CALADIUM VIRUS X, A NEW POTEXVIRUS FROM *CALADIUM BICOLOR* (ARACEAE)

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SUMMARY

An elongated virus with particles measuring 460 nm in length and tentatively named Caladium virus X (CalVX) was isolated from *Caladium bicolor* (fam. *Araceae*) co-infected with *Dasheen mosaic virus*. The effect of this double infection was to induce chlorotic and necrotic spots and rings.

CalVX was easily transmitted by mechanical inoculation but not via seeds or by aphids. The only systemic experimental hosts detected were *Nicotiana benthamiana* and some araceous species. There were no positive reactions in serological tests using antisera to 17 potexviruses. CalVX capsid protein has a M_r of 26,000. Electron microscopy of infected cells revealed aggregates of virus-like particles in the cytoplasm, and electron-dense crystalline inclusions in the cytoplasm and nucleus. Comparison of the sequence of 740 nucleotides encoding the putative C-terminal region of the replicase showed that CalVX was most similar (65.8% identity) to *Cymbidium mosaic virus* and phylogenetic analysis supported its classification in the genus *Potexvirus*. This is the first report of a natural infection of araceous plants by a potexvirus.

Key words: partial characterization, potexvirus, caladium.

INTRODUCTION

Members of the genus *Potexvirus*, family *Flexiviridae*, have flexuous virions measuring 470 to 580 nm in length (Adams *et al.*, 2004). The genus includes 28 definitive species and 18 putative species naturally infecting mono- and dicotyledonous plants as well as the fungus *Boletus edulis* (Adams *et al.*, 2004). Recently, three probable new potexvirus species were described (Koenig *et al.*, 2004).

Potexvirus genomic positive sense ssRNAs typically have five ORFs (Adams *et al.*, 2004). ORF1, at the 5' terminus, encodes a replicase that contains three functional domains: a methyltransferase at the N-terminus, a central helicase, and an RNA-dependent RNA polymerase (RdRp) at the C-terminus (Brunt *et al.*, 2000). Potexvirus replicases have the motifs conserved among the alpha-like supergroup or supergroup III of replicases that also include those of carlaviruses, furoviruses, hordeiviruses, trichoviruses and tymoviruses (Koonin, 1991). The ORFs 2-4 encode a 'triple gene block' (TGB), a conserved module involved in the cell-to-cell and long-distance movement of virus; the motifs conserved in the NTPase/helicase domain in TGB1 are characteristic of helicases of superfamily I (Morozov and Solovyev, 2003), that also include allexiviruses, carlaviruses, foveaviruses, furoviruses, hordeiviruses and tymoviruses (Brunt *et al.*, 2000; Morozov and Solovyev, 2003). ORF 5 encodes the coat protein (Adams *et al.*, 2004).

Plants in the family Araceae (aroids) are found naturally in different habitats, except in the sea and in the extremes of desert, arctic and high alpine regions, but the vast majority of the genera occur in the New World tropics (Bown 1988). In the late 1800s and early 1900s, Brazil produced more than 600 cultivars that were spread throughout the world (Figueiredo, 1936). Although caladiums are found in Brazil, currently over 90% of the world's commercial supply of caladiums is produced in Florida (Zettler and Hartman, 1987).

Dasheen mosaic virus (DsMV), an aphid-borne potyvirus, is a prevalent pathogen in cultivated aroids, probably because most cultivated aroids are propagated vegetatively (Zettler and Hartman, 1987).

In this paper we present the biological and molecular properties of a new virus that infects plants in the Araceae and tentatively name it Caladium virus X (CalVX).

MATERIALS AND METHODS

Virus source and maintenance. CalVX was isolated from *Caladium bicolor* doubly infected with this virus and DsMV (Fig. 1), identified by immunosorbent elec-

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Fig. 1. *Caladium bicolor*, naturally infected by DsMV and CalVX, displaying chlorotic spots and necrotic ringspots.

tron microscopy using specific antiserum raised against DsMV. The plant was grown in a house-garden in São Paulo (Brazil). CalVX was biologically purified by a single passage of a lesion isolated in *Gomphrena globosa*, since DsMV cannot be mechanically transmitted to this plant, and subsequently propagated in *Nicotiana benthamiana*.

