Characterization of *Canna yellow mottle virus* in a New Host, *Alpinia purpurata*, in Hawaii

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ABSTRACT

Canna yellow mottle virus (CaYMV) is an important badnavirus infecting *Canna* spp. worldwide. This is the first report of CaYMV in flowering ginger (*Alpinia purpurata*) in Hawaii, where it is associated with yellow mottling and necrosis of leaves, vein streaking, and stunted plants. We have sequenced CaYMV in *A. purpurata* (CaYMV-Ap) using a combination of next-generation sequencing and traditional Sanger sequencing techniques. The complete genome of CaYMV-Ap was 7,120 bp with

an organization typical of other *Badnavirus* species. Our results indicated that CaYMV-Ap was present in the episomal form in infected flowering ginger. We determined that this virus disease is prevalent in Hawaii and could potentially have significant economic impact on the marketing of *A. purpurata* as cut flowers. There is a potential concern that the host range of CaYMV-Ap may expand to include other important tropical plants.

Flowering ginger, *Alpinia purpurata* (Vieill.) K. Schum., produces small white flowers subtended by large, showy, red or pink bracts. It belongs to the diverse tropical plant family Zingiberaceae (Illg and Faria 1995). In Hawaii (United States), it is grown as an ornamental plant in landscapes and for its cut flowers, which had a commercial value of \$1.6 million in 2005 and 2006 (Paret et al. 2008). In previous studies, we have found *Banana bract mosaic virus* (BBRMV) infecting *A. purpurata* on Oahu, where it causes leaf mosaic and streaking (Wang et al. 2010; Zhang et al. 2016). However, other virus-like symptoms distinct from those produced by BBRMV were also observed on some plants, suggesting the presence of more than one virus.

Recent advances in next-generation sequencing (NGS) have made possible the detection of new infectious plant viruses in the genera *Closterovirus* (Melzer et al. 2013), *Begomovirus* (Idris et al. 2014), and *Ampelovirus* (Marais et al. 2014), as well as *Betaflexivirus* (Elbeaino et al. 2014). Information generated by NGS includes all nucleic acids present in the plant genome and also those from any infecting viruses, highlighting the value of NGS for virus discovery (Marais et al. 2015). This approach was used by us to search for other viruses that may coinfect *A. purpurata* in addition to BBRMV. NGS revealed sequences most similar to *Canna yellow mottle virus* (CaYMV; family *Caulimoviridae*, genus *Badnavirus*). We also detected CaYMV-like sequences in several *A. purpurata* plants that were not infected by any other plant viruses. These plants showed symptoms typical of CaYMV infections in *Canna* species, strongly suggesting that a CaYMV-like agent was associated with the severe symptoms in infected plants.

CaYMV was first reported infecting *Canna* spp. in Japan (Yamashita et al. 1979) before it was found in the United States (Lockhart 1988; Yamashita et al. 1985). The disease is now found

worldwide including in Italy, the Netherlands (Marino et al. 2008), India (Kumari et al. 2014), and Kenya (Agneroh et al. 2015). On the U.S. mainland, CaYMV infects *Canna indica* in several states, including Florida (Momol et al. 2004) and Washington (Pappu et al. 2008). The disease on *Canna* spp. causes substantial yellow mottling and necrosis of the foliage with chlorotic and necrotic vein streaking (Lockhart 1988). These foliar symptoms may often be combined with streaking on the stems and flowers (Marino et al. 2008). Stunting has been associated with infections by this virus (Yamashita et al. 1985), as has yellow vein mosaic of betel vine (*Piper betel*) in India (Kumari and Raj 2015).

CaYMV has not been reported previously infecting plants in Hawaii, nor has *A. purpurata* been reported previously as a host. This is the first report of CaYMV infecting any plants in Hawaii. In this study, we characterized the complete genome of CaYMV in *A. purpurata* and determined its phylogenetic placement among known badnaviruses. We also determined that the episomal form of this CaYMV isolate was associated with the severe symptoms on *A. purpurata*. This information may lead to a more effective, knowledge-based management of the disease in Hawaii. For the sake of discussion, we will refer to the CaYMV isolate in *A. purpurata* as CaYMV-Ap in this manuscript.

MATERIALS AND METHODS

Virus characterization. Leaves of *A. purpurata* with virus-like symptoms were collected from the same site on the University of Hawaii at Manoa campus in Honolulu where BBRMV was first found in 2009 (Wang et al. 2010; Zhang et al. 2016). Double-stranded RNAs (dsRNAs) from symptomatic leaves were isolated and used to synthesize cDNA (Melzer et al. 2010) following the procedures of Morris and Dodds (1979) as modified by Hu et al. (1993). The double-stranded cDNA library was generated using the dsRNA templates as described by Zhang et al. (2016). Size-fractionated PCR products were directly sequenced on an NGS machine, the Ion PGM System (Ion 314 Chip, ThermoFisher Scientific, MA), at the University of Hawaii's Advanced Studies of Genomics, Proteomics, and Bioinformatics

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary files and two supplementary figures are published online.

