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Recent characterization of cowpea aphid-borne mosaic virus (CABMV) in Bahia State, Brazil, suggests potential regional isolation

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Woodiness disease is the most important disorder of passion fruit worldwide. The causal agent in Brazil is the Cowpea aphid-borne mosaic virus (CABMV), and despite the economic relevance of passion fruit for agriculture there have been recently very few studies about this virus in Brazil and worldwide. This work reveals the phylogenetic relationships of 10 newly identified CABMV isolates from Bahia State, the region where CABMV was first identified (in that time reported as PWV) in South America before its outbreak. The coat protein of 10 CABMV isolates (CABMV-Lns1 - CABMV-Lns10) from Livramento de Nossa Senhora Country, Bahia State, were sequenced and presented very close identity between themselves (nucleotide: 97 to 99%, amino acid: 95 to 100%). They are phylogenetically closely related to Brazilian CABMV, however forming an isolated cluster within the Brazilian clade. According to previous evidences, our data demonstrate that CABMV-Lns are more closely related to isolates from Southern rather than from Northern Africa. Other two isolates from Bahia State clustered separately from CABMV-Lns, but together with isolates from other Brazilian regions thus suggesting that CABMV-Lns are a strain likely restricted to Bahia. The characterization of new populations of CABMV enables greater resolution of the evolution of viruses causing woodiness disease in passion fruit vines. Our data shed light on an as yet unexplored population of CABMV in Brazil and contributes to the understanding of its evolutionary history.

Key words: Passion fruit, Livramento de Nossa Senhora, Bahia, phylogenetics, woodiness disease.

INTRODUCTION

The passion fruit is one of the most important crops in Brazil. It belongs to Passiflora genus and Passifloraceae family. Viral diseases are the most severe in terms of passion fruit yield loss (Sokhandan et al., 1997; Gibbs et al., 2008b). This is mainly due to the lack of both quick and accurate diagnosis as well as effective methods for disease control (Andrade and Pio-Ribeiro, 2001). In Brazil and Africa woodiness disease is caused by Cowpea aphid-borne mosaic virus (CABMV) (McKern et al., 1994; Nascimento et al., 2006; Maciel et al., 2009). This is in contrast to Australia where the passion fruit woodiness virus (PWV) is the most common causal agent

(Sokhandan et al., 1997) and also to Asia where the same disease is caused by the East asia passiflora virus (EAPV) (Iwai et al., 2006). Its genus *Potyvirus*, family *Potyviridae*, contains more agriculturally important plant viruses than any other (Adams et al., 2005). Although in the past 15 years molecular studies about Brazilian CABMV have been carried out (Novaes and Rezende, 2003; Nascimento et al., 2004; Barros et al., 2011; Cerqueira-Silva et al., 2012; Nicolini et al., 2012), surprisingly none of them has phylogenetically investigated the virus from the region of largest passion fruit production in Brazil, the Livramento de Nossa Senhora County. Exploring the molecular variation of the coat protein (CP) in CABMV helps to better understand variability among strains, isolates and related species, to elucidate the evolutionary history of CABMV as well as assists to shed light on disease outbreaks in the course of passion fruit dispersion (Iwai et al., 2006; Gibbs et al., 2008a; Gibbs et al., 2008b). Bahia State is the place where passion fruit woodiness disease was firstly identified in Brazil during the 70's (Chagas et al., 1981) before its dissemination to most of the countries' fields. This led us to hypothesize whether Bahia State might host phylogenetically different isolates than the rest of Brazil that could be due to genomic mutation, recombination with other viral species, selective pressure or other evolutionary events. In this study we report a phylogenetic characterization of a population of CABMV from the State of Bahia, in comparison to other viruses causing fruit woodiness disease in passion fruit vines around the world.

MATERIALS AND METHODS

Virus and plant resources

Ten CABMV isolates were collected from different passion fruit crop fields in the farming region of Livramento de Nossa Senhora Country, Bahia State, Brazil (13° 38′ 34″ S, 41° 50′ 27″ W). Ground extract from symptomatic leaves was used for inoculation on healthy passion fruit plants for maintenance, following the method described by Novaes and Rezende (2003). The 10 CABMV isolates here reported were named: CABMV-Lns1 to CABMV-Lns10. Several sequences from passion fruit woodiness disease-related viruses were acquired from public databases (Supplementary Table 1 and Figure 1).