Transmission electron microscopy observations. Virus particle dimensions were determined from leaf-dip preparations, negatively stained with 2% uranyl acetate, of CalVX and *Tobacco mosaic virus* (TMV) infecting *N. benthamiana* and *N. tabacum* 'White Burley', respectively. Leaf fragments were cut from naturally infected caladium or experimentally infected *G. globosa*, fixed in 2.5% glutaraldehyde, postfixed in 0.5% osmium tetroxide, stained in 2.0% uranyl acetate, dehydrated in graded dilutions of acetone, embedded in Spurr's medium and sectioned (Alexandre *et al.*, 2000).

Host range studies. Crude sap from experimentally infected *N. benthamiana* leaves was prepared in 0.01 M sodium-potassium phosphate buffer, pH 8.0 (PB) and mechanically inoculated to a range of herbaceous hosts comprising 55 species and varieties, from 19 families of monocotyledonous and dicotyledonous plants. Virus infection was checked by back-inoculation to *G. globosa* and *N. benthamiana*.

Serological tests. Indirect enzyme-linked immunosorbent assay (ELISA) was performed using extracts of healthy or infected *N. benthamiana* leaves and antisera to the potexviruses: *Alternanthera mosaic virus* (AltMV), *Bamboo mosaic virus* (BaMV), *Cactus virus X* (CVX), *Cassava common mosaic virus* (CsCMV), *Clover yellow mosaic virus* (ClYMV), *Cymbidium mosaic virus* (CymMV), Dioscorea latent virus (DLV), *Foxtail mosaic virus*

(FoMV), *Hosta virus X* (HVX), *Hydrangea ringspot virus* (HdRSV), *Narcissus mosaic virus* (NMV), *Nerine virus X* (NVX), *Papaya mosaic virus* (PapMV), *Potato virus X* (PVX), *Tulip virus X* (TVX), Viola mottle virus (VMoV), *White clover mosaic virus* (WClMV) and PVX-Brazilian isolate. All the antisera were diluted at 1/64, 1/512, 1/4,096, 1/32,768 and 1/262,144.

Aphid and seed transmission tests. In aphid transmission tests, *Aphis* sp. and *Myzus persicae* were starved for 30 min, allowed acquisition feeding for 30 min on experimentally infected *Philodendron* sp., and then allowed to feed for 12 h on healthy caladiums and philodendrons. Ten aphids were used per test plant.

Seed transmissibility was evaluated by collecting 600 seeds from infected *N. benthamiana* and germinating them in steamed soil in a greenhouse.

Virus infection was tested by inoculation to *G. globosa* and *N. benthamiana*.

Partial purification and coat protein analyses. *N. benthamiana* leaves, 14 to 17 days after inoculation, were ground in PB containing 0.5% Na_2SO_3, and the extract was centrifuged at 5,900 g for 10 min. The supernatant fraction was treated with chloroform (1:1, v:v) and virus particles were precipitated from the aqueous fraction by the addition of polyethylene glycol to 4% and NaCl to 0.6%. After centrifugation, the pellet was resuspended in PB, centrifuged threefold at 6,040 g for 10 min to clean the preparation and the three resulting supernatants were mixed and ultracentrifuged at 78,689 g for 120 min to concentrate the virus.

Virus capsid protein from partially purified virus preparations was analyzed by 12.5% SDS-PAGE gel electrophoresis (Laemmli, 1970). Protein bands were visualized by staining with Coomassie blue R-250.