Laboratory (ASGPB). Short-length (<65 nt) and low-quality reads and primer sequences were trimmed, as were low-quality basecalls at the ends of reads. The remaining high-quality reads were assembled using the de-novo assembly program in GeneiousPro 7.1.5 (Biomatters Ltd., Auckland, New Zealand), and the assembled contig sequences subjected to BLAST analysis against nucleotide sequences in NCBI.

Whole genome sequencing of CaYMV-Ap. To determine the complete genome sequence of this DNA virus, 12 primer pairs (Table 1) were designed based on a contig sequence from NGS and were used to amplify overlapping fragments spanning the whole genome. Total DNA was isolated with the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. PCR amplifications were carried out in 20-µl reactions containing 1.0 µl of DNA template and 10 µl of reaction mixture with Phusion High-Fidelity DNA Polymerase (New England Biolabs) and 1.0 µl forward/reverse primers each at 10 µM. The mixture was incubated at 98°C for 3 min, followed by 35 cycles of amplification at 98°C for 30 s, 55°C for 30 s, 68°C for 60 s, with a final extension at 68°C for 10 min. PolyA-tails were added by PCR using Taq DNA polymerase and dATP and the amplicons were then ligated into pGEM-T Easy. The cloned PCR products were Sanger-sequenced at ASGPB.

Phylogenetic analysis. Thirty-eight complete genome sequences of *Badnavirus* (Supplementary File S1) were retrieved from NCBI for phylogenetic analysis using *Cauliflower mosaic virus* (CaMV, GenBank accession NC_001497.1) as the out-group. All sequences were aligned using ClustalW (Higgins et al. 1994). Phylogenetic analysis was performed with Mega 5.0 (Tamura et al. 2011) using maximum likelihood algorithms boot-strapped with 1,000 replications. We also performed a phylogenetic analysis using amino acid sequences from open reading frame (ORF)3 of CaYMV-Ap and multiple badnavirus sequences (Supplementary File S2) together with the out-group, *Rice tungro bacilliform virus* (RTBV, NC_001914.1).

CaYMV-Ap and BBrMV detection by PCR and RT-PCR. Universal primer pair BadnaFP/RP for detecting viruses of the genus

Badnavirus (Yang et al. 2003) and the specific primers pair CaYMV-3/4 (Momol et al. 2004) were used to detect CaYMV-Ap from the plants in this study. We also developed the specific primer pair CaYMV-1931/2598, which can discriminate CaYMV-Ap from other badnaviruses, such as *Taro bacilliform virus* (Supplementary Fig. S1). Twenty microliters of PCR reaction conditions were assembled as follows: 10 µl of 2× GoTaq Green master mix (Promega), 0.5 µl of forward/reverse primers each at 10 µM, 1.0 µl of DNA, and 8 µl of nanopure H₂O. The cycling program was as follows: 95°C for 5 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and then 72°C for 10 min.

To detect CaYMV-Ap by RT-PCR, the specific primer pair CaYMV-1931/2598 was used. The potyvirus-specific primer pair Nib2F/3R (Zheng et al. 2008; Zhang et al. 2016) was used to detect BBrMV by RT-PCR. Total RNA was isolated using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany) including DNase treatment, according to the manufacturer's instructions. First-strand cDNA synthesis was initiated with 2 µl of DNase-treated RNAs and 1.0 µl of random primers (50 ng/µl) (Promega), heated at 70°C for 5 min, and then quenched on ice. Then, 5 µl of M-MLV 5× reaction buffer (Promega), 5 µl of dNTPs (2.5 µM each), 0.5 µl of recombinant RNasin ribonuclease inhibitor (40 U/µl), and 1.0 µl of M-MLV reverse transcription (200 U/µl) were added and the reaction brought to a final volume of 25 µl with nuclease-free water. This assembled reaction was incubated for 60 min at 37°C. The PCR reaction conditions were as follows: 10 µl of 2× GoTaq Green master mix (Promega), 0.5 µl of forward/reverse primers each at 10 µM, 1.0 µl of cDNA, and 8 µl of nanopure H₂O. The cycling program was as follows: 95°C for 5 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and then 72°C for 10 min.

