyses were performed using a chemiluminescent detector Amersham Imager 600 (GE Healthcare Bio-Sciences). Furthermore, the watermelon samples with exacerbated yellowing symptoms were analysed by RT-PCR using generic polerovirus primers, in addition to specific primers (Table S1) to amplify conserved genomic fragments of CABYV, CMV, PRSV, WMV, and ZYMV. The PCR was performed with the NZYTaq II 2× Green Master Mix (Nzytech), with the following cycling conditions: 95 °C for 5 min; 30 cycles of 94 °C for 10 s, 50–60 °C for 30 s, and 72 °C for 90 s; with a final extension of 72 °C for 10 min before cooling to 4 °C.

2.3 | Full-length CABYV genome amplification and construction of CABYV infectious clones

One CABYV-positive sample from a watermelon with symptoms (2019) was randomly chosen to construct a full-length CABYV infectious clone. Concurrently, another CABYV isolate from a melon sample collected in 2014 was also cloned and sequenced for further comparison. Both full-length genome sequences were aligned with other CABYV isolates from the NCBI GenBank in order to examine the phylogenetic relationship among CABYV isolates. The cDNA synthesis of the CABYV genomes was carried out by using reverse transcriptase (Roche) with a specific primer CE-2513 5′-ACACCGAAACGCCAGGGGGAATC-3′. Then, full-length CABYV genomes were amplified with PCRBIO VeriFi Mix Red (PCR Biosystem) following the manufacturer's recommendations and using primers with an overlapping sequence from the pJL 89 vector (Blawid & Nagata, 2015). The primers were CE-2636 Fw 5′-CATTTCATTTGGAGAGGACAAAAGATACGAGCGGGTGATG-3′ and CE-2637 Rv 5′-ATGCCATGCCGACCCTAAAATCCACACCGAAACGCCAGGGGGA-3′ (vector sequences are underlined). The binary pJL 89 vector (c.4,700 bp) (Addgene) was amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific), and then both the vector and the full-length CABYV genome (c.5,600 bp) were purified from a 0.7% agarose gel with the GENECLEAN kit (MP Biomedicals). CABYV genomes were cloned into the binary pJL 89 vector using In-Fusion HD Cloning (Takara Bio) (Blawid & Nagata, 2015) following the manufacturer's protocol. After In-Fusion HD cloning, Stellar competent *Escherichia coli* cells (Clontech Laboratories) were transformed, plasmids were purified, and verified by using the *Eco*RV and *Sal*I restriction endonucleases. Each CABYV clone was named according to the reference sample: CABYV-LP63 (watermelon 2019) and CABYV-MEC12.1 (melon 2014).

2.4 | Sequencing and phylogenetic relationships of both CABYV isolates

Full-length genomes of both CABYV clones (LP63 and MEC12.1) were sequenced by using a set of eight internal primers (Table S2) and the Sanger method (STAB VIDA, Caparica, Portugal). Each primer was able to provide a sequence of up to 800 bp, matching with the contiguous genome sequence. All the contigs were assembled into a consensus sequence using Geneious Prime 2020 software. The complete nucleotide viral sequences of CABYV-LP63 and -MEC12.1 isolates were deposited in GenBank under the accession numbers MW051363 and MW051362, respectively. Then, the phylogenetic tree was constructed based on the complete genome sequences of CABYV-LP63 and -MEC12.1 isolates, along with 28 reported CABYV sequences in the GenBank database, using MEGA X software (Stecher et al., 2020) with 1,000 bootstrap replicates under default settings. The pairwise nucleotide identities (%) of each gene and complete CABYV genomes were determined using Simplot v. 3.5.1 (Systat Software). Plots of nucleotide identity were obtained with the CABYV-Sq/2003/7.3 genome as the query sequence and a sliding window of 200 nucleotides. Potential recombination breakpoints and events among CABYV isolates were analysed using RDP4 (Martin et al., 2015). CABYV sequences of the LP63 and MEC12.1, as well as the sequences available in the NCBI GenBank and melon aphid-borne yellows virus (MABYV) were aligned in MEGA v. 7.0.1 software, and exported to the RDP4 program, which performs different analytical methods; RDP, GENECONV, Chimaera, MaxChi, BOOTSCAN, and SISCAN, which are implemented with different assumptions. The program was used using the default settings and a Bonferroni corrected p value = 0.01. Only recombination events detected by four or more methods were considered as significant.