RNA extraction, RT-PCR and sequencing. Virus RNA was extracted from partially purified virus by using Trizol[TM] (GIBCO-BRL, Rockville, MD, USA) according to the manufacturer's instructions. RT-PCR was performed using the primers and conditions described by Gibbs *et al.* (1998). The amplified fragment was fractionated in 2% agarose gel, purified and concentrated using the CONCERT Rapid Gel Extraction System (GIBCO-BRL, Rockville, MD, USA) and cloned in a pGEM-T vector (Promega Corp., Madison, USA), according to the manufacturer's instructions. The nucleotide sequences were determined by automated DNA sequencing (ABI PRISM 377 DNA Sequencers, PE Applied Biosystems, Foster City, CA, USA).

Sequence analysis and phylogeny. The putative encoded amino acid sequence was deduced from the nucleotide sequence by using a DNA sequence translation program (http://www.expasy.ch/tools/dna.html). Nucleotide and

amino acid sequences were then submitted to the basic local alignment search tool (BLAST) (Altschul *et al.*, 1997). Partial putative CalVX ORF 1 and the corresponding regions from 18 potexviruses and *Potato virus M* (PVM - *Carlavirus*, family *Flexiviridae*) were aligned by eye using the program Se-Al, Version 1.0 alpha 1 (Rambaut, 1996). Nucleotide sequences used for phylogenetic analysis were obtained from GenBank: NC001642 (BaMV), NC002815 (CVX), NC001658 (CsCMV), NC001753 (ClYMV), AF016914 (CymMV), NC001483 (FoMV), NC001441 (NMV), AY366209 (Opuntia virus X - OpVX), NC001748 (PapMV), NC004067 (*Pepino mosaic virus* - PepMV), Z21647 (*Plantago asiatica mosaic virus* - PlAMV), S73580 (*Potato aucuba mosaic virus* - PAMV), AF111193 (PVX), NC003400 (*Scallion virus X* - ScaVX), D12517 (*Strawberry mild yellow edge virus* - SMYEV), NC004322 (TVX), AY366208 (*Zygocactus virus X* - ZyVX), X16636 (WClMV), and NC001361 (PVM).

PAUP* version 4.0b10 for Macintosh (Swofford, 2002) was used to determine the sequence identities.

To determine phylogenetic relationships of the CalVX sequence, two character-based methods were used, maximum parsimony (MP) and maximum likelihood (ML).

PAUP* version 4.0b10 was also employed for the tree phylogenetic reconstructions using nucleotide sequences, assuming a monophyletic origin for the outgroup (PVM). Sites containing gaps were treated as new state ("fifth base").

In MP analysis with full heuristic search, all molecular characters were assessed as independent unordered and equally weighted. The bootstrap percentages were computed after 1,000 resamplings, followed by an MP reconstruction and MAXTREES = 100 supported the robustness of the nodes of the phylogenetic tree.

The nucleotide substitution model, the proportion of invariable sites (I) and the gamma distribution (G) were determined by likelihood ratio test (Huelsenbeck and Rannala, 1997) in Modeltest version 3.06 (Posada and Crandall, 1998).

As the parsimony method did not correct for multiple superimposed substitutions, ML criterion was used. ML analysis was performed using star decomposition, TBR swapping algorithms, and the Tamura and Nei (1993) (TrN+I+G) model of sequence evolution.