Transmission electron microscopy (TEM). Leaf samples from symptomatic *A. purpurata* plants that tested positive for CaYMV-Ap with PCR were examined by TEM using either the leaf-dip method or a modified partial purification method (Ahlawat

TABLE 1. Primers used for virus detection, and Sanger sequencing including overlapping fragments cloning and subsequencing

Primer	Sequence	Use	Reference
BadnaFP	5'-ATGCCITTYGGIAARAAYGCICC-3'	<i>Badnavirus</i> detection	Yang et al. 2003
BadnaRP	5'-CCAYTTRCAIACISICCCCAICC-3'	<i>Badnavirus</i> detection	Yang et al. 2003
CaYMV-3	5'-GACTTCCTGGGTGCAACAAT-3'	CaYMV detection	Momol et al. 2004
CaYMV-4	5'-TCTGTGCAATCTTGGCGTAG-3'	CaYMV detection	Momol et al. 2004
CaYMV-1931	5'-TGCTGGAACACTGGCTTCA-3'	CaYMV detection	This study
CaYMV-2598	5'-CCTCTTCATCCCCACCAAC-3'	CaYMV detection	This study
Nib2F	5'-GTITGYGTIGAYGAYTTYAAYAA-3'	Potyvirus detection	Zheng et al. 2008
Nib3R	5'-TCIACIACIGTIGAIGGYTGNCC-3'	Potyvirus detection	Zheng et al. 2008
CaYMV-1F (6172)	5'-GTGGCATCTGCAAGTGGAAG-3'	Sanger sequencing	This study
CaYMV-1R (6872)	5'-GCCCAACGACACCATAACT-3'	Sanger sequencing	This study
CaYMV-2F (5627)	5'-GAATGCACCTGGGTTCCTCC-3'	Sanger sequencing	This study
CaYMV-2R (6331)	5'-TCTGATTGTGAGCGCCTTCT-3'	Sanger sequencing	This study
CaYMV-3F (4907)	5'-AAGCCTCGAAGGTGGAGTTC-3'	Sanger sequencing	This study
CaYMV-3R (5656)	5'-CCCCCTAAAACAACCGTCCA-3'	Sanger sequencing	This study
CaYMV-4F (4276)	5'-CCCCGAGACGAGATCAAGCAAT-3'	Sanger sequencing	This study
CaYMV-4R (4989)	5'-GAGGCCACAGTGAATGCCT-3'	Sanger sequencing	This study
CaYMV-5F (3707)	5'-CAAATGCTATGCGTGCGGAG-3'	Sanger sequencing	This study
CaYMV-5R (4405)	5'-CTTGTTTTTGGCCTGCTCCT-3'	Sanger sequencing	This study
CaYMV-6F (3036)	5'-AAGAAGTTCCCGACAGCCG-3'	Sanger sequencing	This study
CaYMV-6R (3808)	5'-CACCACCTCCATTCCTGCTT-3'	Sanger sequencing	This study
CaYMV-7F (2469)	5'-CTCGTGGCATTCCTGGATGT-3'	Sanger sequencing	This study
CaYMV-7R (3247)	5'-GTAAGCGGCATCCTGGGTTA-3'	Sanger sequencing	This study
CaYMV-8F (1787)	5'-AGAACGTATGCTGGTGCTCG-3'	Sanger sequencing	This study
CaYMV-8R (2653)	5'-TCCGAATTGCTTGGACTCGT-3'	Sanger sequencing	This study
CaYMV-9F (1104)	5'-ACTAGCGGCGACAACCTCAAG-3'	Sanger sequencing	This study
CaYMV-9R (1863)	5'-ACGTACTCGAATCCTGCCCT-3'	Sanger sequencing	This study
CaYMV-10F (463)	5'-TCGGAGGACGAAGTAGGACA-3'	Sanger sequencing	This study
CaYMV-10R (1256)	5'-AACTTGTCGTCGAGAGAGGC-3'	Sanger sequencing	This study
CaYMV-11F (6951)	5'-CCCTCTCGTTGCTAGGCAT-3'	Sanger sequencing	This study
CaYMV-11R (678)	5'-AGGTCGTTACGATAACACAGAA-3'	Sanger sequencing	This study
CaYMV-12F (6490)	5'-ACGTTCTCGCCGATACACTG-3'	Sanger sequencing	This study
CaYMV-12R (279)	5'-TATACGGCTTCCTCGCCTTC-3'	Sanger sequencing	This study

et al. 1996). Five-gram leaf samples were powdered in liquid nitrogen and extracted with 18 ml of 200 mM phosphate buffer. The extract was filtered through cheesecloth and centrifuged at $12,000 \times g$ for 10 min and the pellet discarded. Triton X-100 was added to the supernatant to a final concentration of 2% and the mixture was layered over a 5-ml cushion of 30% sucrose in 100 mM phosphate buffer, pH 7.2, and centrifuged for 1 h at $100,000 \times g$ in a Beckman type 70Ti rotor. The pellets were resuspended in 100 μ l of 10 mM phosphate buffer and 150 mM NaCl, pH 7.2. The suspension was clarified by shaking briefly with 50 μ l of chloroform, followed by centrifuging for 6 min at $12,400 \times g$. The upper aqueous phase was examined by TEM after staining in 1% uranyl acetate.