RNA extraction, PCR, cloning and sequencing

RNA extraction was performed following the Arabidopsis Functional Genomics Consortium Trizol protocol (Arabidopsis Functional Genomics Consortium – AFGC, 2013). cDNA synthesis and PCR (Promega), DNA purification (PCR Purification Kit – NORGEN, Wizard SV Gel and PCR Clean-Up System – Promega) and cloning (pGEM-T Easy Vector – Promega, Max Efficiency DH5α Competent Cell – Invitrogen) were performed as essentially as described in the manufacturer's protocol. Newly designed primers for both cDNA synthesis and PCR were: CABMV8364-F (5'-CCTTTCCTTCTACGATG-3'), CABMV9389-R (5'-CAACCGGGGTATGGCCTC-3'), CABMV8359–F (5'-GGCATCCTTTCCTTCTATG-3') and CABMV9402–R (5'-GGCATCCTTTCCTTCTATG-3').

Sequencing and phylogenetic analysis

Sequencing of at least three independent clones from each isolate was performed twice by Macrogen (South Korea). The sequences were manipulated using DNA Baser Sequence Assembler v2 (DNA Baser Sequence Assembler v2.x, 2010). Phylogenetic tree was constructed using MEGA5 (Tamura et al., 2011). For nucleotide-based tree, Neighbor-Joining (NJ) and BioNJ pairwise distances matrix were estimated by maximum composite likelihood (MLC), estimated under bootstrap values higher than 70% (1,000 replications).

RESULTS

Analysis of coat protein sequence

Complete CP coding sequence containing 828 nucleotides and 275 amino acids were identified in all ten CABMV-Lns isolates, in agreement with the sequenced CABMV genomes (Mlotshwa et al., 2002; Barros et al., 2011). Very little variation was found amongst the nucleotide sequence of CABMV-Lns isolates (identity from 97 to 99%) (Table 1). Comparison of CABMV-Lns and other CABMV isolates showed sequence identity varying from 78 to 94%, where the lowest identity was found with CABMV-Mor, CABMV-Iba and CABMV-Monguno isolates (78%) and the highest with CABMV-Brs isolate (94%). The identity of amino acid sequences amongst CABMV-Lns isolates ranged from 95 to 100% identity (Table 1). When compared to other CABMV isolates, it ranged from 80 to 96%, when the least identity was found with CABMV-Bnt isolate (80%) and the highest with CABMV-TC1 isolate (96%). The CP conserved motifs of potyviruses were entirely conserved in nearly all CABMV-Lns isolates, with exception of CABMV-Lns6 which has a substitution of aspartic acid by an alanine residue at the DAG motif (Supplementary Figure 2).

Phylogenetic profile suggests CABMV-Lns as a strain restricted to Bahia State

Maximum-likelihood genealogies of nucleotide sequences

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Abbreviations: CABMV, Cowpea aphid-borne mosaic virus; **PWV,** passion fruit woodiness virus; **EAPV,** East asia passiflora virus.

