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SHORT COMMUNICATION

Biological and molecular characterization of isolates of catharanthus mosaic virus infecting *Mandevilla* sp.

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Abstract

This work reports the genome sequence, host responses and transmission assays of two isolates of catharanthus mosaic virus (CatMV) from plants of *Mandevilla* sp. in the state of São Paulo, Brazil. The complete genome sequences of CatMV isolates, M1 and M2, comprised 9593 and 9711 nucleotides, respectively. The deduced polyprotein showed 99% (M1) and 84% (M2) identities with the corresponding amino acid sequence of a CatMV from Australia. CatMV-M2 isolate was mechanically transmitted and systemically infected *Nicotiana benthamiana* and *Catharanthus roseus*. It also caused local lesions on inoculated leaves of *Chenopodium amaranticolor* and *C. quinoa*. This isolate was transmitted by *Myzus persicae* to plants of *Mandevilla* sp. CatMV-M1 isolate, however, did not infect any of the above plants when mechanically inoculated, neither was transmitted by *M. persicae*. Use of virus-free mother plants of *Mandevilla* sp. for seedling production is recommended for disease management.

Keywords *Catharanthus mosaic virus* · *Potyvirus* · Complete genome · *Mandevilla* sp. · Host range

Genus *Mandevilla* belongs to the family *Apocynaceae*, subfamily *Apocynoideae*. This genus is endemic to the American continent, with more than 170 species already reported (Alvarado-Cárdenas and Morales 2014). Among them, at least 50 species occur in Brazil. Most of them grow as vines, although they can occur as shrubs, herbs or epiphytes (Sales et al. 2006). Due to the diversity of their flowers, *Mandevilla* is one of the most cultivated ornamental plants in the world (Cordeiro et al. 2012).

In 2015, the potyvirus catharanthus mosaic virus (CatMV) was reported infecting *Mandevilla* sp. plants grown in nurseries located in the states of Minnesota and Florida, USA. The infected *Mandevilla* sp. plants exhibited symptoms of mosaic, leaf deformation, early leaf senescence and vine dieback (Mollov et al. 2015). CatMV has also been reported infecting *C. roseus* plants in Saudi Arabia (Elbeshehy et al. 2017) and the gymnosperm *Welwitschia mirabilis* in Australia (Koh et al. 2015).

Plants of *Mandevilla* sp. with symptoms of mosaic, leaf malformation and early senescence of leaves were found in flower shops in the cities of Piracicaba, Mogi das Cruzes and Limeira, state of São Paulo. Symptomatic plants were established by vegetative propagation in a greenhouse of the Department of Plant Pathology and Nematology, ESALQ/USP, Piracicaba, Brazil for further characterization.

To observe if virus particles were present, sap from symptomatic *Mandevilla* leaves was negatively stained with uranyl acetate for transmission electron microscopy (JEOL JEM 1011). For visualization of cytopathic effects of potential virus(es) on symptomatic *Mandevilla* plants, small pieces of symptomatic leaves were fixed, post fixed, dehydrated, infiltrated, embedded and thin sectioned for examination with a transmission electron microscope (Kitajima and Nome 1999).

The symptomatic *Mandevilla* plants and *C. roseus* plant infected by CatMV isolate (CatMV-Cr) were subjected to high throughput sequencing (HTS) (Maciel et al. 2011). Total RNA was extracted from plants of each of the above species using the Purelink viral RNA/DNA kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. cDNA libraries were prepared with the Illumina TruSeq Stranded Total RNA Sample Prep Kit (Illumina). Sequencing was done on

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the Illumina HiSeq 2500 System platform at the Center for Functional Genomics (ESALQ/USP, Piracicaba, Brazil) using HiSeq Flow Cell v4 and HiSeq SBS v4 (Illumina) kits in paired-end mode (2 × 100 bp). Reads were trimmed and adapter sequences removed with Trimmomatic v0.36 (Bolger et al. 2014) and BBduk v38.08 (SourceForge: BBMap. 2019). *De novo* contig assembly was done with SPAdes v3.11.1 (Bankevich et al. 2012) and MEGAHIT v1.3.1 (Li et al. 2016). Taxonomic assignment of the contigs was done through tBLASTx (Altschul et al. 1990) searches against the virus RefSeq database from NCBI. Contigs were extended and genome coverage depth was calculated using Geneious Mapper Tool in Geneious 8.1.9 (Biomatters).