Assessment of CaYMV-Ap incidence in *A. purpurata* in Hawaii. To screen for CaYMV-Ap, *A. purpurata* plants with and without virus-like symptoms were collected from seven locations on the island of Oahu and one location on the island of Hawaii. DNA from 87 collected samples was screened by PCR using the primer pair CaYMV-3/4.

RESULTS

Draft genome of CaYMV-Ap by NGS. A total of 289,674 high-quality reads were generated from the cDNA library, with a median length of 217 bp. Of these reads, 44,586 and 19,528 were both de novo assembled into contigs that mapped to the genome of BBBrMV, the complete genome of which was validated to be 9,713 bp (GenBank accession KT456531) (Zhang et al. 2016). Our NGS analysis also generated another 453 high-quality reads that were de novo assembled into a contig of 6,961 bp (Fig. 1). This contig had 99% identity to a partial gene sequence within the RT-RNase H region of the CaYMV polyprotein in GenBank (accessions HE774733.1 and HE774735.1). The draft genome of CaYMV-Ap is double-stranded DNA (dsDNA) and contains a gap between the termini of its noncircular contig sequence (Fig. 1). To confirm that the CaYMV-Ap sequence is indeed present in the symptomatic *A. purpurata* plants, we used the universal primer pair BadnaFP/RP and the specific primer pair CaYMV-3/4 to

screen for the badnavirus in *A. purpurata* showing symptoms of foliar necrosis and yellow mottling. All eight symptomatic samples collected from the same site that the samples used for NGS were collected and tested positive (Fig. 2A and B).

Whole-genome sequencing and genome annotation. The complete genome sequence of CaYMV-Ap from symptomatic *A. purpurata* plants was assembled from NGS (6,961 bp), and completed and confirmed by Sanger sequencing of 12 overlapping PCR fragments. The complete genome (7,210 bp) was generated and deposited in GenBank (accession number KU168312). The CaYMV-Ap genome has an organization typical of other *Badnavirus* species (Fig. 3), beginning with the conserved sequence (TGGTATCA GAGC) of the tRNA^{MET} primer-binding site, followed by three predicted ORFs. The tRNA^{MET} primer-binding site is considered the starting point for minus-strand synthesis during the replication of other badnavirus isolates (Bouhida et al. 1993). Upstream of the tRNA^{MET} primer-binding site, a typical TATA-box (TATATAA) was found at position 6,935 to 6,941 bp. This region is considered part of the

Fig. 1. Sequence coverage of the *Canna yellow mottle virus* (CaYMV)-Ap contig (6,961 bp) revealed by next-generation sequencing and its corresponding site (5'-3': 138 to 7,070 bp) in the complete genome of CaYMV-Ap. The region at the top indicates the gap in the circular genome, and the numbers 138 and 7,070 (arrows indicating the gap termini) correspond to the sites in the complete genome of CaYMV-Ap.

Fig. 2. PCR detection using badnavirus universal primers and *Canna yellow mottle virus* (CaYMV)-specific primers. **A**, PCR detection from eight symptomatic samples using the badnavirus universal primer pair BadnaFP/RP. **B**, PCR detection from eight symptomatic samples using CaYMV-specific primer pair CaYMV-3/4. In both panels, C1 to C8 = symptomatic samples, N = no template control, and M = molecular weight markers.

Fig. 3. Genome organization of *Canna yellow mottle virus* (CaYMV)-Ap. Genome replication is initiated at the tRNA^{MET} primer-binding site followed by three predicted open reading frames (ORFs), and a typical TATA-box located upstream of the tRNA^{MET} primer-binding site. A polypeptide encoded by ORF3 harbored motifs for the movement protein (MP), zf-CCHC in the coat protein (CP), aspartic protease, reverse transcriptases (RT), and RNaseH domains.

promoter region used in badnavirus replication (Medberry et al. 1992). ORF1, ORF2, and ORF3 are located at sites 542 to 1,060 bp, 1,060 to 1,461 bp, and 1,461 to 6,677 bp, respectively, with an overlap at the ends of adjoining ORFs (both junctions had the sequence TG ATG). ORF1 putatively encodes a protein of 173 amino acids with a molecular weight of 19.8 kDa; ORF2 putatively encodes a protein of 134 amino acids with a molecular weight of 14.7 kDa; and ORF3 putatively encodes a protein of 1,739 amino acids with a molecular weight of 198.5 kDa. The ORF1 protein is predicted to be in the DUF1319 super family containing a number of viral proteins of approximately 200 residues, the function of which is not known (NCBI's conserved domain database). The protein encoded by ORF2 is predicted to contain putative DNA binding sites as observed in *Cacao swollen shoot virus* (Jacquot et al. 1996). ORF3 encodes a

polyprotein harboring motifs for the movement protein (MP; Arg127 to Arg215), zf-CCHC in the coat protein (CP; Cys749 to Asn766), aspartic protease (Ile1055 to Leu1151), reverse transcriptases (RT; Lys1278 to Gly1461), and RNaseH domains (Ile1560 to Val1687) (Fig. 3).