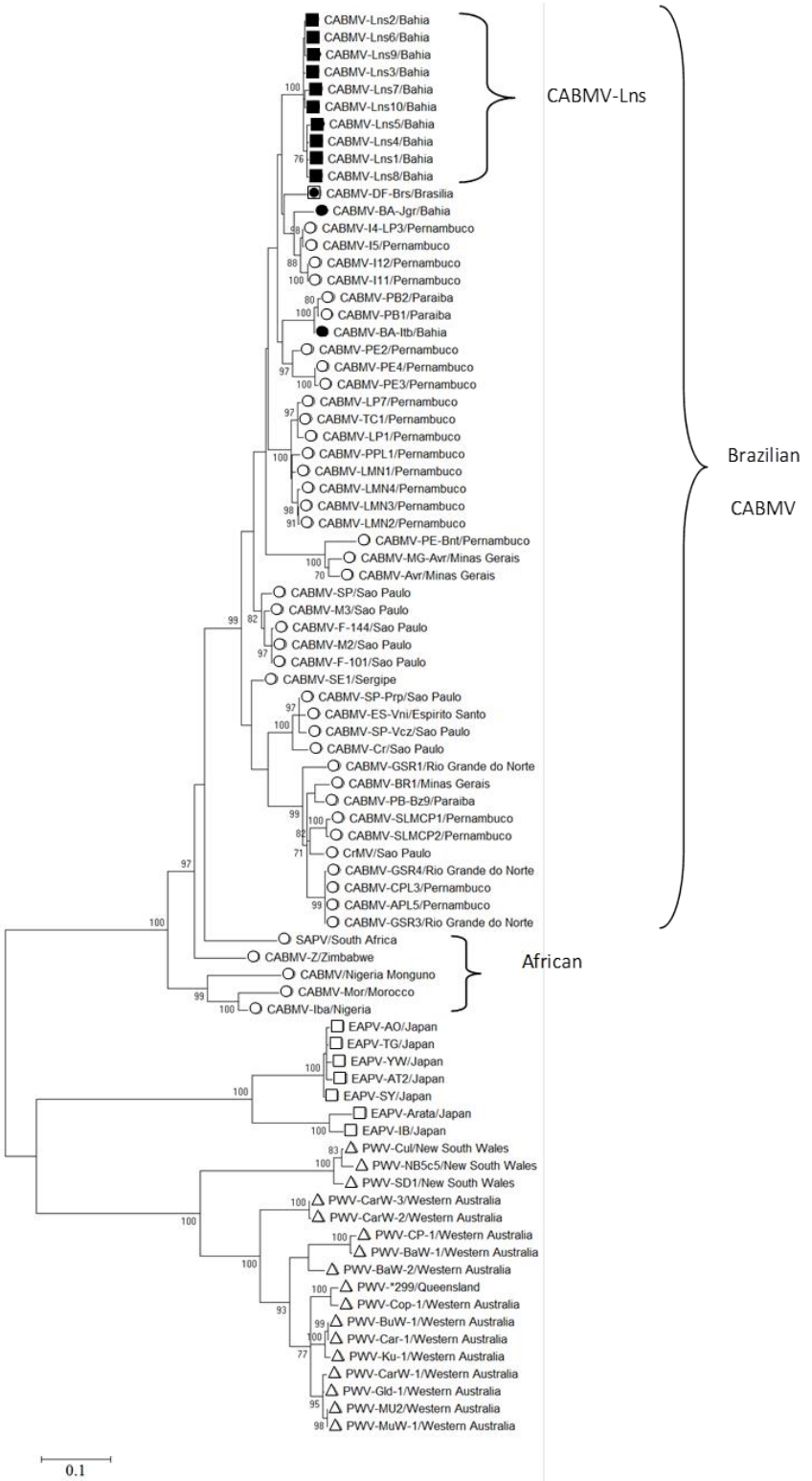

Figure 1. Molecular phylogenetic tree of viral species that cause woodiness disease in Passion fruit plants. The nucleotide coding sequence for the Coat Protein was used to generate the tree. The relationships were inferred using the Maximum Likelihood method based on the General Time Reversible model (highest log likelihood -10235.3876 is shown). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8776)). The branch length is measured by number of substitutions per site. ■ CABMV-Lns, ● CABMV from Bahia State, CABMV from Brasília, ○ Other CABMV, □ EAPV and ∆ PWV.

Table 1. Percentage identity of nucleotide (above the diagonal) and amino acid (below the diagonal) sequences of the coding *CP* gene from ten CABMV-Lns isolates.

	CABMV-Lns1	CABMV-Lns2	CABMV-Lns3	CABMV-Lns4	CABMV-Lns5	CABMV-Lns6	CABMV-Lns7	CABMV-Lns8	CABMV-Lns9	CABMV-Lns10
CABMV-Lns1	---	99	99	99	99	97	98	99	99	99
CABMV-Lns2	99	---	99	99	99	98	99	99	99	99
CABMV-Lns3	99	99	---	99	99	98	99	99	99	99
CABMV-Lns4	100	99	99	---	99	97	98	99	99	99
CABMV-Lns5	98	98	98	98	---	97	98	99	98	98
CABMV-Lns6	96	97	97	96	96	---	97	97	98	98
CABMV-Lns7	98	98	98	98	97	95	---	98	98	99
CABMV-Lns8	99	98	99	99	98	96	97	---	99	99
CABMV-Lns9	99	100	99	99	98	97	98	98	---	99
CABMV-Lns10	99	99	100	99	98	97	98	99	99	---