2.5 | Agroinoculation of cucurbit plant species

Agrobacterium tumefaciens C58C1 was transformed with the purified plasmids that contained either LP63 or MEC12.1 isolates. The cultures were incubated overnight at 28 °C, centrifuged, and resuspended in the same volume of MES buffer (Gómez et al., 2009). Two groups of *Nicotiana benthamiana* plants were agroinoculated with the CABYV-LP63 and -MEC12.1 clones, and the viral infection was verified after 21 days post-agroinoculation (dpai) by symptom expression and PCR detection. Then, after both CABYV clones were successfully tested, 12 healthy plants of five cucurbit species: melon (cv. Piel de Sapo), zucchini (cv. Black Beauty), cucumber (cv. Marketer), squash (cv. Butternut), and watermelon (cv. Sugar Baby), were agroinoculated with CABYV-LP63 and -MEC12.1 clones, including three mock-inoculated plants per group of plant species.

The inoculations were carried out in the leaves of the cotyledons 2 weeks after potting. The plants were grown in a greenhouse (16 hr photoperiod and 24 °C in a day/night cycle). Note that virus-like symptoms suggestive of CABYV infection were observed during the experiment in older leaves, and in watermelon plants, LP63 symptoms were only observed after 31 dpai. Thus, to ensure Koch's postulates with this LP63 isolate, another group of watermelon plants was inoculated and maintained for a longer term, and the yellowing severity symptoms were confirmed at 50 dpai.

2.6 | Viral load quantification

All leaves above the cotyledons were harvested in groups of three replicated plants for each group of plant species and virus infection at 6, 12, 21, and 31 dpai. All material was ground in a mortar using liquid nitrogen. Total RNA was extracted using TRI reagent, purified by phenol-chloroform extraction, and treated with DNase I (Sigma-Aldrich). Thereafter, CABYV viral load was estimated by measuring the viral RNA accumulation via real-time quantitative RT-PCR (RT-qPCR) with an AB7500 System (Applied Biosystems) using the One-step NZYSpeedy RT-qPCR Green kit, ROX plus (NZYTech). Two specific primers, CE-2879 Fw 5′-GAGAGCCCAGCATTCAGC-3′ and CE-2880 Rv 5′-TGCAGTGGGGGTCCAA-3′, were designed to target the CP region (3,805–3,941 nucleotides), and their specificity was monitored with melting curve analysis. The reaction mix was prepared according to the manufacturer's instructions (NZYTech), using 2 µl of RNA (70 ng/µl). Both non-template controls and mock-inoculated plants were included to ensure product-specific amplifications and the absence of primer-dimers. The CP gene fragment (c.598 nucleotides) from MEC12.1 isolate was cloned into pGEM T-Easy vector by using the CE-9 and CE-10 primers (Table S1) in order to synthesize viral RNA from a plasmid. After linearizing the plasmid with *Sph*I and RNA transcription using SP6 RNA polymerase, the RNA transcripts were quantified twice with a Qubit 3.0 fluorometer following the manufacturer's instructions (Thermo Fisher Scientific). The initial RNA concentration was used in serial dilutions (10-fold) to generate external standard curves for RT-qPCR from the CP gene. From each sample, the RNA concentration (ng of viral RNA per 100 ng of total RNA) was estimated by plotting the cycle threshold (C_t) values from each biological assay (n = 3, at each time point) with three experimental replicates for each biological replicate.

2.7 | Statistical analysis

The analysis of the viral load for each plant species was performed with a general linear model (GLM). Values from each plant were independent among treatments, data were transformed with a logarithmic function to meet the assumption of normality and homoscedasticity of variance. We thus fitted the viral isolate type, the five cucurbit plant species, and the time of infection (dpai) as three-factor fixed effects, including replicates as random effects (REML). For the analysis of each CABYV clone effect, virus accumulation from each plant species was considered separately and analysed using one-way repeated-measures analysis of variance (ANOVA). All analyses were performed with JMP v. 9 software (SAS institute). Plot graphs of the viral RNA accumulation for each isolate and plant species were drawn using R v. 4 software (R Core Team).

3 | RESULTS

3.1 | Occurrence of aphid-borne viruses in watermelon and squash plants

In general, CABYV was the most common virus found in the plants showing yellowing symptoms from watermelon and squash crops (Figure 1). Overall, CABYV was detected in 29% of the samples, WMV in 23%, with the low occurrence of PRSV and ZYMV (<3%) in both species, while CMV was not detected. In watermelon, CABYV was detected in 14% of the samples from Castilla La-Mancha, 21% from Alicante, and 36% from Murcia, while WMV was detected in 14% of the samples from Castilla La-Mancha, 37% from Alicante, and 36% from Murcia (Figure 1a). For squash, CABYV appeared to occur in 50% of the samples from Castilla La-Mancha in 2019, ranging between 25% and 50% for Alicante, and 67% for Murcia in 2020. WMV was detected in 13% of the squash samples from Alicante in 2019, and in 25% for Murcia in 2018 (Figure 1b). Watermelon crops from the three locations exhibited mixed infections, with the combination of CABYV + WMV being commonly (8%–32%) found. ZYMV was undetected as a single infection in watermelons, and the combination of CABYV + ZYMV was recorded (8%) but only in Murcia in 2019. In squash, ZYMV was found in 33% of the samples as single infections in Murcia in 2020, and in mixed infections (with PRSV) in 13% of the plant samples from Alicante in 2019.