Phylogenetic analysis. A phylogenetic analysis was conducted using the whole genome sequences of 38 *Badnavirus* species (Fig. 4A), with CaMV as the out-group. The phylogenetic tree confirmed the taxonomic placement of CaYMV-Ap within the genus *Badnavirus*. CaYMV-Ap was most closely related to *Sugarcane bacilliform virus* (ScBV), *Banana streak Mysore virus* (BSMysV), and *Pineapple bacilliform comosus virus* (PBCoV). These viruses constituted a clade that also included other isolates of *Banana streak virus* (BSV). They showed a high genome nucleotide divergence, however, with

Fig. 4. Phylogenetic analyses of **A**, the complete genome sequence and **B**, amino acid sequences of the ORF3 polyprotein from members of the genus *Badnavirus*. The virus names and sequences are presented in Supplementary Files S1 and S2. The analysis used maximum likelihood algorithms bootstrapped with 1,000 replications. Numbers at nodes indicate the level of bootstrap support for each node.

CaYMV-Ap presenting overall nucleotide identities of only 57.3, 53.1, and 50.2% with ScBV, BSMysV, and PBCoV, respectively. ORF1 of CaYMV-Ap shared 55.5, 49.4, and 55.3% nucleotide identity, respectively, with ScBV, BSMysV, and PBCoV; ORF2 shared 60.7, 54.5, and 43.2% nucleotide identity, respectively, with ScBV, BSMysV, and PBCoV; and ORF3 shared 64.9, 59.2, and 60.03% nucleotide identity, respectively, with ScBV, BSMysV, and PBCoV. Within the RT-RNase H region of ORF3, CaYMV-Ap shares 76% identity with ScBV, 75% identity with PBCoV, and 70% identity with BSMysV. A phylogenetic analysis was also conducted using the amino acid sequences of ORF3 (Fig. 4B). Again, CaYMV-Ap was most closely related to ScBV, BSMysV, and PBCoV using this approach.

Evidence of the episomal form of CaYMV-Ap in *A. purpurata*. We used PCR and RT-PCR to investigate whether CaYMV-Ap was present in the episomal form. Leaves from symptomatic plants all tested positive by PCR using the specific primer pair CaYMV-3/4 (Fig. 5A, lanes 1 to 7) and the newly designed specific primer pair CaYMV-1931/2598 (Fig. 5B, lanes 1 to 7). Asymptomatic leaves from other plants produced no amplicons even though they were collected from the same location as leaf samples that tested positive (Fig. 5A and 5B, lane H). Six of seven symptomatic samples tested positive using RT-PCR (Fig. 5C).

In addition, partially purified preparations from symptomatic *A. purpurata* leaves that were also used to generate the NGS contigs and tested positive for CaYMV-Ap in PCR were examined by TEM. Typical badnavirus-like bacilliform particles of approximately 120 × 30 nm were observed (Fig. 6).

Symptoms on *A. purpurata* associated with CaYMV-Ap infections. To explore the association between disease symptoms on *A. purpurata* and samples that tested positive for CaYMV-Ap, we collected additional samples from the previously sampled collection sites and the other sites. Most samples from the previous collection sites tested positive for CaYMV-Ap using PCR (Fig. 5A, B, lanes 1 to 15). Of these PCR-positive samples (1 to 15), CaYMV-Ap could be detected in 13 samples using RT-PCR (Fig. 5C). Only one site (collection site 1) contained plants that were also positive for BBrMV by RT-PCR (data not shown). The CaYMV-Ap-positive samples had foliar symptoms similar to those described previously for CaYMV on *Canna* spp. The symptoms included yellow mottling and necrosis (Fig. 7A), and vein streaking (Fig. 7B) of leaves (Lockhart 1988). Additionally, these plants were stunted (Fig. 7C), with a reduced

flower size. All asymptomatic plants (Fig. 7D) tested negative for CaYMV-Ap using PCR and RT-PCR.

