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Characterization of viruses and viroids by deep sequencing of small RNAs in *Caladium spp.*

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Abstract Insert your abstract here. Include keywords, PACS and mathematical subject classification numbers as needed.

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1 Introduction

Caladium spp. (family Araceae) is produced in a very narrow area of Florida, Lake Placid the capital of Caladium. In a surface of X acres in the middle of the state. The little county produce up to the 90-95% of the caladium production in the world. Shorten plants, smaller leaves and less vigorous and weaker root system has been reported in Caladium. There is a few viral species infecting the Araceae family, potyviruses *Dasheen mosaic virus* (DsMV), *Konjac mosaic virus* (KoMV) (family *Potyvirus*), Taro virus, etc. etc. etc. In Florida just has been reported in Caladium, Cholocasia and Zandenteschia (Hartman, 1974; Pappu et al., 1994). We sort out a deep sequence analysis to identify virus diversity in Caladiums in Florida.

2 Materials and methods

2.1 *Caladium* samples

C. x hortulame cultivars were collected from Lake Placid (Highlands County), Florida in 2011 to 2013 in central Florida. Caladium with multiple eyes, short and decoulored leaves were assigned as symptomatic specimens from 8 different cultivars and six

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cultivars as asymptomatic, used for RNAseq. In total 18 cultivars were sampled and potted in the facilities of the University of Florida in Gainesville and Balm.

Table 1 Cultivars for NGS

Cultivar	Farm	ID

[1]Software: Illumina Pipeline (CASAVA) v1.8.2
[2]Fastq Quality Encoding: Sanger Quality (ASCII Character Code = Phred Quality Value+33)

2.2 RNA *Caladium* extraction for diagnosis and metagenomics

Caladium RNA extraction for was done using three different protocols. 1) The method for small and high quality RNA extraction was modifying the Flóres and Llácer (1989) and Ochoa et al., (1996) using 20 g of tissue pulverized with liquid Nitrogen, adding 36 ml 2X GPS buffer [0.2 M Glycine, 0.1 M Na_2HPO_4 and 0.6 M NaCl (p.H. 9.6)], 4 ml 10% SD, 9.6 ml Phenol:Chloroform 1:1, 400 μl of β-Mercaptoethanol, 400 μl Isopropanol and 10 mg of Bentonite powder and was chilled for 1 hour, shaking eventually. The samples were centrifuged at 6,000 xg for 30 minutes at 4C. The upper natant was recovered and adjusted to 40 ml with 2X GPS buffer and precipitated with 1/3rd of Ethanol and mixed by inversion. 1 gr of CF-11 cellulose powder (Whatman) was added to the mixture and vigorously shake and incubate at 4C with rotation. Centrifuged up to 6,000 xg to precipitated the CF-11 cellulose powder, the supernatant was discarded. The CF-11 cellulose powder was washed up to 8 times with 25 ml of 1X STE buffer [50 mM Tris-HCl (p.H. 7.2), 100 mM M NaCl and 1mM EDTA (p.H. 6.8)] and 25 % Ethanol. The CF-11 cellulose powder was packaged into a column and eluted with 10 ml of 1X STE buffer. The previous eluting was precipitated with 25 ml isopropanol overnight at -20C and centrifuged at 12,000 xg for 30 minutes at 4C, the pellet was dried out and resuspended with 200 μl DEPC treated water. 2) A second RNA extraction protocol was implemented for Caladiums that allows us to test viruses and possible viroids, using 1.5 gr of tissue with 2% of PVPP plus 20 mL of prewarmed at 65 C grape buffer (4 M guanidine thiocyanate, 100 mM Tris-HCl (p.H. 8), 25 mM sodium citrate and 0.5 % N-Lauryl sarcosyne), plus 0.1 volumes of sodium chloride and 1 % β-Mercaptoethanol. Samples were vortex and incubated on ice for 5 minutes. The samples were washed two times with 1 volume of chloroform: isoamyl alcohol (24:1), shake for 5 to 10 minutes at room temperature and centrifuged at 14,000 xg for 15 minutes at 4C. Sample were precipitated with one volume of isopropanol with 0.1 volume of NaCl 5M overnigth and washed two times with 75 % etanol. Samples were rehydrate with prewarmed DEPC treated water. This methods allow us to recover small RNAs under 500 nucleotides. 3) A third method for plant RNA extraction we used the RNeasy plant mini kit, using the RC buffer supplemented with β-Mercaptoethanol as suggested for plants with high polysaccharides concentration, this protocol was intended for test for molecules over 400 nucleotides.