of the CP demonstrate that all CABMV isolates clustered within a monophyletic clade that comprehends sequences from Brazil and Africa. CABMV-Lns isolates formed an isolated cluster inside the CABMV monophyletic clade (Figure 1). Interestingly they clustered separately from CABMV-Jgr and CABMV-Itb isolates also identified in Bahia State, which are more closely related to isolates from the States of Pernambuco and Paraíba, located around 1,200 km and 1,350 km away from Livramento de Nossa Senhora, respectively. PWV and EAPV (Iwai et al., 2006), which are well known different species from CABMV, formed a distinct clade also separately from CABMV-Lns (Figure 1).

DISCUSSION

The high identity of nucleotide and amino acid sequences amongst the ten CABMV-Lns isolates is strong evidence that they belong to the same strain of CABMV. This information is of great relevance for purposes of disease control, once most of the attempts to overcome woodiness disease in passion fruit such as premunization with mild strains of CABMV/PWV or pathogen-derived resistance have been hindered by variant strains of the same virus (Novaes and Rezende, 2003; Alfenas et al., 2005, Trevisan et al., 2006; Cerqueira-Silva et al., 2014). On the other hand, CABMV-Lns clustered separately from the isolates CABMV-Jgr and CABMV-Itb, which were also collected in Bahia State. As CABMV-Jgr and CABMV-Itb clustered together with isolates from rather far regions, the data suggest that CABMV-Lns are likely a strain restricted to Bahia State. This finding proposes either a single introduction of this strain to the area or a constraint in dissemination of this strain to other places by plant material, since there is no evidence that the virus is seed-transmitted in Passion fruit. According to the demarcation criteria for the *Potyvirus* genus (King et al., 2011), our data support the hypothesis that CABMV-Lns isolates are a different strain from CABMV-Prp, CABMV-Cr, CABMV-Vni, CABMV-Z, CABMV-Iba, CABMV-Monguno, CABMV-Mor, CABMV-MG-Avr, CABMV-PE-Bnt (80 to 89% amino acid identity). Additionally, our data confirm the higher sequence identity and closer phylogenetic relationship of CABMV-Lns with Southern African CABMV rather than isolates from the North of Africa, which was also previously suggested (Nascimento et al., 2006). The slave trade between Africa and South America is the likely event for introduction of CABMV in Brazil (Gibbs et al., 2008b), by which infected biological material could have been transported from the South rather than the North of Africa. However, we are aware that the limited number of CABMV sequences currently available, associated to the little information about African CABMV infecting Passion fruit plants, significantly narrow down the possibilities of drawing more precise conclusion on this regard. With reference to the important CP conserved motifs of potyviruses, the DAG motif is related to transmissibility of potyviruses by insects (Abdullah et al., 2009). Isolate CABMV-Lns6 shows a substitution of aspartic acid by alanine it to the DAG motif (Supplementary Figure 1). Mutations in this region significantly reduced transmissibility by insects in previous studies (Abdullah et al., 2009; López-Moya et al., 1999). However, unfortunately transmissibility experiments to investigate the consequences of the mutated DAG motif on the transmissibility capacity of CABMV-Lns6 were not suitable before the submission of this work.

Conclusion

Our data precisely demonstrate that CABMV-Lns belong also to CABMV species. These isolates can be classified as a strain of CABMV thus far occurring only in Bahia State and phylogenetically more distant from other isolates occurring in the same region. In addition

and accordingly to previous reports, they are more closely related to Southern rather than Northern African CABMV. The exploitation of a larger number of viral samples from other as yet unexplored places in the promising State of Bahia will help to shed light on the events contributing to the observed regional isolation of CABMV-Lns. The whole genome sequencing of CABMV-Lns is ongoing and will allow us to comprehend their evolutionary history with regard to other related viral species around the world.

Conflict of interests

The authors did not declare any conflict of interest.

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Brand RJ, Burger JT, Rybicld EP (1993). Cloning, sequencing, and expression in Escherichia coli of the coat protein gene of a new potyvirus infecting South African Passiflora. Arch. Virol. 128(1-2): 29-41.

