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BRIEF REPORT

Construction of an infectious full-length cDNA clone of a recombinant isolate of cucurbit aphid-borne yellows virus from Brazil

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Abstract

In Brazil, the main viral disease of melon plant is severe yellowing disease called "Amarelão do Meloeiro," and a polerovirus, cucurbit aphid-borne yellows virus (CABYV) was considered one of the etiological agents. This virus is a recombinant strain originated from CABYV and unknown polerovirus. Due to unsuccessful mechanical inoculations of CABYV to host plants, the study of its biological characterization is hampered. Therefore, an infectious clone of the recombinant strain of CABYV was constructed using the Gibson Assembly technology. The full-length cDNA clones produced in this study showed to be infectious in three cucurbit species; melon (*Cucumis melo*), squash (a hybrid of *Cucurbita maxima* × *C. moschata*), and West Indian gherkin (*Cucumis anguria*) plants, but not in watermelon, cucumber, and zucchini plants. This insusceptibility of watermelon plants to the infectious clone corroborates the observation that this virus was never found in watermelon plants often located next to the infected melon plants. This infectious clone provides important tools for future study in developing resistant melon variety to CABYV infection.

Keywords *Cucumis melo* · CABYV · Infectious clone · Gibson Assembly · Cucurbit whitefly-borne yellows virus

Cucurbit aphid-borne yellows virus (CABYV) is a positive-sense single-stranded RNA virus belonging to the *Polerovirus* genus of the *Solemoviridae* family [1]. In Brazil, the occurrence of CABYV has been reported in passion fruit plants showing multi-symptom, such as blisters, mosaic, and leaf deformation [2]. Later, a totally different type of CABYV isolate infecting melon plants, showing severe yellowing disease (called "Amarelão do meloeiro" in Brazil), was reported [3]. This isolate was recombinant one in 3′ region including the capsid protein (*cp*) and readthrough domain (*rtd*) protein genes (i.e., P3/P5 region). The distribution of this recombinant isolate expands from Ceará to Bahia states in Brazil, showing the establishment of this recombinant strain in melon fields widely in Brazil. In addition, this virus was very peculiar polerovirus, and hence its transmissibility by whitefly was reported later [4]. Thus, the name of "cucurbit whitefly-borne yellows virus" (CWBYV) was suggested for this recombinant isolate [4].

To date, there are no efficient management strategies available against this disease. There are still no melon-resistant varieties to this recombinant strain of CABYV. To develop the resistant or immune melon variety, virus inoculation to test plants is essential; however, our trials of mechanical inoculation of CABYV to host and test plants failed [3]. Thus, the full-length cDNA of the recombinant strain of CABYV was constructed to have inoculum source and for reverse genetic study.

The CABYV-M3 isolate, collected in Icapuí county, Ceará state, Brazil [3] was used to prepare cDNA fragments of the genomic RNA of the CABYV recombinant strain. The total RNA, from the infected leaves stored at freezer – 70 °C, was extracted using silica resin-based protocol according to Rott and Jelkmann [5]. The cDNA of the complete genome of the M3 isolate was amplified dividing into two fragments, the 5′ and 3′ regions with an

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overlapping region of 20 nts, by RT-PCR. At first, cDNA was synthesized with SuperScript IV reverse transcriptase (Thermo Fisher Scientific, Waltham, USA) using the specific primer which anneals the genomic 3′ end (CABYV_Infectious_pJLRz_Rev: 5′ GGA GAT GCC ATG CCG ACC CAC ACC GAA ACG CCA GGG 3′) (Fig. 1a). However, the consequence PCR amplification with primer pair (CABYV_Infectious pJLRz_Rev and CABYV_3325_For: 5′ TCC ATT CTC AAT GAA TTG CGG TC 3′) was not successful in many trials. With the hypothesis that the highly stable secondary structure in the 3′ end region of the genome disturbs the PCR, the cDNA synthesis method was changed. Before reverse transcription reaction, the poly(A) tail was added to the extracted RNA using poly(A) polymerase (New England Biolabs, Ipswich, USA), then cDNA was synthesized using oligo dT_{50} primer [3]. With this cDNA, almost half of the genome from the 3′ end was amplified using LongAmp Taq DNA polymerase (New England Biolabs) and specific primers (CABYV_Infectious pJLRz_Rev and CABYV_3325_For). To amplify the 5′ region of the viral genome, cDNA was synthesized with random primers using SuperScript IV reverse transcriptase and PCR was performed using CABYV_Infectious_35s_For (5′ GTT CAT TTC ATT TGG AGA GGA CAA AAG ATA CGA GCG GGT G 3′) and CABYV_3348_Rev (5′ AGA CCG CAA TTC ATT GAG AAT G 3′) primers. These cDNA fragments were cloned into pCR4 TOPO plasmid vector (Thermo Fisher Scientific) in *Escherichia coli* DH10B strain (Thermo Fisher Scientific) and cultured at 28 °C for up to 2 days. The plasmids were purified using Wizard Plus SV Minipreps