Identities of the consensus nucleotide sequences were determined using BLASTn. Polyprotein cleavage sites were manually identified by comparison with those described for CatMV isolates from Australia (GenBank accession number KP742991) and the United States (KP343681) (Koh et al. 2015; Mollov et al. 2015). The deduced amino acid sequences were obtained using ExPASy (http://ca.expasy.org/tools/dna.html). Sequence alignment was done using Geneious 8.1.9. Phylogenetic relationships were inferred using the Maximum Likelihood method based on the JTT model (Jones et al. 1992), implemented in MEGA 7 (Kumar et al. 2016), with 1000 bootstrap.

For diagnostic purposes, two pairs of primers were designed based on the nucleotide sequences of the potyvirus isolates of *Mandevilla* sp. obtained in this work. One pair was designed flanking nucleotides 348 and 651 (304 nt) of the P1 protein gene (P1-F 5'-TACAAGTGATGCAA TTCATGTG-3′; and P1-R 5'-TGCTGTAGCTCGTA CCAAT-3′), whereas the other was flanking nucleotides 2644 and 3474 (831 nt) of the P3 protein gene (P3-F 5'-CACATGCATAGATTTATGGATA-3′; and P3-R 5'-GCAAGATGTGGTTTTTCCTCAAC-3′). Total RNA was extracted with the Purelink viral RNA/DNA kit (Thermo Fisher Scientific). RT-PCR was done in 25 μL, containing 3 μL of total RNA, 12.5 μL of 2X PCR Master Mix (Promega, Madison, USA), 1.25 μL of 20 mM of each of the primers, 2 U of AMV Reverse Transcription Enzyme (Promega) and 6.8 μL of nuclease free water. Thermal cycler procedure was as follows: one cycle at 42 °C for 30 min, one cycle at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. When primers P3-F and P3-R were used, the only modification was an increase of the annealing temperature to 58 °C. Amplicons were analyzed by 1% agarose gel electrophoresis, containing 0.5 x TBE buffer (45 mM Tris base, 45 mM boric acid, 0.05 mM EDTA pH 8.0), stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) and visualized in a transilluminator. Some amplicons were directly sequenced at Macrogen Inc. (Seoul, South Korea).

The experimental host range of the two potyvirus isolates infecting *Mandevilla* sp. was evaluated by mechanical transmission to two healthy plants of each of the following species: *Chenopodium amaranticolor*, *C. quinoa*, and *Gomphrena globosa* (*Amaranthaceae*); *C. roseus* (*Apocynaceae*); *Carica papaya* (*Caricaceae*); *Cucumis sativus*, *Cucurbita moschata* cv. Menina Brasileira, and *C. pepo* cv. Caserta (*Cucurbitaceae*); *Glycine max*, *Phaseolus vulgaris* cv. Carioca, and *Vigna unguiculata* cv. Gourgeia (*Fabaceae*); *Passiflora edulis* (*Passifloraceae*); and *Capsicum annuum*, *Datura stramonium*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. tabacum* cvs. Havana, Turkish and TNN, and *Solanum lycopersicum* (*Solanaceae*). One plant of each species was mock inoculated to serve as negative control. For biological comparison, the same plant species were mechanically inoculated with the CatMV-Cr isolate. The inoculated plants were kept in a greenhouse for symptoms expression and RT-PCR confirmation.

Transmission tests were done with the aphid *Myzus persicae* reared on sweet pepper plants. Symptomatic *Mandevilla* sp. and *C. roseus* plants were separately used as sources of inocula. Aphids were first fasted for 30 min, then transferred onto leaves of each of the source plants using a fine brush and then kept for a virus acquisition access period (AAP) of 10 min. Thereafter, groups of 10 aphids were transferred to two healthy plants of *Mandevilla* sp. and *C. roseus* for a virus inoculation access period (IAP) of 24 h. The insects were then manually eliminated, and the plants were kept in a greenhouse for symptom evaluation and virus detection by RT-PCR.