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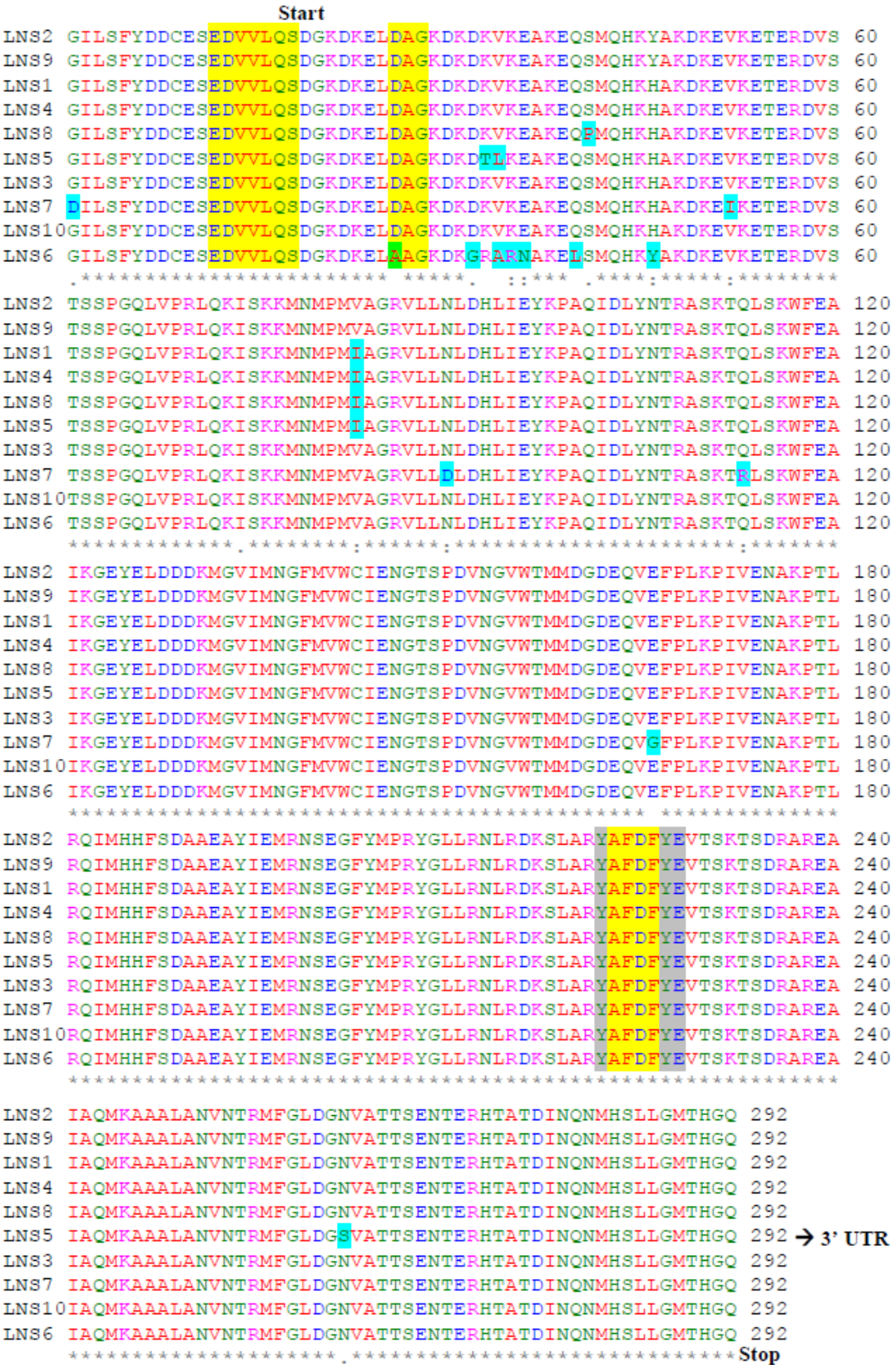
Supplementary Table 1. Accession details of all sequences analyzed in this study, available in GenBank (National Center for Biotechnology Information - NCBI, 2013).