Plants from the location (collection site 1) that were coinfecting with BBrMV and CaYMV-Ap displayed different symptoms (Fig. 8A). Samples 1, 2, and 8 had diffuse chlorotic and necrotic spots on mottled leaves and were CaYMV-Ap positive and BBrMV negative with PCR and RT-PCR, respectively (Fig. 8B and C). Samples 9 and 10 showed foliar mosaic and streaking typical of BBrMV infections (Wang et al. 2010) and also tested BBrMV positive by RT-PCR and weakly CaYMV-Ap positive by PCR. Samples 3, 4, 11, and 12 were all positive for both CaYMV-Ap and BBrMV and displayed the most severe symptoms. These results suggested that on *A. purpurata* CaYMV-Ap may exacerbate the disease symptoms caused by BBrMV alone.

Assessment of CaYMV-Ap incidence in *A. purpurata* in Hawaii. To investigate the distribution of CaYMV-Ap in Hawaii, we collected samples of *A. purpurata* with and without CaYMV-Ap-associated symptoms from the islands of Oahu and Hawaii. Plant samples from 4 of 7 locations (locations 1, 2, 3, and 7) on Oahu with severe previously described symptoms of CaYMV-Ap were positive for CaYMV-Ap in PCR analyses (Table 2). Asymptomatic plants collected from the other locations on Oahu (locations 4, 5, and 6) and the location on the island of Hawaii were negative for CaYMV-Ap in PCR analyses.

DISCUSSION

In previous work, we used dsRNA isolated from a single common green ti plant (*Cordyline fruticosa* L.) in Hawaii and obtained the genomes of four closely related closteroviruses (Melzer et al. 2013). In the present study, we used NGS to analyze virus infections in *A. purpurata* and were able to obtain the complete genome sequence of the potyvirus BBrMV infecting *A. purpurata* (Zhang et al. 2016). NGS analysis of diseased *A. purpurata* plants in Hawaii produced 289,674 sequence reads from a cDNA library generated from symptomatic plants. Unexpectedly, we found 453 reads matching the genus *Badnavirus*. These reads may have been produced from residual DNAs present in the dsRNA preparations since these preparations were not treated with DNase. Assembly of these reads into a large contig (6,961 bp) revealed a sequence highly similar to partial sequences of a polyprotein gene from CaYMV. We also performed rolling-circle amplification (RCA) analyses to identify circular dsDNA sequences present in the symptomatic *A. purpurata* plants. The RCA results revealed

Fig. 5. PCR and RT-PCR detection of *Canna yellow mottle virus* (CaYMV)-Ap from samples collected from different locations. **A**, PCR detection using the specific primer pair CaYMV-3/4. **B**, PCR detection using the specific primer pair CaYMV-1931/2598. **C**, RT-PCR detection using the specific primer pair CaYMV-1931/2598. M = molecular weight markers. Lanes 1 to 21 = tested samples, H = asymptomatic samples, P = CaYMV-Ap positive control, and N = no template control. Of the three collection sites on the University of Hawaii at Manoa campus, plants from site 1 (sample numbers 1 to 4, H, 8 to 12) were coinfecting with both *Banana bract mosaic virus* and CaYMV-Ap, and plants from site 2 (sample numbers 5, 6, 13, and 14) and from site 3 (sample number 15) were infected with CaYMV-Ap only. Off-campus collection sites included site 4 (sample number 7) and sites 5 to 8 (negative sample numbers 16 to 21).

circular fragments of about 7 kb in length, corresponding to the genome size of badnaviruses (data not shown). Based upon the detection of a circular double-stranded DNA sequence, we then proceeded to complete the whole genome (7,210 bp) of CaYMV-Ap by designing primers using the NGS data and filling gaps in the contig using traditional Sanger sequencing.

Typical symptoms on *Canna* caused by CaYMV include chlorosis, mottling, necrosis, and streaking on leaves (Lockhart 1988), as well as stunted growth (Yamashita et al. 1985). We observed a similar range of symptoms on *A. purpurata* in Hawaii, suggesting that the CaYMV-Ap detected might also be involved in this disease of *A. purpurata*. Although the presence of BBrMV coinfecting with CaYMV-Ap in some symptomatic plants complicated visual diagnosis, results of our molecular analyses indicate that CaYMV-Ap is more prevalent than

Fig. 6. Bacilliform virions of *Canna yellow mottle virus* (CaYMV)-Ap negatively stained with 1% uranyl acetate. Scale bar = 200 nm. Arrows indicate putative badnavirus particles.

BBrMV in symptomatic plants (Fig. 8). Coinfection by both CaYMV-Ap and BBrMV in *A. purpurata* may produce more severe foliar symptoms than infections with either virus alone (Fig. 8). Mixed infections between badnaviruses and other viruses have been reported (Laney et al. 2012), including BSV and *Cucumber mosaic virus* (CMV) in banana (Brioso et al. 2000; Carnelossi et al. 2014), and *Rubus yellow net virus* and other unrelated viruses in *Rubus* spp. (Jones et al. 2002). CaYMV has also been found in mixed infections with CMV and a flexuous rod-shaped virus that may have been *Bean yellow mosaic virus* (BYMV) or *Canna yellow streak virus* (CaYSV) (Lockhart 1988). CaYMV has also been found in triple infections with BYMV and CaYSV (Chauhan et al. 2015).