3.2 | Field symptoms and genetic characterization of a novel CABYV variant

During the first inspection (2018) of watermelon crops, we observed plants with an exacerbated yellowing symptom that had a patchy distribution in the field (Figure 2a). The plants showing leaf yellowing symptoms with intensive variegated green mosaics and vein clearing (Figure 2b,c) were combined with blistering and abortion of the fruits (Figure S1). Only CABYV was detected as a causal agent by dot-blot hybridization and confirmed by RT-PCR. Similar diseased plants were observed during the following inspections, and one watermelon sample with symptoms from 2019 season was selected randomly. The phylogenetic tree showed that isolates LP63 and MEC12.1 grouped together with previously reported CABYV isolates from Spain belonging to the Mediterranean clade (Figure 3a). Likewise, the nucleotide sequence analysis of both full-length LP63 and MEC12.1 genomes showed a 96% similarity, varying between 95% and 99% of similarity between each open reading frame (ORF).

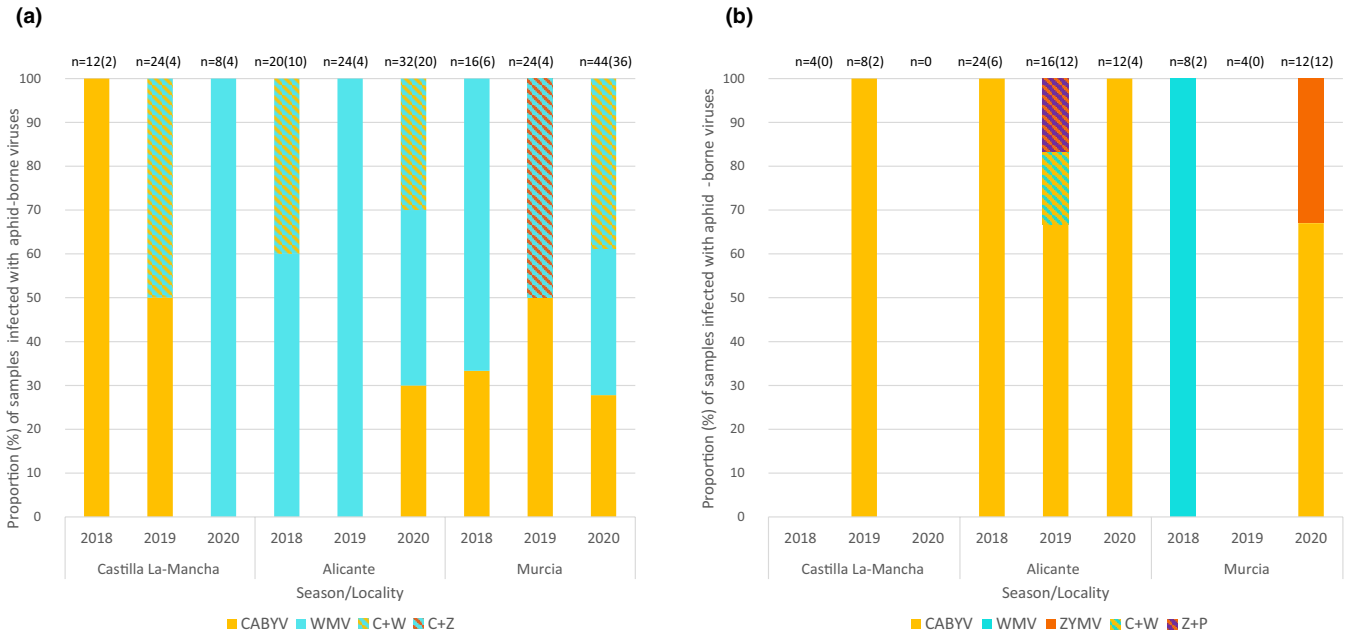

FIGURE 1 Proportion of aphid-borne viruses detected from watermelon (a) and squash plants (b) showing yellowing symptoms. Four samples with symptoms were collected from different plots in three cultivation areas (Castilla La-Mancha, Alicante, and Murcia) for three seasons (2018–2020). Two replicates per leaf sample were tested by dot-blot hybridization and the number of leaf samples tested (and positive samples) is indicated at the top of each bar. Tested RNA aphid-borne viruses included: CABYV, CMV, PRSV, WMV, and ZYMV [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Virus symptoms observed in watermelon fields. Yellowing watermelon plants (a,b), and older leaves showing the yellowing and interveinal chlorotic mottling (c) [Colour figure can be viewed at wileyonlinelibrary.com]