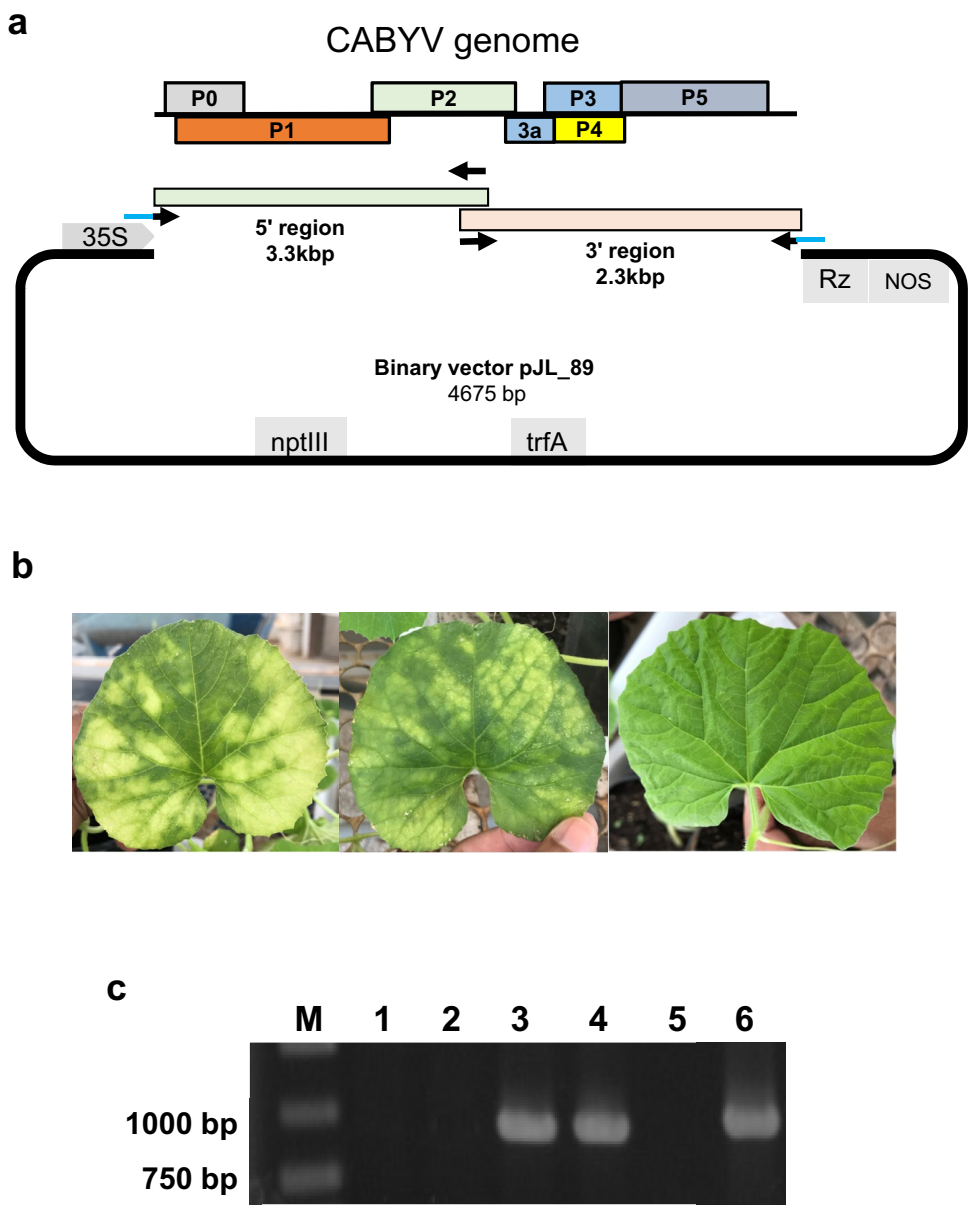

Fig. 1 Genomic map, symptom, and electrophoresis of amplicons of recombinant strain of CABYV. **a** Genomic map and the scheme of RT-PCR and cloning. *3a* P3a, *35S* cauliflower mosaic virus 35S duplicated promoter, *Rz* HDV ribozyme, *NOS* nopaline synthase terminator, *nptIII* neomycin phosphotransferase III, *trfA* replication-initiation protein. **b** Symptoms in melon plants infiltrated with CABYV-M3 clone at 15 dpi. Chlorosis caused by CABYV-M3a clone (left), CABYV-M3b clone (middle), and control plant (right). **c** Amplicons observed agarose gel electrophoresis. The expected size is 1066 bp. *M* marker, 1 Kb DNA ladder (Thermo Fisher Scientific); wells 1 and 2, agroinfiltrated plants with empty vector; wells 3 and 4, agroinfiltrated plants with CABYV-M3a and CABYV-M3b, respectively; wells 5 and 6, negative and positive controls, respectively

DNA Purification Systems (Promega, Madison, USA) and sequenced by Sanger method for the insert cDNA confirmation. The selected clones were re-amplified by PCR to join the two fragments into one using "Gibson Assembly" protocol [6]. The binary vector backbone of pJL89, containing dual promoter of cauliflower mosaic virus 35S and hepatitis delta virus ribozyme prior to nopaline synthase terminator, was prepared by PCR using a primer pair of pJL89_Gibson_rev (5′ CCT CTC CAA ATG AAA TGA ACT TCC 3′) and pJL89_Gibson_for (5′ GGG TCG GCA TGG CAT CTC 3′). These three fragments share the overlapping sequences of 20 nts in