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Full Length Article

Molecular Detection of Viral Diseases in Chewing Cane (*Saccharum officinarum*) from Southern China

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Abstract

One hundred and six samples of chewing cane (*Saccharum officinarum* L.) with suspected symptoms of mosaic disease were collected from Southern China in 2016. RT-PCR or PCR was used to determine the types of viral diseases and pathogenic viruses as well as the occurrence of the infections to provide a scientific basis for the use of viral detection to ensure virus-free seedling production of chewing cane. The results showed that sugarcane mosaic disease and sugarcane yellow leaf disease usually occurred in chewing cane planted in Southern China, and three types of viruses were detected in all the 106 samples of chewing cane, namely SrMV (97.2%), SCMV (98.1%), and SCYLV (95.3%). Among them, the co-infection rates of SrMV and SCMV; SrMV and SCYLV; SCMV and SCYLV; and SrMV, SCMV, and SCYLV were 95.3, 92.5, 93.4 and 90.6%, respectively. No positive samples of SCSMV, JGMV, MDMV, or SCBV were detected in any of the samples. The dominant pathogenic virus of mosaic disease in the chewing cane of Southern China was SCMV or the co-infection of SCMV and SrMV. This study indicates that it is necessary to carry out detoxification and detection of SCMV, SrMV, and SCYLV in chewing cane seedlings in Southern China. These findings will be helpful for the development of virus-free chewing cane seedlings in the future.

Keywords: Chewing cane; Molecular detection; *Sorghum mosaic virus*; *Sugarcane mosaic virus*; *Sugarcane yellow leaf virus*; Viral disease

Introduction

Sugarcane (*Saccharum* spp. hybrids) is a promising renewable biomass energy crop that uses buds as breeding bodies and is easily infected by viruses. Sugarcane mosaic disease (SMD), sugarcane yellow leaf disease (SYLD), sugarcane bacilliform virus disease (SBVD) etc. are important viral diseases of sugarcane worldwide (Xu *et al*., 2008; Puchades *et al*., 2016). SMD is the most commonly occurring sugarcane viral disease, and is caused by *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV), *Sugarcane streak mosaic virus* (SCSMV), *Johnson grass mosaic virus* (JGMV), *Maize dwarf mosaic virus* (MDMV), and *Zea mosaic virus* (ZeMV) (Seifers *et al*., 2000; Mayo, 2005). Only SCMV and SrMV can infect sugarcane under natural conditions (Xu *et al*., 2008).

Generally, SBVD is caused by *Sugarcane bacilliform virus* (SCBV, genus *Badnavirus*, family *Caulimoviridae*). While the disease was primarily found in Cuba in 1985, it has been reported in more than 20 countries and regions since that time (Karuppaiah *et al*., 2013; Xu *et al*., 2015). SYLD, caused by sugarcane yellow disease virus (SCYLV, genus *Polerovirus*, family *Luteoviridea*) (Mayo, 2005), was first reported in Hawaii in 1988 (Schenck *et al*., 1997). It has now spread into the major sugarcane growing countries and regions across the world (Lockhart and Cronje, 2000; Lin *et al*., 2015).

SMD, SYLD, and SBVD have all been found in Southern China (Xu *et al*., 2014; Wu *et al*., 2016); among them, SMD generally occurred, while SYLD and SBVD were sporadic (Li *et al*., 2011; Wang *et al*., 2012; Xu *et al*., 2014). According to reports, SCMV, SrMV, and SCSMV were the pathogens that caused SMD in Southern China; among them, SrMV was the dominant pathogen and the other viruses were secondary (Xu *et al*., 2014; Zhang *et al*., 2015; Jing *et al*., 2015). In Southern China, there were different degrees of two or more type of viruses that were co-infecting sugarcane in the fields, including the co-infection of SrMV and SCMV (Xu *et al*., 2014; Jing *et al*., 2015); the co-infection of SrMV and SCYLV (Xiong *et al*., 2011; Xu *et al*., 2014); the co-infection of SCMV and SCYLV (Xie *et al*., 2009; Xu *et al*., 2014); the co-infection of SrMV and SCSMV (Zhang *et al*., 2015; Luo *et al*., 2016); and the co-infection of SrMV, SCMV, and SCYLV (Xu *et al*., 2014). There were even three kinds of RNA viruses (SrMV, SCMV, and SCYLV) and one DNA virus (SCBV) complex infection; however, the incidence of co-infection is generally low in the field (Tang *et al*., 2015).