ORF0 had the highest level of similarity, followed by ORF1, ORF3, and ORF4 that shared a 97% similarity. However, ORF2 had 96%, and ORF3a and ORF5 95% nucleotide similarity. Accordingly, the highest amino acid similarity between both isolates was found in ORF0 (99%), followed by ORF3 and ORF5 (98%), and then by ORF3a and ORF1 (97%), ORF2 (96%), and ORF4 with the lowest amino acid similarity (93%) recorded, suggesting the lack of any frameshift mutation.

We then explicitly carried out an analysis of the number of mutations for each ORF that were exclusive to the MEC12.1 and LP63 genomes, compared to two other full-length genomes sourced from GenBank (JF939812 and JF939814) that also belonged to the Mediterranean subpopulation (Table 1). We found 87 positions with single nucleotide polymorphisms (SNPs) in the LP63 genome, with 58 and 29 synonymous and nonsynonymous SNP mutations, respectively. In the MEC12.1 genome, we found 79 SNPs, with 55 synonymous and 24 nonsynonymous SNP mutations. This indicated a moderate genetic variability in both isolates, although interestingly, ORF2 (replication-associated protein) from LP63 had a higher number of nonsynonymous mutations as compared to the MEC12.1 isolate (17 versus 5), as well as to the rest of the ORFs (Table 1). Furthermore, the detection of evidence pointing to recombination was attempted, using the full-length genomes of both CABYV-LP63 and -MEC12.1 isolates, including 28 sequences used for phylogenetic analysis. This recombination analysis suggested that CABYV-LP63 may have originated from two breakpoints in the P0-P1 region (40–1,068 nucleotides), and regions P1, 2, and 3a (1,069–3,464 nucleotides). In the first potential recombination event, all the methods used significantly identified ($p < 0.01$) the sq/2004/1.9 isolate as the major parent and the MEC12.1 isolate as the minor parent. In the second recombination event, the methods identified the MEC12.1 isolate as the major parent and an unknown minor parent (Figure 3b).