In the family *Caulimoviridae*, the genera *Badnavirus* and *Tungrovirus* have bacilliform virions with particle lengths of 100 to 130 × 30 nm (Hull et al. 2005), similar to what we found in *A. purpurata* preparations in this study. In this study, we determined the complete genome of CaYMV-Ap infecting *A. purpurata*, and found the sequence to be highly similar to the partial CaYMV sequences deposited in GenBank from previous studies (Marino et al. 2008; Kumari et al. 2014; Agneroh et al. 2015). Partial sequences of CaYMV isolates infecting *Piper betel* were also found to be highly homologous (96 to 99% identity) to the published partial sequences of CaYMV (Kumari and Raj 2015). This result, together with our findings that CaYMV-Ap shares high sequence homology to the published partial sequences of CaYMV in *Canna*, indicate that CaYMV from different hosts can still share a high degree of similarity in their genome sequences. The complete genomic sequences of three badnavirus isolates from *Canna* spp., CaYMV isolate 0028 (accession KX066020.1), and defective isolates GAP5RC (accession KX255725.1) and GAP6RC (accession KX255726.1) were recently deposited into GenBank. In addition, two associated subviral elements EVS0036 (accession KX255723.1) and EVR00206 (accession KX255724.1) that code for a portion of a nonfunctional polyprotein (Wijayasekara et al. 2016) and that are very similar to all other previously reported CaYMV isolates have also recently been submitted to GenBank. However, those complete genome sequences of isolates 0028, GAP5RC, and GAP6RC share

Fig. 7. Typical symptoms associated with *Canna yellow mottle virus* (CaYMV)-Ap infections: **A**, yellow mottling and necrosis of the leaves; **B**, vein streaking; **C**, stunting; **D**, CaYMV-Ap-negative asymptomatic plants.

less than 80% nucleotide sequence identity with the previously reported sequences from all other CaYMV isolates (Supplementary Fig. S2). In contrast, the complete genome sequence of the *A. purpurata* isolate reported in our study has a nucleotide sequence identity of 99% with the previously reported partial sequences of the RT-RNase H region from *Canna* spp. and *P. betel*. Because <80% nucleotide identity in the RT-RNase H regions is a threshold criterion for species demarcation in the genus *Badnavirus* (Geering and Hull 2012), these three new genomic sequences appear to represent a *Badnavirus* species distinct from the previously reported CaYMV isolates and the *A. purpurata* isolate. This indicates that the variation in CaYMVs is similar to that among BSVs in banana and suggests that CaYMV is a group of species, as BSV is a group of species (Kumar et al. 2015). We have therefore decided to follow the nomenclatural system for BSVs and add the host plant name to the original virus isolate. We therefore propose the name CaYMV-Ap for the CaYMV isolate that occur in *A. purpurata* and suggest naming the CaYMV-0028 isolate from *Canna indica* as CaYMV-Ci (J. Verchot, *personal communication*).

BSV is known to integrate its genome sequences into the host genome (Geering et al. 2001). Other integrated badnaviruses, known as endogenous pararetroviruses (EPRVs), are integrated into the genomes of several plant species (Hany et al. 2014; Laney et al. 2012; Seal et al. 2014; Wang et al. 2014). The risk of introducing new viruses through infected plants is increased if information on episomal viruses and EPRVs is lacking for a host species or if the tools needed to detect episomal viruses or EPRVs are unavailable (Bousalem et al. 2009). Prior to our study, it was not known whether or not endogenous or episomal forms of CaYMV were present in plant hosts (Chauhan et al. 2015). Badnavirus particles have been found in *Canna* spp. infected with CaYMV (Momol et al. 2004). We have also found typical bacilliform particles in the same symptomatic leaves of *A. purpurata* (Fig. 6) that were used for the NGS experiments. Additionally, the

episomal form of CaYMV-Ap could also be detected in *A. purpurata* using PCR and RT-PCR (Fig. 5). Most symptomatic plants that tested positive for CaYMV-Ap by PCR also tested positive for the virus using RT-PCR, while asymptomatic plants tested negative for CaYMV-Ap in both PCR and RT-PCR assays.