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Chewing cane (*Saccharum officinarum* L.) is a type of sugarcane that is low in fiber content but rich in sugar, water, amino acids, protein, Ca, and Fe, and other necessary nutrients for the human body (Pan *et al*., 2007). Chewing cane has been planted for more than 200 years and has become one of the main economic crops in Southern China (He *et al*., 2016). Badila (*S. officinarum*) is one of the main cultivars of chewing cane and has been planted for decades in Southern China. SMD generally occurs in the field of chewing cane planting (Wang *et al*., 2014); however, up to now, there has been no systematic study on the molecular detection of viruses that cause diseases in chewing cane in Southern China. The objective of this study was to detect the pathogens of viral diseases of chewing cane so as to clarify the types and occurrence of the viruses from chewing cane in Southern China, and to provide a scientific basis for the development of virus-free chewing cane seedlings.

Materials and Methods

Chewing Cane Sample Collection

One hundred and six samples of young chewing cane leaves with suspected symptoms of SMD were collected in China in June 2016, including 95 samples from Guangdong, 7 samples from Sichuan, and 4 samples from Jiangxi. Leaf samples were stored at -80°C for further use.

Extraction of Total Nucleic Acid

Total nucleic acid (including genomic DNA and RNA) was extracted using the cetyl trimethyl ammonium bromide method, as described by Xie *et al*. (2009). The total nucleic acid samples were diluted to prepare a working solution of 50 ng/μL for RT-PCR or PCR analysis and they were stored at -20°C for further use.

RT-PCR Detection of RNA Viruses

SCMV, SrMV and SCSMV were detected using the primers reported by Xie *et al.* (2009) (Table 1). The expected amplified products were 906 bp, 860 bp, and 1160 bp, respectively. JGMV and MDMV were detected using primers reported by Jiang *et al.* (2009) (Table 1). The expected amplified products were 865 bp and 1107 bp. SCYLV was detected using the primers reported by Xu *et al*. (2005) (Table 1). The expected amplified product was 634 bp. The above primers were commercially synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). The total nucleic acid prepared from the leaf tissues was amplified by RT-PCR using a HiScript II One Step RT- PCR Kit (Vazyme Biotech Co., Ltd., Nanjing, China) following the manufacturer's protocol. The PCR reaction was performed in a final volume of 20.0 μL containing 1.0 μL total nucleic acid (~50 ng), 1.0 μL Enzyme Mix (5.0 U/μL), 10.0 μL 2× One Step Mix, and upstream and downstream primers that were each 1.0 μL (5.0 μmol/L). The final volume was adjusted to 20.0 μL with 6.0 μL RNase-Free ddH_2O. RT-PCR amplification was performed using the MyCycler™ thermal cycler (BioRad, Hercules, CA, USA). The program for RT-PCR was 50°C for 30 min, 94°C for 2 min, followed by 35 cycles each of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, an extension at 72°C for 1.0 min, and a final extension phase of 72°C for 5.0 min. The amplification products were electrophoresed on 1.0% agarose gel with 0.5× TBE buffer at 100 V for 1.0 h together with the DL 2000 DNA markers. The separated DNA fragments were stained with 5.0% GoldView™ and photographed with the Gene Genius Bioimaging System.