RESULTS

Morphology and cytopathology. CalVX has flexuous filamentous particles mostly measuring 420 to 479 nm. In naturally infected *C. bicolor*, cells contained two different inclusions. One was a cytoplasmic cylindrical inclusion, like those induced by members of the family *Potyviridae* that was serologically identified as being DsMV-specific (data not shown). The other inclusions were masses of aggregates of virus-like particles in the cytoplasm (Fig. 2). In *Gomphrena* mesophyll cells, CalVX induced masses of virus particles and also electron-dense crystalline inclusions in the cytoplasm (Fig. 3A) and nucleus (Fig. 3B).
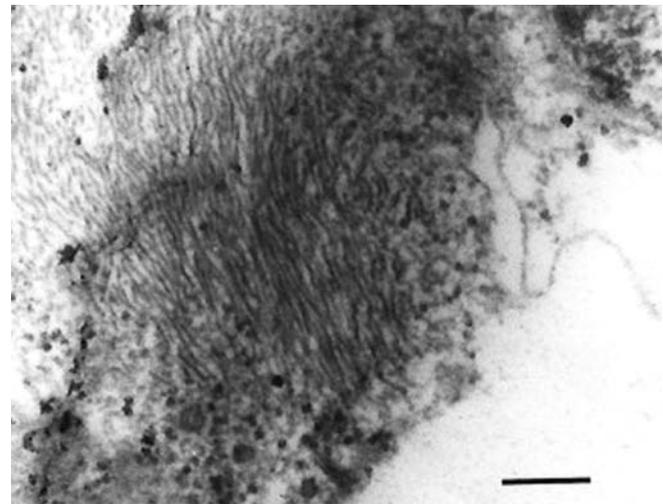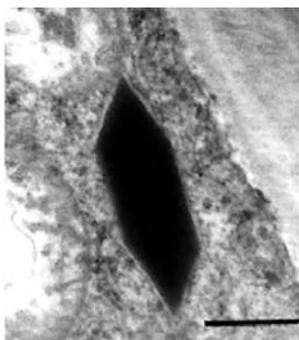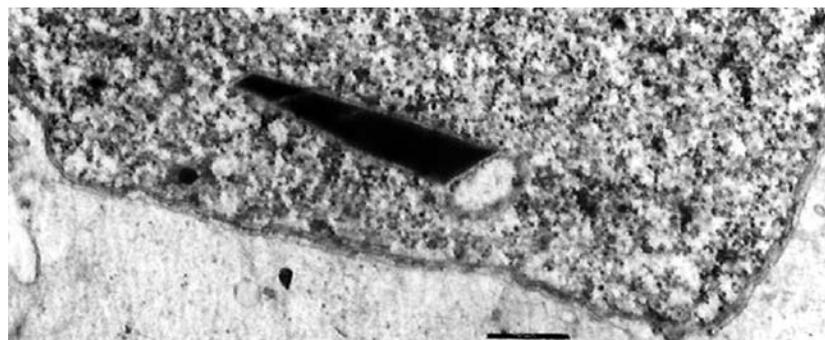

Fig. 2. Cross section of CalVX-naturally infected caladium mesophyll cell showing a mass of elongated virus-like particles in the cytoplasm. Bar equals 200 nm.

Fig. 3. Cross sections of electron-dense crystalline inclusions in mesophyll cells of a local lesion area from *Gomphrena globosa* inoculated with CalVX; **A**) inclusion in the cytoplasm; **B**) inclusion in the nucleus. Bar equals 500 nm.

Table 1. Host range and symptoms induced by CalVX

| Host	Symptoms [a]
Amaranthaceae	
Gomphrena globosa	L: necrotic spot with red halo
Araceae	
Caladium bicolor	S: chlorotic spots, green rings, mosaic
Caladium humboldtii	S: red pin-points, degeneracy
Colocasia esculenta	L: dark green rings and line-patterns
	S: chlorotic line-patterns
Philodendron sp.	L: chlorotic spots
	S: yellow vein-banding, chlorotic spots and rings
Chenopodiaceae	
Chenopodium amaranticolor	L: necrotic spots
Chenopodium murale	L: chlorotic spots, necrotic points
Chenopodium polyspermum	L: necrotic ringspots
Chenopodium quinoa	L: chlorotic spots
Plantaginaceae	
Plantago sp.	L: necrotic rings
Portulacaceae	
Portulaca oleracea var. *sativa*	L: necrotic spots
Solanaceae	
Datura stramonium	L: necrotic pin-points
Nicotiana benthamiana	L: necrotic ringspots and spots
	S: necrotic streak, necrotic pin-points, mild leaf deformation