2.3 Sequencing and bioinformatics

The RNA for metagenomics was prepared for a small RNA library preparation and sequenced by HiSeq 2000, 50 bp single-end lane (Macrogen, Seoul, Ko). The data was analyzed using FASTX-toolkit (Pearson et al., 1997), for quality and tag removal from both data sets, reads and the trimming (filter) results are displayed in table 2. The sequences were assembled using different k-mer values by velvet (Zerbino et al 2009;), the RNAseq was analyzed using PFOR to identify viroid like-sequences (Wu, et al. 2012). The resulted contigs ¿ 100 nt were analyzed by 1) by using BLASTN and BLASTX non redundant data base from the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/, Alstchul et al., 1990)). 2) to analyze any subviral pathogen we used the subviral RNA database (Rochealeau and Pelchat 2006). The top plant virus hits over 10_^{5} and ¡90 nt were used to design primers and map back to a reference sequence to corroborate the results. Fo mapping back the reads to its reference sequence using BOWTIE (Langmead et al., 2009).

Table 2 Sequence results

RNAseq						
Sample	Total bases	Read count	Filter reads	Contigs Velvet	Contigs PFOR	(%)
Healthy Cal2	2,985,755,424	58,544,224	0.0042	51.79	98.46	
Grassy Cal	2,773,957,269	54,391,319	0.0041	50.91	98.65	

[3]Software: Illumina Pipeline (CASAVA) v1.8.2
[4]Fastq Quality Encoding: Sanger Quality (ASCII Character Code = Phred Quality Value+33)

2.4 Molecular diagnosis

2.4.1 Reverse Transcription -PCR

Reverse Transcription (RT) -PCR were diagnosed for RNA viruses (Promega, Madison Wisconsin and Twist DX, Some, Where). For RT-PCR, 500 ng of RNA were tested using the following primers; Rubisco and Actin as house keeping genes (HKG) for Caladium. The BLAST hits and the mapping of the viral agents were used for primer design. Screening potyviruses, Tospovirus, Cauilomoviruses and virods (table 2).

Further analyzed by RT-PCR for potyviruses, viroids and Tospvirus broad spectrum primer (Eiras et al 2001). For RPA analysis we used the RNA extraction kit (fulanito de tal, Some, Where), RNA was stored at -20°C or 80°C.

PCR protocols for HKG.

Rubisco:

1 cycle at 95C - 2 min, 30 cycles at 95C - 30 sec, 50-60 - 30 sec, 72C - 30 sec, 72 - 30 sec, 1 cycle at 72C - 7 min.

β-actin:

Table 3 List of primers.

Name	Id	sequence 5'- 3'	size	Author
HKG				
Rubisco	JAP1053	AGGCCCGCCTCACGGTATCC	500	this paper
	JAP1054	CTGCATGCATTGCGCGGTGG		
Actin	JAP1055	ATGAAGATCCTGACGGAGCG	360	this paper
	JAP1056	CCACTGAGAACGATGTTGCC		
Potyvirus				
DMV	JAP1037	TAAAGGAGTGCGAGCTTCAGC	1000	this paper
	JAP1038	TTTACCAGACCTTTACTGCGG		
KMV	JAP1039	GACCGTGATGCTAATGAGGAGG	1000	this paper
	JAP1040	AAGGCAGGCTCGTCCAGAG		
Caulimovirus				
BSV-like	JAP1172	GGGTTGGTATTAAGCCCAAC	875	this paper
	JAP1173	CCAGTTCCTGTGATGTAATCC		
Tospovirus				
	algo	XXXXXXXXXXXXXX	800	Eiras et al. 2001
	algo 2	xxxxxxxxxx		

1 cycle at 95C - 2 min, 30 cycles at 95C - 30 sec, 50-54 - 30 sec, 72C - 30 sec, 72 - 30 sec, 1 cycle at 72C - 7 min.

2.4.2 Reverse Transcription -Recombinase Polymerase Amplification

We developed a quick assay for screening KoMV from caladiums using a new available probe, Recombinase Polymerase Amplification coupled to Reverse Transcription (RT-RPA). The RNA extraction was done using the following procedure. Primers must be larger than 30 nucleotides JAP1242 5' -GCTCTATCTAGACCGTGATGCT AATGAGGAGG- 3' and JAP1244 5' -TTTGCTAAATCCGCTTGCTCTGGATTATA TTGGG- 3' giving an amplicon smaller than 500 bp. The mixture was incubated at 42 °C and cleaned up by heating at 95°C for 15 minutes. 25 ul of the sample was runned in agarose gels 1 % at 80 volts for 50 minutes.

2.4.3 Biological experimentas, transmission of viral and viroids

Bla bla bla bla

$$a^2 + b^2 = c^2 \tag{1}$$

3 Results

3.1 Bioinformatics analysis of small RNAs

The small RNAs sequences were de novo assembly using velvet and a circular de novo assembly: PFOR. Velvet results for the non symptomatic plants were 2157 contigs with a mean length of 156 nt and max length of 1460 nt and for the symptomatic

plants the number of contigs were 92228 with a mean length of 117 nt and max length of 1324 nt. PFOR resutls for the non sympotamcis plants were 3 circles and for the symptomatic plants were 94 circles. The contigs were analyzed by blastn against the nr and viroid database, blast results are shown in table 3.