PCR Detection of DNA Virus

SCBV was detected using specific primers reported by Wu *et al*. (2016) (Table 1). The expected amplified product was 726 bp. The primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) and *r*Taq DNA polymerase was purchased from TaKaRa Bio Inc. (Dalian, China). The PCR reaction was performed in a final volume of 25.0 μL containing 1.0 μL total nucleic acid (~50 ng), 0.25 μL *r*Taq DNA polymerase (5.0 U/μL), 2.5 μL 10× PCR reaction buffer (with Mg^{2+}), 2.0 μL 2.5-mM dNTP mixture, and 2.0 μL 10.0-μM SCBV-specific primers (each primer 1.0 μL). The final volume was adjusted to 25.0 μL with 16.8 μL ddH_2O. PCR amplification was performed using the MyCycler™ thermal cycler (BioRad, Hercules, CA, USA), with an initial denaturation at 94°C for 5.0 min, followed by 35 cycles each of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, an extension at 72°C for 45 s, and a final extension phase of 72°C for 10 min. The amplification product detection was the same as that for RT-PCR above.

Sequencing and Analysis of the Nucleotide Identity of RT-PCR Products

Several target amplification products were chosen based on the host and geographic origin for further CP gene sequence analysis. RT-PCR products were directly sequenced by Sangon Biotech Co. Ltd. (Shanghai, China) using an ABI3730 DNA sequencer. The partially sequenced PCR products were analyzed by BLAST alignment in NCBI and the DNA Star 5.01 package (DNA STAR Inc., USA).

Results

(RT-) PCR Detection of Viruses

Using primer pairs SCMV-F/SCMV-R, SrMV-F/SrMV-R, SCYLV-P1/SCYLV-P2, SCSMV-F/SCSMV-R, JGMV-F/JGMV-R, and MDMV-F/MDMV-R, and the total nucleic acids of leaves as templates, 106 samples of chewing cane were detected by RT-PCR.

Table 1: Primers used for virus detection in this study

Virus	Primer	Sequence (5'-3')	Product size (bp)	Reference
SCMV	SCMV-F	GAAGAWGTYTTCCAYCAAKCWGGAAC	906	Xie *et al*. (2009)
	SCMV-R	AGCTGTGTGTCTCTCTGTATTCTC		
SrMV	SrMV-F	ACAGCAGAWGCAACRGCACAAGC	860	
	SrMV-R	CTCWCCGACATTCCCATCCAAGCC		
SCSMV	SCSMV-F	ATTTTGCCGTCCCGTTTTACATC	1160	
	SCSMV-R	AGCGCGTTGTCTTTCTTCTTCAGTCA		
MDMV	MDMV-F	TCCCTCAATACCGTCTTGARGC	1107	Jiang *et al*. (2009)
	MDMV-R	CAGCTGTGTGNCKYTCTGTATT		
JGMV	JGMV-F	TCRGGCAATGARGAYGCTGG	865	
	JGMV-R	CAGCTGTGTGNCKYTCTGTATT		
SCYLV	SCYLV-P1	AATCAGTGCACACATCCGAG	634	Xu *et al*. (2005)
	SCYLV-P2	GGAGCGTCGCCTACCTATT		
SCBV	SCBV-F	GTTCATCGCHGTNTAYATTGATGAC	726	Wu *et al*. (2016)
	SCBV-R	GAAGGYTTRTGTTCTVCACTCTTGTTG		

Note: W= A/T, Y=C/T, K=G/T, R=A/G, H= A/C/T, V=A/C/G, N=A/C/G/T

The single amplification bands were obtained using SCMV-F/SCMV-R, SrMV-F/SrMV-R, and SCYLV-P1/SCYLV-P2 as the primers, respectively, and the amplified products of the positive control and most of the samples were the expected sizes, which were about 906, 860 and 634 bp, respectively. Furthermore, the negative control (virus-free seedling) had no amplified bands (Fig. 1). However, with SCSMV-F/SCSMV-R, JGMV-F/JGMV-R, and MDMV-F/MDMV-R as the primer pairs, no expected bands were amplified in the negative control and all of the samples after electrophoresis detection. In addition, 106 samples were detected by PCR using the SCBV-F/SCBV-R primer pair; the target fragments were not amplified in the negative control or in all of the samples.