[a] L= Local symptom, S= Systemic symptom.
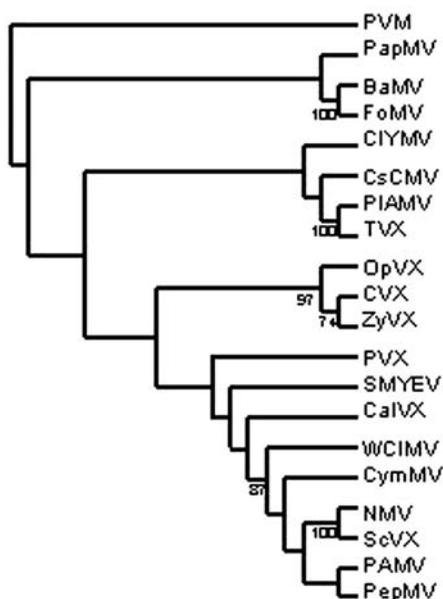
Fig. 4. Maximum likelihood phylogram. Values near nodes are bootstrap. The likelihood value obtained was –lnL= 12109.3887. PVM used as the outgroup.

Transmission tests. CalVX was easily transmissible mechanically from both natural and experimental hosts. CalVX was transmitted to 13 species (Table 1) but not to *Dieffenbachia* sp. and *Anthurium andraeanum*. It is noteworthy that the symptoms observed in *C. bicolor* experimentally infected by CalVX were different from the original symptoms, varying from mosaic to green rings. The symptoms in experimentally infected aroids characteristically tend to disappear. The virus did not infect species from the families Aizoaceae (*Tetragonia expansa*), Amaranthaceae (*Amaranthus hybridus*), Apocynaceae (*Catharanthus roseus*), Asteraceae (*Coreopsis lanceolata*, *Galinsoga* sp., *Lactuca sativa*, *Zinnia elegans*), Balsaminaceae (*Balsamina balsamina*, *Impatiens walleriana*), Brassicaceae (*Raphanus sativus*, *Sinapis alba*), Caesalpinaceae (*Cassia occidentalis*, *Senna macranthera*), Cucurbitaceae (*Citrullus vulgaris*, *Cucumis sativus*, *Luffa acutangula*), Euphorbiaceae (*Phyllanthus niruri*), Fabaceae (*Glycine javanica*, *Glycine max*, *Phaseolus vulgaris*, *Phaseolus lunatus*, *Vigna sinensis*), Liliaceae (*Hemerocallis flava*), Poaceae (*Zea mays*), Rosaceae (*Fragaria vesca*), Scrophulariaceae (*Anthirrhinum majus*) and Solanaceae (*Capsicum annuum*, *Datura metel*, *Lycopersicon esculentum*, *Nicandra physalodes*, *Nicotiana clevelandii*, *Nicotiana debneyi*, *Nicotiana glutinosa*, *Nicotiana rustica*, *Nicotiana sylvestris*, *Nicotiana tabacum* 'Samsun' and 'White Burley', *Petunia hybrida*, *Physalis alkekengi*, *Physalis floridana*, *Solanum luteum*, and *Solanum melongena*).

Aphids did not transmit CalVX to caladium or philodendron plants. No seed transmission was observed in tests from *N. benthamiana* experimentally infected by CalVX.

Serology and physicochemical properties. CalVX did not show serological relationships with the 17 potexviruses assayed. The capsid protein migrated as a single polypeptide species with an apparent molecular mass of 26,000. CalVX has a nucleic acid content estimated at 6.7%. In crude sap, CalVX has a thermal inactivation point of 60°C to 65°C, a dilution end-point of 10^{-2} to 10^{-3} and was infective for 6 days at room temperature.