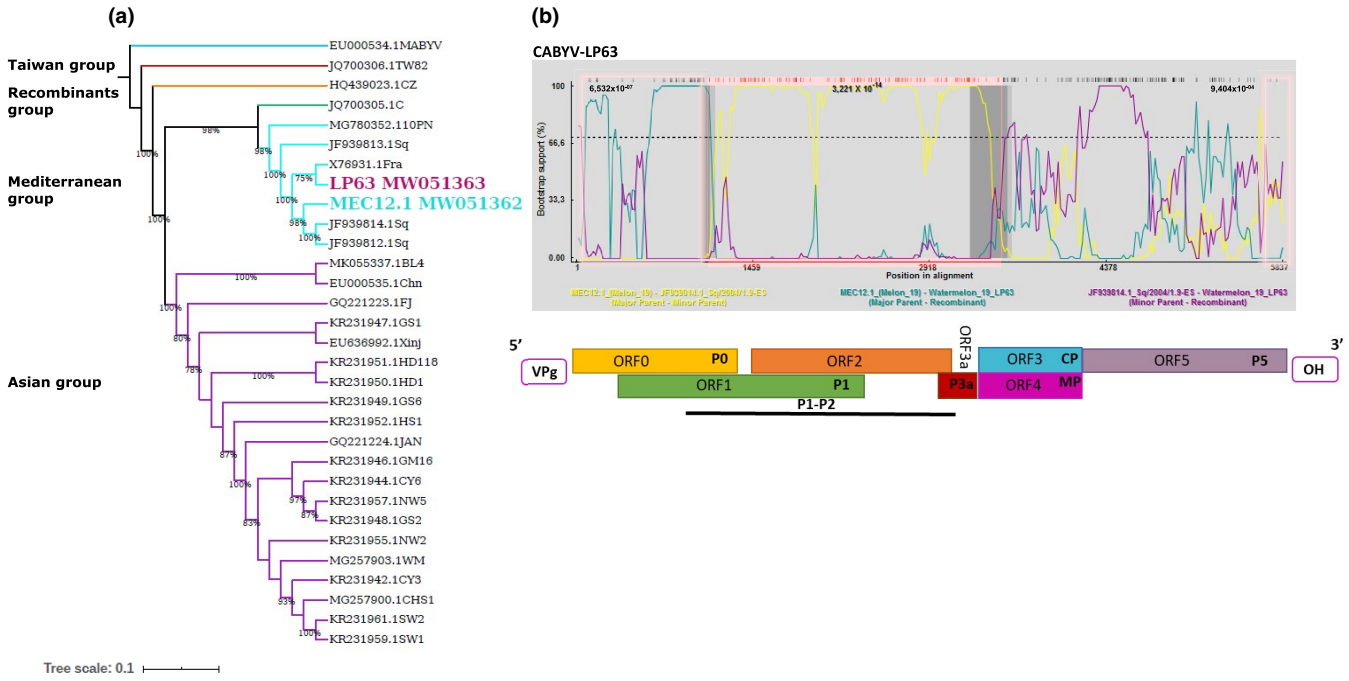

FIGURE 3 (a) Phylogenetic relationships between CABYV isolates. The evolutionary history was inferred by using the complete genome sequences of the MEC12.1 and LP63 isolates, including the full-length CABYV genome sequences of another 28 isolates retrieved from the NCBI GenBank database (referenced according to their accession number), and performing the maximum-likelihood (ML) method. The evolutionary distances were computed using the maximum-composite likelihood method with 1,000 bootstrap replications and using the TIM2 + F + I + G4 model assigned by IQ-Tree based upon the Bayesian information criterion (BIC) minimal score. The ML phylogenetic tree was inferred using ITol v. 5. The tree was rooted with the complete sequence of MABYV (melon aphid-borne yellows virus; EU000534). The genetic distances are shown as the lengths of the branches, and only branches with bootstrap values >70% are shown. The scale bar indicates 0.1 nucleotide substitutions per site. Four phylogenetic groupings are indicated by different colours: Asian (purple), Taiwan (red), Mediterranean (light blue), and Recombinants (orange and green). (b) Recombination analysis between CABYV isolates. The recombination analysis of the LP63 isolate was performed by RDP4 using the full-length genome sequence, showing the potential breakpoints in P0-P1 and P1,2-3a [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Amino acid changes through each open reading frame (ORF) from the complete genomes of CABYV-MEC12.1 and -LP63 isolates

	Isolate	
ORF	MEC12.1	LP63
ORF0	I146 M	
ORF1	F106C, P290S, K544E	Q432 K, T505I, K546R, S547P
ORF2	F106C, P290S, H542Y, E544G, T888I	Q432 K, E442D, G519S, N520D, P527S, E546 K, V547 T, T560A, A677 V, K724Q, V7470D, E753D, A829 T, E887 K, E1016D, I1019 V, A1023 T
ORF3	P21L, D25E, I33 V	
ORF3a	A33S	
ORF4	E7G, A15 V, T16S, P145L, R189Q	V143G, M169 T
ORF5	P21L, D25E, I33 V, V353A, E453A, S457P	T213A, S455P, D460E, S466F, K472E, T545A

Note: Each amino acid change is based on nonsynonymous mutations that are unique and different from the other CABYV Mediterranean isolates (JF939812 and JF939814) at the same genome position.

3.3 | Comparison of symptom expression and viral accumulation of the CABYV isolates

To examine whether genetic differences of the CABYV-LP63 isolate were attributed to biological differences in cucurbit plants, a group of cultivated plant species (cucumber, melon, squash, watermelon, and zucchini) were independently agroinoculated with either CABYV-LP63 or -MEC12.1 clones. At 14 dpai, all infected plants showed symptoms in the inoculated leaves, with the subsequent expression of yellowing symptoms in older leaves. It was striking that

the yellowing symptom varied according to the CABYV clone infection and plant species. The yellowing symptom was more severe in the CABYV-LP63 infected plants than -MEC12.1 (Figure 4) after approximately 30 dpai. In particular, watermelon, zucchini, and melon leaves of the infected plants displayed yellowing and interveinal chlorotic mottling symptoms that were stronger in the LP63- than the MEC12.1-infected plant. The LP63 infection was also accompanied by a marked fruit abortion in the watermelon plants (Figure S1). All cultivated plant species were steadily infected by LP63 and MEC12.1. The viral load increased up to 21 dpai, with a moderate decline observed at 31 dpai (time effect; $F[1, 98] = 39.26$, $p < 0.001$, Figure 5). The viral RNA load of the CABYV-LP63 clone was significantly higher than that of MEC12.1 in all plant species (CABYV clone effect; $F[1, 98] = 246.61$, $p < 0.001$), but the load was different according to plant species; cucumber and watermelon plants had the highest viral load values (plant species effect on LP63 accumulation; $F[4, 48] = 5.60$ $p < 0.001$). However, the viral RNA level of the CABYV-MEC12.1 clone was similar among the plant species (species plant effect on MEC12.1 accumulation; $F[4, 48] = 0.356$, $p < 0.838$). The analysis indicates that the LP63 viral load was higher than that of MEC12.1 for each plant species.