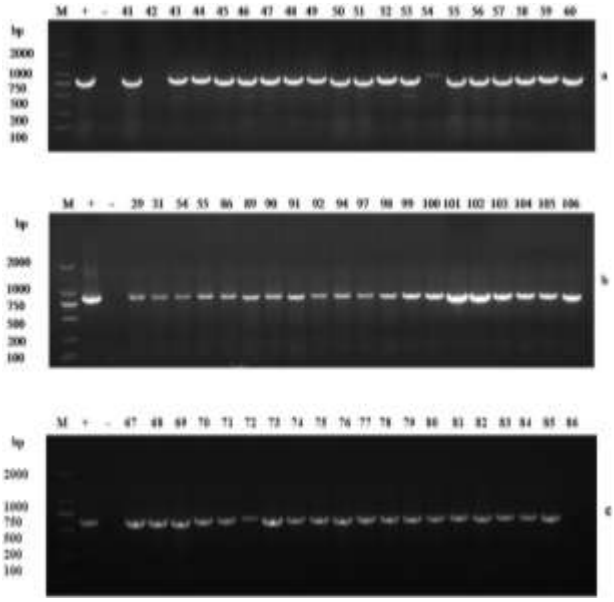

Fig. 1: Electrophoresis analysis of amplified products from SCMV (a), SrMV (b), and SCYLV (c) based on RT-PCR detection

Note: M: DNA marker DL 2000; +: positive control; -: negative control (virus-free seedling); the other lanes are the sample numbers in Table 2

Occurrence of Viruses

The detection results of each sample are shown in Table 2. According to Table 3, there were 103 SrMV-positive samples with the positive rate of 97.2% (103/106); there were 104 SCMV-positive samples with the positive detection rate of 98.1% (104/106); and there were 101 SCYLV-positive samples with the positive rate of 95.3% (101/106). There were 96 samples that were co-infected by SCMV, SrMV, and SCYLV with the co-infection rate of 90.6% (96/106); 101 samples were co-infected by SCMV and SrMV with the co-infection rate of 95.3% (101/106); 98 samples were co-infected by SrMV and SCYLV and the co-infection rate was 92.5% (98/106); and 99 samples were co-infected by SCMV and SCYLV with the co-infection rate of 93.4% (99/106). No positive samples of SCSMV, JGMV, MDMV, or SCBV were detected.

Sequence Identity Analysis

The partially sequenced PCR products were analyzed by BLAST alignment and DNA star software. The results showed that a comparison of the nucleotide sequences of the SCMV sample (GenBank accession number MF356403) and the corresponding nucleotide sequence of the SCMV strains or isolates from Chinese Fujian, Argentina, and Chinese Yunnan (GenBank accession numbers KJ944724, EU196448, and FM997890) indicated that they were 95%~97% identical, and confirmed that the amplified sequences were the SCMV genomic fragment. The nucleotide sequence of the SrMV sample (GenBank accession number MF356432) ranged from 97%~99% identity with the corresponding nucleotide sequence of the SrMV strains or isolates (GenBank accession numbers DQ991390 and KC179680) from Chinese Guangdong and Chinese Yunnan, indicating that the amplified sequence was the SrMV genome fragment sequence.