Sequence analyses and phylogeny. A fragment of 740 bp (GenBank accession number AY727533) was obtained from CalVX RNA. The fragment contained the characteristic motifs conserved among the alpha-like supergroup of RNA polymerases (Koonin 1991), shared significant nucleotide identity with the RdRp domain of potexviruses, ranging from 65.8% (CymMV) to 60.3% (CsCMV). Analyses of the corresponding 246 amino acids showed that identity between CalVX and other potexviruses varied from 68.0% (SMYEV) to 58.4% (FoMV).

Phylograms based on MP or ML criteria, produced similar subtrees. Subtrees involving (NMV, ScaVX), (PlAMV, TVX), (BaMV, FoMV), and viruses from Cac-

taceae (CVX, ZyVX, and OpVX) were present in MP (data not shown) and ML trees (Fig. 4), and bootstraps resampling indicated strong support for the monophyly of these subtrees. The group formed by lineages CymMV, PAMV, PepMV, WClMV, NMV and ScVX shared a common ancestor with CalVX (Fig. 4).

DISCUSSION

Many virus diseases of ornamental plants can be attributed to a synergistic interaction between two or more viruses, whether related or not, in the same plant. In aroids, DsMV, when in mixed infection, has been reported only with other potyviruses (Lesemann and Winter, 2002), thus this paper is the first report of DsMV in mixed infection with a potexvirus.

The first evidence of mixed infection came from electron microscope observations. Ultrathin sections from naturally infected leaf tissues of *C. bicolor* revealed the presence of the cylindrical inclusions typical of potyvirus-infected cells (data not shown), in the same cells as, or in different cells from, those in which putative CalVX-particles aggregates were present. Moreover, the chlorotic-spot and necrotic ringspot symptoms observed in *C. bicolor* leaves could be a synergistic effect between DsMV and CalVX, because *C. bicolor* singly infected with CalVX did not show the symptoms observed in the original plant.

The distribution curve for the lengths of CalVX particles was compatible with that of potexvirus particles, which are the smallest for viruses in the family *Flexiviridae* (Adams *et al.*, 2004).

Potexviruses infect hosts in a wide range of families, although the host range of each species of the genus is limited (Brunt *et al.*, 2000). Monocotyledonous families containing plants naturally infected by potexviruses include: Alliaceae, Amaryllidaceae, Asparagaceae, Commelinaceae, Cyperaceae, Dioscoreaceae, Hostaceae, Liliaceae, Musaceae, Orchidaceae, and Poaceae (Purcifull and Edwardson, 1981; Che *et al.*, 2002). Thus, this is the first report of a plant in the Araceae being infected by a potexvirus.

Our results indicate that CalVX is a previously unknown distinct species of the genus *Potexvirus*, family *Flexiviridae*, so far found only in a *C. bicolor* plant.

The virus has a limited host range, locally infects Gomphrena (as do most potexviruses) and has a host range to similar to that of CsCMV, a species first reported in Brazil (Silberschmidt, 1938). However, CalVX shows no serological relationships with CsCMV or 16 other potexviruses. Cytopathic alterations include masses of virus particles in the cytoplasm, as in cells infected with most potexviruses, and electron-dense crystalline inclusions in nuclei and cytoplasm, as observed in BaMV infection (Kitajima *et al.*, 1977), another potexvirus also reported in Brazil.

Using primers specific for viruses in the genus *Potexvirus* (Gibbs *et al.*, 1998), it was possible to amplify a product from RNA extracts of plants infected with CalVX whose characteristics are consistent with those of the RdRp domain and that shows a clear sequence relationship between CalVX and potexviruses.

Adams *et al.* (2004) established identity values for *Potexvirus* species demarcation using replicase sequences of between 45.2% and 70.1% at the nucleotide level and between 40.4% and 73.3% at the amino acid level. The results presented here confirm the position of CalVX in the genus *Potexvirus*.

Considering biological and physico-chemical properties, and the fact that the origin of CalVX shares a common ancestor with six lineages of *Potexvirus* (CymMV, PAMV, PepMV, WClMV, NMV and ScVX), we propose that CalVX belongs to the genus *Potexvirus.*

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