GenBank accession number	Country of origin	State / area	Isolate	Source / publication
KF725706	Brazil	Bahia	CABMV-Lns1	This study
KF725707	Brazil	Bahia	CABMV-Lns2	This study
KF725708	Brazil	Bahia	CABMV-Lns3	This study
KF725709	Brazil	Bahia	CABMV-Lns4	This study
KF725710	Brazil	Bahia	CABMV-Lns5	This study
KF725711	Brazil	Bahia	CABMV-Lns6	This study
KF725712	Brazil	Bahia	CABMV-Lns7	This study
KF725713	Brazil	Bahia	CABMV-Lns8	This study
KF725714	Brazil	Bahia	CABMV-Lns9	This study
KF725715	Brazil	Bahia	CABMV-Lns10	This study
DQ397527	Brazil	Bahia	CABMV-Jgr	Nascimento et al. (2006)
DQ397528	Brazil	Bahia	CABMV-Itb	Nascimento et al. (2006)
AY253911	Brazil	Sergipe	CABMV-SE1	Nascimento et al. (2006)
AY433950	Brazil	São Paulo	CABMV-SP	Gioria et al. (2004)
AY433951	Brazil	São Paulo	CABMV-F101	Gioria et al. (2004)
AY433952	Brazil	São Paulo	CABMV-M2	Gioria et al. (2004)
AY434454	Brazil	São Paulo	CABMV-M3	Gioria et al. (2004)
AY505342	Brazil	São Paulo	CABMV-F144	Novaes e Rezende (2005)
DQ397530	Brazil	São Paulo	CABMV-SP-Vcz	Nascimento et al. (2006)
DQ397531	Brazil	São Paulo	CABMV-Prp	Nascimento et al. (2006)
EU004070	Brazil	São Paulo	CABMV-Cr	Kitjima et al. (2008)
AF368424	Brazil	São Paulo	CrMV	Freitas et al. (2002)
AY253907	Brazil	Paraíba	CABMV-PB1	Nascimento et al. (2006)
AY253910	Brazil	Paraíba	CABMV-PB2	Nascimento et al. (2006)
DQ397529	Brazil	Espírito Santo	CABMV-Vni	Nascimento et al. (2006)
DQ397532	Brazil	Brasília-DF	CABMV-Brs	Nascimento et al. (2006)
AY253909	Brazil	Pernambuco	CABMV-PE4	Nascimento et al. (2006)
AY253906	Brazil	Pernambuco	CABMV-PE2	Nascimento et al. (2006)
AY253908	Brazil	Pernambuco	CABMV-PE3	Nascimento et al. (2006)
DQ397526	Brazil	Pernambuco	CABMV-PE-Bnt	Nascimento et al. (2006)
JF833415	Brazil	Pernambuco	CABMV-LMN1	Nicolini et al. (2011)
JF833416	Brazil	Pernambuco	CABMV-CPL3	Nicolini et al. (2011)
JF833419	Brazil	Pernambuco	CABMV-SLMCP1	Nicolini et al. (2011)
JF833420	Brazil	Pernambuco	CABMV-LMN4	Nicolini et al. (2011)
JF833421	Brazil	Pernambuco	CABMV-LMN3	Nicolini et al. (2011)
JF833422	Brazil	Pernambuco	CABMV-SLMCP2	Nicolini et al. (2011)
JF833423	Brazil	Pernambuco	CABMV-PPL1	Nicolini et al. (2011)
JF833424	Brazil	Pernambuco	CABMV-APL5	Nicolini et al. (2011)
JF833426	Brazil	Pernambuco	CABMV-I4-LP3	Nicolini et al. (2011)
JF833427	Brazil	Pernambuco	CABMV-LP7	Nicolini et al. (2011)
JF833428	Brazil	Pernambuco	CABMV-I12	Nicolini et al. (2011)
JF833429	Brazil	Pernambuco	CABMV-LP1	Nicolini et al. (2011)
JF833431	Brazil	Pernambuco	CABMV-LMN2	Nicolini et al. (2011)
JF833432	Brazil	Pernambuco	CABMV-TC1	Nicolini et al. (2011)
JF833433	Brazil	Pernambuco	CABMV-I11	Nicolini et al. (2011)
JF833434	Brazil	Pernambuco	CABMV-I5	Nicolini et al. (2011)
JF833417	Brazil	Rio Grande do Norte	CABMV-GSR1	Nicolini et al. (2011)
JF833418	Brazil	Rio Grande do Norte	CABMV-GSR3	Nicolini et al. (2011)
JF833425	Brazil	Rio Grande do Norte	CABMV-GSR4	Nicolini et al. (2011)
DQ397525	Brazil	Minas Gerais	CABMV-MG-Avr	Nascimento et al. (2006)

Supplementary Table 1. Contd.