Table 2: Detection of SrMV, SCMV, and SCYLV in chewing cane from Southern China

Sample number	Sampling site	Varieties	SrMV	SCMV	SCYLV
1	Guangzhou, Guangdong	Badila	+	+++	+++
2			+	+++	+++
3			+	+++	+++
4			+	++	+++
5			++	++	+++
6			+	+++	+++
7			+	+++	+++
8			+	+++	+++
9			-	+++	+
10			+	+++	+
11			+	+++	+++
12			+	+++	+++
13			+++	++	+++
14			+	+++	+++
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39			+	+++	+
40			+	+++	+
41			+	+++	+
42			+	-	+
43			+	+++	+
44			+	+++	+
45			+	+++	+
46	Yingde, Guangdong	Badila	+	+++	+
47			+	+++	+
48			+	+++	+
49			+	+++	+
50			+	+++	+
51			+	+++	+
52			+	+++	+
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80			+	+++	++
81			+	+++	++
82			+	++	++
83			+	+++	++
84			+	++	++
85			+	+++	++
86	Ganzhou, Jiangxi	Badila	+++	++	-
87			++	++	-
88			+	+++	+
89			+++	-	+
90	Neijiang, Sichuan	Neijiang mizhe	+++	+	+
91		Tiancheng 22	+++	+	+
92		Tiancheng 99	+++	+	+
93		Tiancheng 22	+++	+	-
94		Tiancheng 21	++	+	+
95		Tiancheng 19	+++	+	+
96		Tiancheng 99	+++	+	+
97	Guangzhou, Guangdong	Qianzhe 08-1497	++	+	+
98		Qiantang 3	++	+	+
99		Guangdong huangpi	+++	+	+
100		Tiancheng 22	+++	+	+
101		Neijiang mizhe	+++	+	-
102		Yunnan hongpi	+++	+	+
103		Qianzhe 08-688	++	+	++
104		Tiancheng 21	++	+	+
105		Qiantang 5	+++	+	+
106		Tiancheng 99	+++	+	-

Note: " + " is positive, " - " is negative. The greater the number of " + ", the brighter the target fragment

A comparison of the nucleotide sequences of the SCYLV sample (GenBank accession number MF363047) and the corresponding nucleotide sequence of the SCYLV strains or isolates from Chinese Yunnan, Chinese Fujian, Chinese Guangxi, and India (GenBank accession numbers HQ245347, GU190160, GU190161, and JF925155) indicated that they were 95~99% identical, and confirmed that the amplified sequences were the SCYLV genomic fragment.

Discussion

In the present study, SCMV, SrMV and SCYLV were detected, but we failed to find positive samples of SCSMV, JGMV, MDMV, or SCBV in the 106 samples of chewing cane leaves. SCMV and SrMV are pathogens of SMD, causing SMD, while SCYLV is a pathogen of SYLD, causing SYLD. Therefore, the main viral diseases of chewing cane are SMD and SYLD in Southern China.

Table 3: Occurrence of viruses in chewing cane from Southern China

Virus or co-infection	SrMV	SCMV	SCYLV	SrMV+SCMV+SCYLV	SrMV+SCMV	SrMV+SCYLV	SCMV+SCYLV
Number of positive or co-infection samples	103	104	101	96	101	98	99
Positive rate or co-infection rate (%)	97.2	98.1	95.3	90.6	95.3	92.5	93.4

Xu *et al.* (2015) reported that the positive detection rate of SCBV was 58.1% in 43 chewing cane samples from Chinese Guangxi using the universal primer pair Badna FP/RP based on baculovirus DNA (Badnavirus); however, no chewing cane sample was collected from Guangxi in the present study. Therefore, the occurrence of SCBV in chewing cane in Southern China should be studied further. Jing *et al.* (2015) reported that of 43 sugarcane samples from China that were detected by RT-PCR, no sample with JGMV and MDMV was discovered. In the present study, of the 106 chewing cane samples from Southern China, JGMV and MDMV were also not detected. This may be related to JGMV and MDMV not infecting sugarcane or chewing cane in the natural conditions in the field (Xu *et al*., 2008).