Transmission electron microscopic analysis revealed the presence of elongated flexuous virus-like particles, *ca.* 700–880 nm in length, in negatively stained sap of symptomatic leaf of *Mandevilla* sp. (Fig. 1a). Observation of ultrathin sections of infected tissue showed the presence of pinwheel-shaped cytoplasmic inclusions, typical of infections caused by potyvirus, in the cytoplasm of epidermal and mesophyll cells (Fig. 1b).

A total of 53,624,572 reads was generated for the symptomatic *Mandevilla* sp. sample. After trimming and adapter sequences removal, 32,425,390 paired reads were retained. Blast search revealed the presence of two contig sequences comprising the near-complete genome of two distinct isolates of CatMV. They are 9593 (accession number: MN356037) and 9711 (MN356038) nucleotides long, with a mean coverage depth of 76× and 4869×, respectively. These two isolates share 98% and 78% nucleotide identities with a CatMV isolate from Australia (KP742991), respectively. Deduced amino acid sequences of both isolates are 99% and 84% identical to the corresponding amino acid sequence of the Australian CatMV isolate. According to the demarcation criteria of *Potyvirus* species (Wylie et al. 2017), the two potyvirus isolates infecting *Mandevilla* sp. correspond to the species *Catharanthus mosaic virus*. They were named CatMV-M1 and CatMV-M2, respectively.

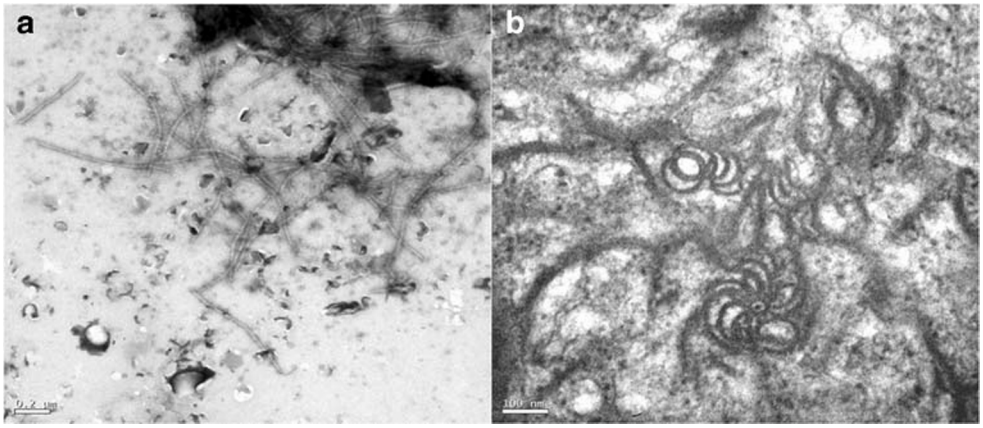

Fig. 1 **a**) elongated flexuous virus particles in leaf extract of symptomatic *Mandevilla* sp. Scale bar 0.2 μm. **b**) pinwheel-shaped cytoplasmic inclusions observed in epidermis and mesophyll cells of *Mandevilla* sp. Scale bar 100 nm

The designed pair of primer P1-F and P1-R specifically detected CatMV-M1 in RT-PCR, whereas P3-F and P3-R primers only detect CatMV-M2 isolate. The specificity of the primer pairs was confirmed by nucleotide sequencing of the obtained amplicons (data not shown). Further RT-PCR tests showed that symptomatic *Mandevilla* sp. plants from the flower shops in Mogi das Cruzes and Piracicaba Counties were infected with CatMV-M1-type and CatMV-M2-type, whereas those from Limeira County were infected only with CatMV-M2-type.

A total of 58,136,090 reads were generated for the *C. roseus* sample, of which 58,083,928 were retained after trimming and adapter sequence removal. The genome of CatMV-Cr has 9750 nucleotides (MN356039), and was assembled with a mean coverage depth of 521,024×. A portion of the CatMV-Cr genome (1654 nt), comprising part of the replicase gene (NIb), the coat protein gene (CP) and the 3'UTR are 97% identical to the corresponding nucleotide sequence of the previously identified CatMV isolate (DQ365928) infecting *C. roseus* plants in Brazil (Maciel et al. 2011).