In a previous study of fig (*Ficus carica*), badnavirus-1, which is known to integrate into the fig genome, produced DNA fragments of multiple sizes in Southern hybridizations (Laney et al. 2012). However, we found no evidence of such fragments in digested

TABLE 2. Detection of *Canna yellow mottle virus* (CaYMV)-Ap in *Alpinia purpurata* collected from eight locations on the islands of Oahu and Hawaii

Island	Collection area	Site	Samples	Presence of CaYMV
Oahu	Location 1	1	7	7
		2	6	6
	Location 2	1	3	3
		2	2	2
		3	2	2
		4	4	4
		5	2	2
	Location 3	6	3	3
		1	5	0
		2	5	0
		3	6	0
	Location 4	4	6	6
		1	2	0
	Location 5	1	5	0
	Location 6	1	5	0
Hawaii	Location 7	2	6	0
		1	7	3
	Location 8	1	7	0
		2	4	0

Fig. 8. Symptoms on leaves of *Alpinia purpurata* infected with *Canna yellow mottle virus* (CaYMV)-Ap, *Banana bract mosaic virus* (BBrMV), or both. **A**, Leaves collected from the University of Hawaii at Manoa, showing typical symptoms of CaYMV-Ap infections only (samples 1, 2, and 8), typical symptoms of BBrMV infections only (samples 9 and 10), and complex symptoms of both CaYMV-Ap and BBrMV infections (samples 3, 4, 11, and 12). **B**, CaYMV-Ap detected in *A. purpurata* leaves using specific primer pair CaYMV-3/4. All symptomatic leaves tested positive, but samples 9 and 10 were only weakly positive. **C**, BBrMV detected in *A. purpurata* leaves. Samples 1, 2, and 8 tested negative, while samples 3, 4, 9, 10, 11, and 12 tested positive, though sample 11 was only weakly positive. M = molecular weight marker; Numbers = ID of samples showing symptoms; H = samples from asymptomatic leaves; P = positive control (BBrMV).

genomic DNA from CaYMV-Ap-infected *A. purpurata*, suggesting that its sequences might not be integrated into the *A. purpurata* genome (data not shown). Symptomatic leaves (Fig. 5, samples 1 to 4) tested positive for CaYMV-Ap by PCR, but asymptomatic samples (Fig. 5, lane H) collected from different plants at the same location were negative in our assay. We found similar results in samples collected from another location (Table 2, location 3), where only the six symptomatic samples from one of the four collecting sites tested positive for CaYMV-Ap using PCR. If CaYMV-Ap integrates into the host genome, the plants from all collection areas might have tested positive in PCR analyses. In addition, we completed the genome sequence of CaYMV-Ap from symptomatic *A. purpurata* plants and confirmed the sequence by Sanger sequencing of 12 overlapping PCR fragments; we did not identify any host sequences in the PCR products. However, more thorough studies are needed to conclusively determine if the integration of CaYMV sequences into its host genome can occur.

There are no vectors that are known to transmit CaYMV (Momol et al. 2004), but many badnaviruses are transmitted by mealybug species of the family *Pseudococcidae* (Harper et al. 2004). Examples include BSV transmitted by the citrus mealybug (*Planococcus citri*), the sugarcane mealybug (*Saccharicoccus sacchari*), and the pineapple mealybug (*Dysmicoccus brevipes*) (Kubiriba et al. 2001); ScBV transmitted to banana by *S. sacchari* (Lockhart and Autrey 1988); *Piper yellow mottle virus* (PYMV) transmitted by the mealybug, *Ferrisia virgata* (Bhat et al. 2003); and *Dioscorea alata bacilliform virus* transmitted efficiently both mechanically and by the mealybug *P. citri* (Kenyon et al. 2008). Badnaviruses can also be transmitted by insect vectors other than mealybugs. *Rubus yellow net virus* and *Gooseberry vein banding virus*, closely related phylogenetically (Fig. 4), are both transmitted by aphids (Bhat et al. 2016; Stace-Smith and Jones 1987). However, CaYMV-Ap is most closely related to the mealybug-transmitted badnaviruses ScBV, PBCoV, and BSV (Fig. 4), as well as PYMV (Lockhart et al. 1997). Several mealybug species are known to colonize *A. purpurata* including the citrus mealybug (Hansen et al. 1992). Therefore, mealybug species may be capable of vectoring CaYMV-Ap.

We have identified CaYMV-Ap in a new host *A. purpurata* in this study. In addition to the previously reported isolates infecting *Canna* spp., the isolates infecting *P. betel* are also very similar (97 to 98% identity) to the CaYMV-Ap isolates (Kumari and Raj 2015). It is possible that the host range of CaYMV-Ap may eventually expand to include other important tropical plants. Additional studies on CaYMV-Ap are needed, including surveys of its distribution within the Hawaiian Islands and proactive screening of potential other plant hosts for the presence of this virus. These efforts will provide the information necessary to develop management strategies to mitigate the spread of CaYMV-Ap in Hawaii.

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