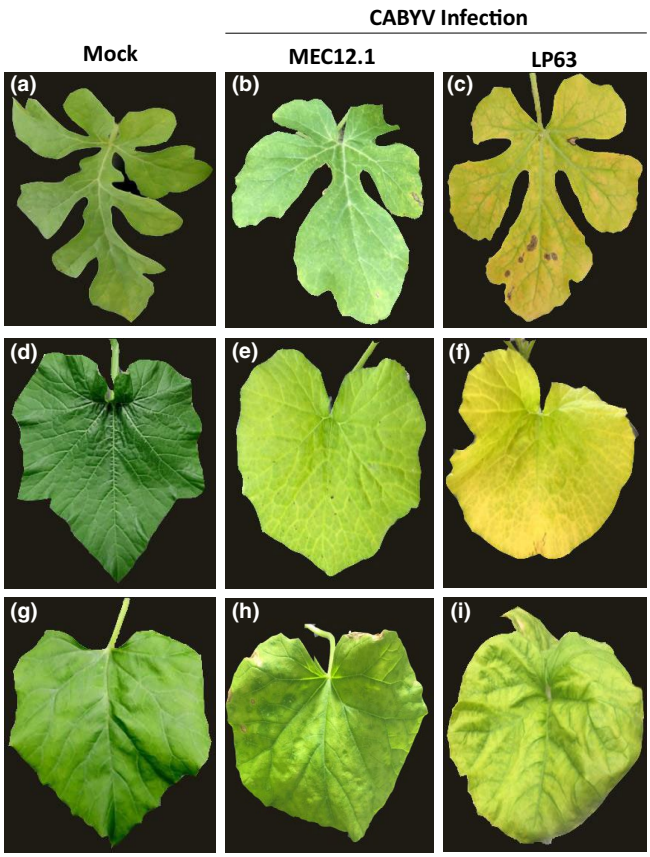

FIGURE 4 Mock-inoculated plants of watermelon (a), zucchini (d), and melon (g). CABYV symptoms induced by the inoculation of MEC12.1 in watermelon (b), zucchini (e), and melon (h) plants, and the inoculation of LP63 in watermelon (c), zucchini (f), and melon (i) plants. All plants were grown in a greenhouse at 24°C and symptom expression was evaluated at 31 days post-agroinoculation [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Cucurbit crops are economically important, and Spain is one of the main producers of cucurbits in Europe, with around 83,000 ha under cultivation and cucurbit production of approximately 4 million tonnes (MAPAMA, 2020). This production can be seriously threatened by viral diseases. In our assessment of the current status of aphid-borne viruses in watermelon and squash samples showing yellowing symptoms, CABYV was the most common pathogen in

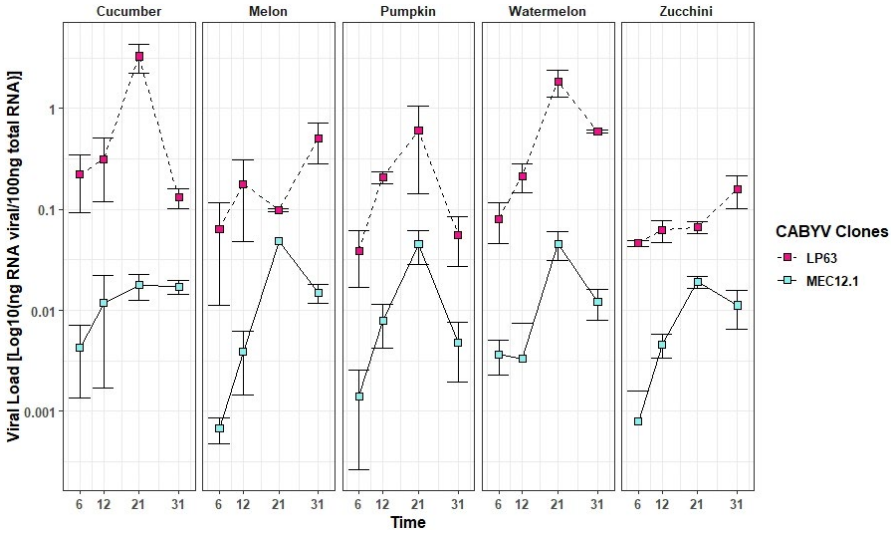

FIGURE 5 Viral load (mean and *SE* error bars, $n = 3$) of two CABYV infectious clones (LP63, dark-pink squares; MEC12.1, light blue squares) in five different cucurbit plant species during a time course experiment of 31 days. Viral accumulation of each clone was inferred by quantitative reverse transcription PCR. RNA transcripts of the coat protein (CP) gene were used in serial dilutions (10-fold) to generate external standard curves. RNA concentration in each sample (ng of viral RNA per 100 ng of total RNA) was estimated from the cycle threshold (C_t) values obtained from each independent biological assay, with three biological replicates at each time point [Colour figure can be viewed at wileyonlinelibrary.com]