GenBank accession number	Country of origin	State / area	Isolate	Source / publication
HQ880242	Brazil	Minas Gerais	CABMV-BR1	Barros et al. (2011)
HQ880243	Brazil	Minas Gerais	CABMV-Avr	Barros et al. (2011)
AJ132414	Nigeria	Ibadan	CABMV-Iba	Boxtel et al. (2000)
Y17822	Nigeria	Monguno	CABMV-Moguno	Boxtel et al. (2000)
NC_004013	Zimbabwe	Harare	CABMV-Z	Mlotshwa et al. (2002)
Y18634	Morocco	N/A	CABMV-Mor	Boxtel et al. (2000)
D10053	South Africa	N/A	SAPV	Brand et al. (1993)
AB627437	Japan	Kagoshima	EAPV-SY	Fukumoto et al. (2012a)
AB627436	Japan	Kagoshima	EAPV-YW	Fukumoto et al. (2012a)
AB627435	Japan	Kagoshima	EAPV-Arata	Fukumoto et al. (2012a)
AB604610	Japan	Kagoshima	EAPV-IB	Fukumoto et al. (2012a)
AB246773	Japan	Kagoshima	EAPV-AO	Iwai et al. (2006)
AB690441	Japan	Kagoshima	EAPV-TG	Fukumoto et al. (2012b)
AB690440	Japan	Kagoshima	EAPV-AT2	Fukumoto et al. (2012b)
AJ430527	Australia	Queensland	PWV-#299	Dietzgen et al. (2002 Unpublished)
DQ898218	Australia	Western Australia	PWV-Cop-1	Webster et al. (2007)
DQ898215	Australia	Western Australia	PWV-Gld-1	Webster et al. (2007)
JF427623	Australia	Western Australia	PWV-BuW-1	Coutts et al. (2011)
DQ898216	Australia	Western Australia	PWV-Car-1	Webster et al. (2007)
DQ898217	Australia	Western Australia	PWV-Ku-1	Webster et al. (2007)
JF427621	Australia	Western Australia	PWV-CarW-1	Coutts et al. (2011)
HQ122652	Australia	Western Australia	PWV-MU2	Wylie andJones (2011)
JF427620	Australia	Western Australia	PWV-MuW-1	Coutts et al. (2011)
JF427622	Australia	Western Australia	PWV-BaW-2	Coutts et al. (2011)
JF427619	Australia	Western Australia	PWV-CP-1	Coutts et al. (2011)
JF427618	Australia	Western Australia	PWV-BaW-1	Coutts et al. (2011)
JF427617	Australia	Western Australia	PWV-CarW-3	Coutts et al. (2011)
JF427616	Australia	Western Australia	PWV-CarW-2	Coutts et al. (2011)
U67149	Australia	New South Wales	PWV-Cul	Sokhandan et al. (1997)
U67150	Australia	New South Wales	PWV-SD-1	Sokhandan et al. (1997)
U67151	Australia	New South Wales	PWV-NB5c5^{+}	Sokhandan et al. (1997)

Supplementary Figure 1. Location of Brazilian CABMV isolates used in this study. Number in brackets represents the amount of sequences per location. RN: Rio Grande do Norte, PB: Paraíba, PE: Pernambuco, SE: Sergipe, BA: Bahia, LNS: Livramento de Nossa Senhora, DF: Distrito Federal (Brasília), MG: Minas Gerais, ES: Espírito Santo, SP: São Paulo. Maps were adapted from original draws available in Wikimedia Commons and Free World Maps websites.



Supplementary Figure 2. Multiple alignment of 5' > 3' predicted amino acid sequence that encodes CP gene flaked by NIb at 5' end and 3' UTR at 3' end. Letter with green and blue backgrounds highlights amino acid substitutions. Letter with yellow background highlights conserved motifs. Letter with grey background shows recently suggested conserved motifs. Start: Start codon, Stop: stop codon, UTR: untranslated region.