The genomes of CatMV-M1, CatMV-M2 and CatMV-Cr encode polyproteins of 3052, 3052 and 3053 amino acids, respectively. Conserved cleavage sites, potentially generating 10 proteins, were found in all three polyproteins: P1, HC-Pro, P3, 6 K1, CI, 6 K2, VPg, NIa, NIb and CP. The small pretty interesting potyvirus protein (PIPO), located inside the gene encoding the P3 protein, was also found in the genome of the three CatMV isolates.

Comparisons of the nucleotide and deduced amino acid sequences of each gene of CatMV-M1, CatMV-M2 and CatMV-Cr genomes show that CatMV-M1 and CatMV-Cr have higher nucleotide and amino acid identities with each other, than to CatMV-M2 (Table 1). The genes encoding the proteins P1, P3 and PIPO are more divergent, while those encoding the CP, CI, NIa and NIb are less divergent. Coverage depth varied significantly between these viruses (76×, 4869× and 521,024× for CatMV-M1, CatMV-M2 and CatMV-Cr, respectively), although a direct comparison of the sequencing depth between isolate CatMV-Cr and isolates CatMV-M1 and CatMV-M2 should be done with caution given the impossibility of data normalization. Considering that

Table 1 Comparison of the nucleotide (nt) and deduced amino acid (aa) sequences of CatMV-M1, CatMV-M2 and CatMV-Cr isolates

Genome region	Identity (%) nt/aa		
	CatMV-M1 CatMV-M2	CatMV-M1 CatMV-Cr	CatMV-M2 CatMV-Cr
Polyprotein	77/84	82/90	77/84
P1	65/60	76/78	67/61
HC-Pro	76/85	82/92	77/84
P3	73/70	79/80	74/73
6 K1	76/87	85/100	76/87
CI	79/92	84/96	79/92
6 K2	73/80	75/83	79/82
VPg	77/85	82/94	79/87
NIa	81/93	84/93	79/92
NIb	79/89	82/93	79/88
CP	84/90	83/91	84/90
PIPO	77/66	81/74	80/69

Table 2 Comparison of the nucleotide (nt) and deduced amino acid (aa) sequences of different isolates of catharanthus mosaic virus (CatMV)

	Identity (%) nt/aa				
	CatMV-M1	CatMV-M2	CatMV-Cr	CatMV (AUS)	CatMV (EUA)
CatMV-M1	100/100	77/84	82/90	98/99	97/98
CatMV-M2	77/84	100/100	77/84	77/84	77/84
CatMV-Cr	82/90	77/84	100/100	81/90	81/90
CatMV (AUS)	98/99	77/84	81/90	100/100	97/98
CatMV (EUA)	97/98	77/84	81/90	97/98	100/100

the coverage depth of CatMV-M2 is much higher than that of CatMV-M1, it is possible that both CatMV isolates are interacting in an antagonistic manner within the *Mandevilla* sp. host, where the isolate CatMV-M1 has lower fitness or is being excluded by the super-infection exclusion phenomenon (Syller and Grupa 2016).

Contrasting the nucleotide and deduced amino acid sequences of the three CatMV isolates with corresponding sequences of different CatMV isolates available in GenBank, it was observed that CatMV-M1 showed high identities with the CatMV isolates from Australia and the United States. CatMV-M2 showed the lowest identity among the CatMV isolates evaluated (Table 2). The phylogenetic tree inferred using the deduced polyprotein amino acid sequences of different CatMV isolates (Fig. 2) clearly shows that CatMV-M1 groups with CatMV isolates from the United States and Australia. The CatMV-M2 is the most genetically distant, while the CatMV-Cr grouped in an intermediate clade, compared to the other isolates of this potyvirus.

Among the plant species mechanically inoculated with CatMV isolates, only four became infected. Plants of *C. amaranticolor* and *C. quinoa* exhibited local lesions on the leaves inoculated with CatMV-M2 and CatMV-Cr isolates. The same isolates systemically infected *N. benthamiana* and *C. roseus*. CatMV-M2 induced only mild mosaic on leaves of *C. roseus*, whereas CatMV-Cr caused severe mosaic leaf distortion. The partial host range results of CatMV-M2 and CatMV-Cr isolates of the present work are consistent with those obtained by Maciel et al. (2011) when evaluated the previously identified CatMV isolate infecting *C. roseus* plants. CatMV-M1 isolate did not infect any of the inoculated plants.