both crops in the three major producing areas in Spain. This result is in agreement with previous studies conducted in southeastern Spain (Juárez et al., 2013; Kassem et al., 2007, 2013) that confirmed CABYV as the most prevalent aphid-borne virus in these crops. CABYV seems to be widespread throughout the Mediterranean basin and Europe (Desbiez et al., 2020; Mehle et al., 2019; Menzel et al., 2020; Minicka et al., 2020; Mnari-Hattab et al., 2009). In fact, CABYV appears to be the most common virus affecting cucurbit crops across different climatic regions, temperate, Mediterranean, and subtropical (Lecoq et al., 1992). This high occurrence and prevalence of CABYV could be linked to either cultivated or wild plant species that may provide the source of inoculum for spread, in addition to the aphid-associated transmission, because CABYV is transmitted in a persistent and circulative manner by at least two aphid species, *A. gossypii* and *M. persicae* (Kassem et al., 2013; Lecoq et al., 1992). Furthermore, it has been reported that the aphid feeding behaviour can be influenced by CABYV-infected plants in a manner in which virus acquisition is stimulated, and viruliferous aphids also prefer to settle on healthy plants (Carmo-Sousa et al., 2016). Therefore, it is quite plausible that the prevalence of CABYV observed in those cucurbit crops could be explained by the lack of CABYV-resistant cucurbit species, along with the potential presence of wild plants near the cultivation fields, and seasonally overlapping cucurbit crops systems, in addition to the role of CABYV in the aphid-mediated transmission.

Similarly, WMV was found in several samples of diseased watermelon plants. However, WMV was only detected in Murcia in 2018 in squash crops. This differential presence was unexpected, as the squash fields were often located close to watermelon fields, and the aphid populations can feed on both crops. Additionally, ZYMV occurrence was very low and CMV was undetected in both plant species. The occurrence of WMV and the other viruses could be explained in part by reasons similar to those for CABYV. First, differences in the viral host range may constrain opportunities, and hence the abundance of host diversity, including alternative wild plants, may have contributed to the occurrence of the viruses evaluated in this study. Secondly, it is likely that the use of commercial cultivars that are resistant/tolerant to potyviruses may be constraining these potyviral populations, at least in squash crops. Thirdly, it is also worth mentioning that we sampled plants showing yellowing virus-like symptoms, which may introduce a bias considering that the collection of samples was carried out during late seasons and the vegetative status of the crop may influence the misinterpretation of plant symptoms, such as yellowing associated to possible plant nutritional deficiency. Also, symptom expression can differ among cultivated species, plant cultivar, growing conditions, or even the presence of pathogen affecting the same plant. Finally, the vector transmission rate must be considered and further investigated. On the one hand, WMV is transmitted by at least 35 aphid species in a nonpersistent manner (Desbiez & Lecoq, 2008), and this could be favouring the occurrence of WMV in those yellowing plants. On the other hand, although the impact of aphid-vector performance on the ecology and evolution of CABYV and WMV in mixed infections is still unexplored, it is worth mentioning that mixed-infection melon plants, infected with WMV and cucurbit yellow stunting disorder crinivirus (CYSDV), may prompt ecological advantages that allow for the coexistence of both viruses in the field (Domingo-Calap et al., 2020). Thus, it is likely that this combination of CABYV and WMV may be influencing physiological and chemical plant changes, with an effect on the vector behaviour that supports the occurrence and distribution of these aphid-borne virus diseases in cucurbit crops (Carmo-Sousa et al., 2016; Mauck, 2016; Schoeny et al., 2020).