Table 4 Blast results

Sample	Contig	Length	hit	e-value
Non-symptomatic				
	nn	#	virus 1	e-10
Symptomatic				
	nn	#	virus 1	e-10

3.2 Subsection title2

as required. Don't forget to give each section and subsection a unique label (see Sect. 4).

Paragraph headings Use paragraph headings as needed.

Table 5 Blast results

Cultivar	KoMV	DsMV
Aaron	0/20	18/20
Brandywine	0/21	15/21
Candidum	6/21-	15/21
Carolyn Whorton	6/21-	13/21
Freida Hemple	0/21	9/21-
Pink Beauty	4/21-	17/21
Postman Joyner	2/21-	13/21
Red Flash	10/20-	9/20-
White Xmas	1/21-	17/21
White Queen	5/21-	19/21
Whiten Wing	0/20	11/21-
Rosebud	0/20	9/21-
Cherry Tart	1/21-	18/21
Fairytale Princess	0/21	18/21
Tapestry	0/21	18/21

3.3 Subsection title3

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Paragraph headings Use paragraph headings as needed.

$$a^2 + b^2 = c^2 \tag{2}$$

3.4 Subsection title4

as required. Don't forget to give each section and subsection a unique label (see Sect. 4).

Paragraph headings Use paragraph headings as needed.

$$a^2 + b^2 = c^2 \tag{3}$$

3.5 Subsection title5

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Paragraph headings Use paragraph headings as needed.

$$a^2 + b^2 = c^2 \tag{4}$$

3.6 Subsection title6

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Paragraph headings Use paragraph headings as needed.

$$a^2 + b^2 = c^2 \tag{5}$$

3.7 Subsection title7

as required. Don't forget to give each section and subsection a unique label (see Sect. 4).

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$$a^2 + b^2 = c^2 \tag{6}$$

4 Discussion

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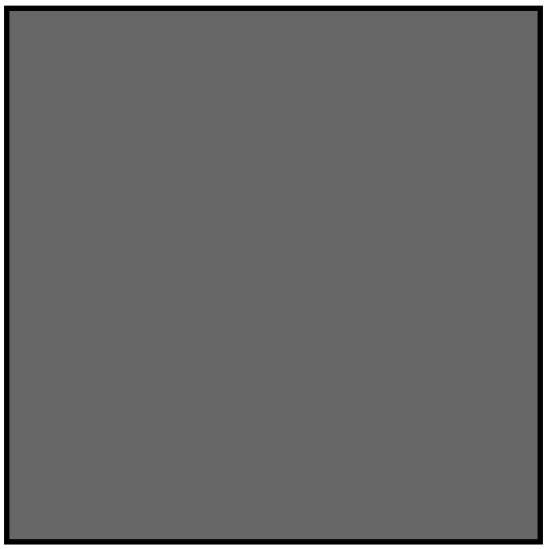

Fig. 1 Please write your figure caption here

Table 6 Please write your table caption here

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4.1 Subsection title

as required. Don't forget to give each section and subsection a unique label (see Sect. 4).

Paragraph headings Use paragraph headings as needed.

$$a^2 + b^2 = c^2 \tag{7}$$

References

1. Author, Article title, Journal, Volume, page numbers (year)
2. Author, Book title, page numbers. Publisher, place (year)

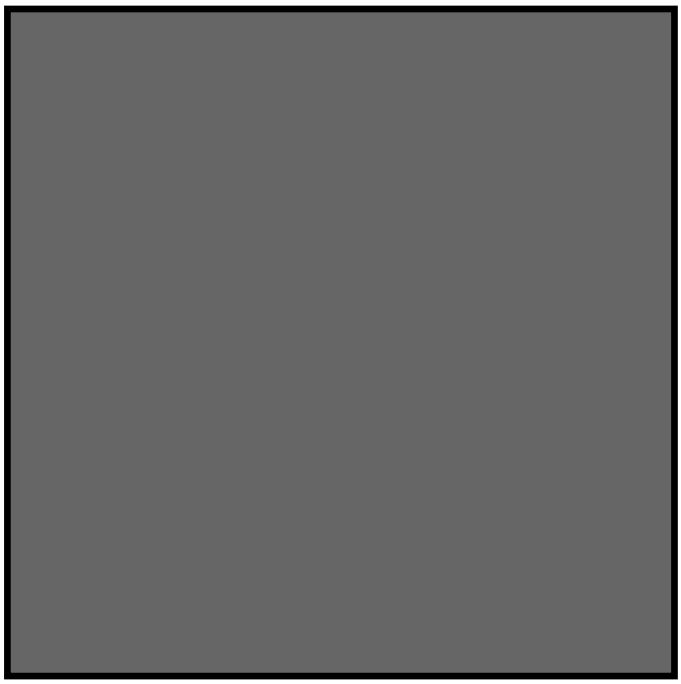

Fig. 2 Please write your figure caption here