both 5′ and 3′ ends to join with Gibson Assembly (Fig. 1a). Again, the insert of the selected two plasmids were totally sequenced by Sanger method. *Agrobacterium tumefaciens* strain GV3101:pMP90 was transformed with the CABYV-M3 constructs.

For the first trial to evaluate the infectivity of CABYV-M3 clones, two CABYV clones in *A. tumefaciens* cells named CABYV-M3a and CABYV-M3b were agroinfiltrated according to Blawid and Nagata [6] in five plants (seedlings with cotyledons and one true-leaf stage) of *Cucumis melo* (cv. Goldex, which is a susceptible variety for this virus) for each clone. As a control, *A. tumefaciens* containing pJL89 vector without insert was agroinfiltrated. After 7 to 10 days post infiltration (dpi), agroinfiltrated plants with the CABYV-M3a and M3b clones showed symptoms of interveinal chlorosis in the inoculated leaves. At 30 dpi, melon leaves showed strong interveinal chlorosis (Fig. 1b). Control plants were asymptomatic (Fig. 1b). The upper leaves (not-infiltrated) from five inoculated plants with CABYV-M3a or CABYV-M3b clones were prepared as pooled samples separately for RNA extraction and RT-PCR detection using specific primers, CABYV_2663 For (5′ AAC GCC CTC GGA ATT GAT CAC C 3′) and CABYV_3728 Rev (5′ TTG CTG AAT ACA AAT GCT TGC ATC 3′). The amplified DNA fragments of the expected size (1066 bps) in both samples were confirmed by agarose gel electrophoresis (Fig. 1c). The amplicon was sequenced by Sanger method and the infection by the M3a and M3b clones was confirmed.