Multiple infections are more common in nature than what would be expected to occur by chance (Alcaide et al., 2020; Moreno & López-Moya, 2020; Syller, 2012). We observed that the frequency of the combination of CABYV + WMV varied between 8% and 32% of the infected plants, depending on the species and cultivation area. This combination of CABYV + WMV infections has also previously been described in the Mediterranean basin (Desbiez et al., 2020; Juárez et al., 2013; Kassem et al., 2007). The epidemiological consequences of mixed virus infections are unclear and seem to be contingent upon agroecological factors (Alcaide et al., 2020; Moreno & López-Moya, 2020). Thus, the presence or absence of mixed infections is difficult to explain. Given that aphid vectors are also polyphagous pests, feeding in a gregarious way on cultivated and wild plants, one could speculate that the occurrence of mixed virus infections may depend on the plant cropping systems, as different cultivated plant species are often overlapped spatiotemporally, and this could favour virus dispersal within and between crops. It is thus that mixed infections are gaining considerable significance in crop cultivations, and further research is required.

Indeed, our first observation of diseased watermelon plants with an exacerbated yellowing symptom made us suspect that a mixed viral infection could be the causal agent. However, it appeared that only CABYV was present. Hence, we characterized a novel CABYV variant (LP63). Its phylogenetic inferences showed that this isolate grouped within the Mediterranean clade, despite it showing more severe symptoms and higher RNA accumulation than MEC12.1. The genetic diversity of CABYV populations may be associated with specific hosts and/or geographic origin, having Spanish and Chinese isolates phylogenetically closer than other isolates from Italy, France, or Tunisia (Mnari-Hattab et al., 2009). In this study, both CABYV isolates were phylogenetically closely related to the Mediterranean clade. However, it should be taken into consideration that different subgroups within the CABYV populations have been previously reported from a study on Spanish CABYV isolates (sampled in 2003–2005; Kassem et al., 2013). The nucleotide sequence analysis for both CABYV isolates revealed that the ORF2 genomic region exhibited a higher substitution of nonsynonymous mutations of the LP63 isolate, suggesting a positive selection. Yet, recombination signals were detected in the LP63 isolate, pointing to the presence of CABYV recombinants in its Mediterranean populations. At present, several polerovirus recombinant isolates, including CABYV isolates, have been defined to be agronomically important (Costa et al., 2020; Knierim et al., 2010), and this demands further research on the genetic variability of CABYV populations in cucurbit crops.

The construction of the two full-length infectious cDNA clones and the inoculation experiment revealed that the CABYV plant infection was complete for each isolate in all cucurbit plant species. In fact, we sought to test Koch's postulates in the host where symptoms were observed, and this test was successfully completed for the CABYV-LP63 isolate, verifying the involvement of LP63 on the development of the new yellowing disease in watermelon plants. Likewise, all CABYV-LP63-infected plants showed more severe symptoms and higher viral RNA accumulation as compared to CABYV-MEC12.1-infected plants. Furthermore, the five infected cucurbit plants showed different symptoms. Remarkably, watermelon plants infected with LP63 showed severe yellowing and interveinal chlorotic mottling symptoms accompanied by a marked rate of fruit abortion. However, it is worth mentioning that this new CABYV symptomatology was not observed either in Castilla La-Mancha or in Alicante during the last field watermelon inspection in the 2020 season. This indicates a temporal variation that may be a consequence of agroecological, environmental, and/or biotic factors shaping the incidence of this disease. In our agroinoculation experiment, the analysis of the viral load revealed that CABYV-LP63 had a higher fitness than -MEC12.1 in all cucurbit plants. In particular, LP63 accumulation peaked at 21 dpai in cucumber and watermelon plants, with a subsequent decline. This LP63 accumulation was in agreement with the symptom expression that was more severe with the higher viral RNA accumulation. Because the level of LP63 accumulation in cucurbit plants was higher than MEC12.1, it is likely that this novel variant has a competitive advantage in cucurbit crops, with new outcomes on the CABYV spread and distribution, especially in areas of watermelon production. In this sense, the occurrence of CABYV and genetic diversity of the viral populations needs to be further investigated.

In conclusion, despite the extent of this new yellowing disease in watermelon and other cucurbit crops being unknown, our findings suggest that this novel CABYV variant may be currently threatening cucurbit crops. It is thus fundamental to consider that plant viral epidemics are often initiated and spread through different host plants and varieties, along with different agroecological practices that may alter the viral disease epidemiology (Moya-Ruiz et al., 2021; Jeger, 2020; Juárez et al., 2019; Valverde et al., 2020). This exchange of viral diseases between overlapping crops at spatial/temporal scales could play an important role, as it may hinder the early detection of the disease. Therefore, there is a need to establish a comprehensive detection programme to monitor the occurrence, distribution, and genetic diversity of CABYV populations in different cultivated and wild plant species to facilitate the design and implementation of control measures against this disease.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in DIGITAL-CSIC at https://digital.csic.es, reference number http://dx.doi.org/10.20350/digitalCSIC/13757.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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