M. persicae that fed on leaves of *Mandevilla* sp. doubly infected with CatMV-M1 and CatMV-M2 transmitted only the later isolate to two inoculated *Mandevilla* sp. plants. Both isolates were not transmitted by *M. persicae* to *C. roseus*. The aphid was also able to acquire CatMV-Cr isolate from infected *C. roseus* plants and transmit it to 50% (1/2) of *Mandevilla* sp. and 100% (2/2) of the inoculated *C. roseus* plants.

The three Brazilian CatMV isolates have distinct biological and molecular features. CatMV-M1 isolate was closest in genome sequence to the CatMV isolates from Australia and the United States, but it was not possible to make biological comparison between them because there are no reports on the host range of these two isolates. However, CatMV isolate occurring in *W. mirabilis* was mechanically transmitted to *N. benthamiana* and caused symptoms of chlorosis, leaf malformation, and plant stunting (Koh et al. 2015). Interestingly, the CatMV-M1 isolate, despite being the closest genetically to the CatMV isolate from Australia, was the only one that did not infect *N. benthamiana* plants among the CatMV isolates evaluated in the present work.

CatMV-M1 was the only isolate which was not transmitted by *M. persicae* to plants of *Mandevilla* sp. This result is difficult to understand, because this isolate has the DAG (coat protein), KTIC, and PTK (HC-Pro) motifs considered essential for aphid transmission (Thornbury et al. 1990; Huet et al. 1994; Wang et al. 1996). A possible explanation for the absence of aphid transmission maybe related to the interference of CatMV-M2 isolate on the transmission of CatMV-M1 by *M. persicae*, as the *Mandevilla* plant was doubly infected. It is known that in plants with mixed infections the transmission

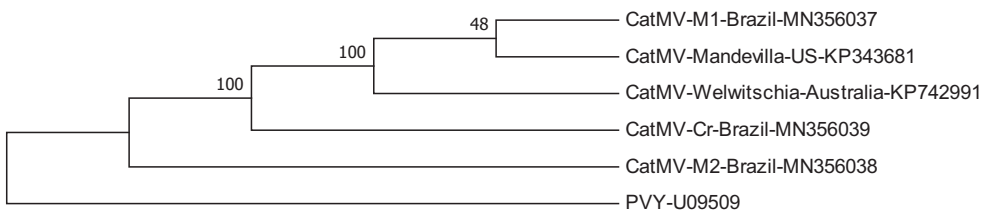

Fig. 2 Phylogenetic tree constructed with the deduced amino acid sequences of the polyprotein of different isolates of catharanthus mosaic virus (CatMV). Potato virus Y (PVY; Potyvirus) was used as outgroup sequence. The corresponding GenBank accession number of each virus sequence is given in the figure

rate of one of the viruses can be altered (Wintermantel et al. 2008; Macedo et al. 2015). However, this hypothesis could not be evaluated due to the absence of plants infected with CatMV-M1 isolate alone. Besides *M. persicae*, there are also reports of *Aphis gossypii* and *M. nicotianae* as vectors of the CatMV (Maciel et al. 2011).

Presence of CatMV-infected *Mandevilla* sp. plants in florists located in different counties of São Paulo state indicates that this potyvirus is being spread in this host plant since the crop is vegetatively propagated. This fact may compromise the production and trade of this ornamental species, as infected plants have a shortened life span and, due to the symptoms presented, may become less attractive to consumers. *Mandevilla* sp. plants are vegetative propagated. Consequently, this cultural practice may be more efficient for CatMV dissemination than aphid transmission. Therefore, the most important measure to minimize the spread and damage caused by CatMV is the establishment of virus-free mother plants of *Mandevilla* sp. for seedling production. As the host range of this virus seems restricted, the chance of aphid introduction of the virus into nurseries may be reduced.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest. This article does not contain any studies with animals performed by any of the authors.

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