In addition, CABYV-M3a was used for host response studies in two independent inoculation trials. The following seedlings with cotyledons and one true-leaf stage were used in this host response study: squash (*Cucurbita maxima* × *C. moschata* cv. Tetsukabuto and *C. maxima* cv. Moranga Coroa), cucumber (*Cucumis sativus* cv. Caipira 201), watermelon (*Citrullus lanatus* cv. Crimson Sweet), zucchini (*Cucurbita pepo* cv. Caserta), West Indian gherkin (*Cucumis anguria* cv. Isla 163), and melon (*C. melo* cv. Calábria and Goldex). At 15 dpi, the upper leaves (not agroinfiltrated) were collected for CABYV-M3a detection by RT-PCR using primers CABYV_2663 For and CABYV_3728 Rev.

Of six cucurbit species tested in the host response experiment, the CABYV-M3a was detected in three cucurbitaceae crops; squash (hybrid of *C. maxima* × *C. moschata*, 7 positive/8 inoculated plants, and *C. maxima* cv. Moranga Coroa, 6/8), West Indian gherkin (*Cucumis anguria*, 6/6), and melon (*C. melo* cv. Calábria, 10/12 and cv. Goldex, 12/12) (Table 1). The virus was undetectable in cucumber (*C. sativus*), zucchini (*C. pepo*), and watermelon (*C. lanatus*) plants by RT-PCR and no symptom was observed in these plants. After 30 dpi, the leaf margins of squash and West Indian gherkin showed chlorosis symptoms in younger leaves. The symptoms of melon plants (cvs. Goldex and Calábria) inoculated were similar to infected melon plants observed in the fields [3].

Table 1 Detection of the CABYV-M3a by RT-PCR and symptoms

Species of cucurbits	Cultivar	Detection[a]	Symptoms
Cucumis anguria	Isla 163	6/6	Leaf edge chlorosis
Cucumis melo	Goldex	12/12	Severe yellowing
C. melo	Calábria	10/12	Severe yellowing
C. sativus	201 Caipira	0/8	
Cucurbita maxima	Moranga Coroa	6/8	Leaf edge chlorosis
C. pepo	Caserta	0/12	
Cucurbita maxima × *C. moschata*	Tetsukabuto F1	7/8	Leaf edge chlorosis
Citrullus lanatus	Crimson Sweet	0/6	

[a]Number of positive plants in virus detection/number of total inoculated plants

In a previous report of whitefly transmission tests, both squash and West Indian gherkin were susceptible when inoculated with M1 isolate collected in the field [4]. Here, we demonstrated that the infectious CABYV clone showed the same infectivity to these plants. Also confirming the previous result [4], watermelon plants were not infected with the infectious clone, which may explain the failure to detect CABYV in watermelon fields, even when located next to melon planted fields.

In conclusion, the full-length genomic cDNA clones of CABYV produced using the Gibson Assembly procedure were fully infectious, and showed very similar properties as the field-collected CABYV M1 isolate [4]. These clones would be important tools for future studies in developing resistant melon varieties to CABYV infection.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Lecoq H, Bourdin D, Wipf-Scheibel C et al (1992) A new yellowing disease of cucurbits caused by a luteovirus, cucurbit aphid-borne yellows virus. Plant Pathol 41:749–761. https://doi.org/10.1111/j.1365-3059.1992.tb02559.x
2. Vidal AH, Sanches MM, Alves-Freitas DMT et al (2018) First world report of cucurbit aphid-borne yellows virus infecting passionfruit. Plant Dis 102:2665. https://doi.org/10.1094/PDIS-04-18-0694-PDN
3. Costa TM, Blawid R, Aranda MA et al (2019) Cucurbit aphid-borne yellows virus from melon plants in Brazil is an interspecific recombinant. Arch Virol 164:249–254. https://doi.org/10.1007/s00705-018-4024-2
4. Costa TM, Inoue-Nagata AK, Vidal AH et al (2020) The recombinant isolate of cucurbit aphid-borne yellows virus from Brazil is a polerovirus transmitted by whiteflies. Plant Pathol 69:1042–1050. https://doi.org/10.1111/ppa.13186
5. Rott ME, Jelkmann W (2001) Characterization and detection of several filamentous viruses of cherry: adaptation of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol. Eur J Plant Pathol 107:411–420. https://doi.org/10.1023/A:1011264400482
6. Blawid R, Nagata T (2015) Construction of an infectious clone of a plant RNA virus in a binary vector using one-step Gibson Assembly. J Virol Methods 222:11–15. https://doi.org/10.1016/j.jviromet.2015.05.003