SCMV and SrMV belong to genus *Potyvirus*, family *Potyviridae*, and they are the pathogenic viruses of SMD. In this study, the positive rates of SCMV and SrMV were 98.1 and 97.2%, respectively, and the SCMV content was significantly greater than SrMV in most of the samples (based on the brightness of the target fragment). Therefore, SCMV was the dominant pathogenic virus associated with chewing cane mosaic disease in Southern China. It has been reported that the dominant pathogen of SMD in Southern China was SrMV (Xu *et al*., 2014; Zhang *et al*., 2015; Jing *et al*., 2015), but our results indicated that the dominant virus of mosaic disease in chewing cane and sugarcane in Southern China was different. In addition, in the present study, it was also found that the SrMV content was obviously higher than SCMV in some varieties that can be used both as fruits and as raw materials for sugar production, such as Tiancheng 19, Tiancheng 21, Tiancheng 22, Tiancheng 99, and Qiantang 5, indicating that the dominant pathogen of mosaic disease was SrMV in the above varieties, which was the same as the dominant pathogen of SMD in Southern China. The possible reason was that the varieties with the dual-purpose of fruit and sugar were low-generation progeny of *S. officinarum* L. based on a hybrid or backcross with wild germplasm. Therefore, they were similar to sugarcane varieties (hybrids), but the fiber content was significantly lower than sugarcane varieties. However, Badila, a main cultivar of chewing cane in Southern China, was the *S. officinarum* L. species that lacked resistance genes. Therefore, it was inferred that SCMV might be less toxic to *Saccharum* spp. than SrMV, and with the introduction of resistance genes from wild germplasm to *Saccharum* spp. hybrids, SCMV gradually lost its infectivity.

In the present study, it was found that most of the chewing cane samples showed a co-infection with two or three types of viruses. The co-infection rate of SrMV and SCMV was 95.3%, and the co-infection rates of SrMV and SCYLV; SCMV and SCYLV; and SrMV, SCMV, and SCYLV were 92.5%, 93.4% and 90.6%, respectively. The above results showed that the co-infection of two or three types of viruses usually occurred in chewing cane from Southern China. Co-infection of viruses in sugarcane from Southern China have been reported; however, the co-infection rate of viruses was generally low (about 5%) in the field, and co-infection mainly involved two types of viruses (Xie *et al*., 2009; Xu *et al*., 2014; Zhang *et al*., 2015), a co-infection with three or more types of viruses was rarely found (Xu *et al*., 2014; Tang *et al*., 2015). The viral co-infection in sugarcane in Southern China only happened by chance compared with the chewing cane in Southern China; this may be related to host resistance. Chewing cane cultivars were mainly *S. officinarum* L. with low resistance, while sugarcane varieties were hybrid progenies of *S. officinarum* L. and wild species, and the resistance gene of wild germplasm was introduced to sugarcane varieties; therefore, the resistance of sugarcane varieties was much stronger than the chewing cane varieties (Chen, 2003). In the present study, the pathogenic viruses SrMV and SCMV, which cause SMD, and SCYLV, which causes SYLD, belong to different virus families (the former is the family *Potyviridae*, while the latter is the family *Luetoviridae*). Their relationship in the co-infection of chewing cane and whether their co-infection plays a synergistic role in the infection process are worthy of further study.

SCYLV has been widely reported in sugarcane from Southern China; however, the positive detection rate was only 20–50% in the field (Wang *et al*., 2012; Yan *et al*., 2014; Lin *et al*., 2015). This study showed that the positive detection rate of SCYLV in chewing cane from Southern China was 95.3% (101/106), and most of the samples did not show symptoms of SYLD (this may be due to low viral content or masked symptoms). For this phenomenon, the effects on the yield and quality of chewing cane should be further studied.

There are different genotypes of SrMV, SCMV, and SCYLV in sugarcane worldwide (Viswanathan *et al*., 2009; Wang *et al*., 2012; Lin *et al*., 2015). The aforementioned viruses in the chewing cane generally occurred, but whether there were different genotypes, and how the relationships between the corresponding virus genotypes influenced the infection in sugarcane need further study.

Conclusion

In conclusion, the main viral diseases are SMD and SYLD, and three pathogenic viruses (SrMV, SCMV and SCYLV) usually occurred in chewing cane from Southern China.

In addition, the dominant pathogenic virus of mosaic disease is SCMV and a co-infection of the abovementioned two or three types of viruses were universally detected, but no positive samples of SCSMV, JGMV, MDMV, or SCBV were found in chewing cane from Southern China. These findings are helpful for the development of virus-free chewing cane seedlings in Southern China.

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