Simultaneous detection and identification of four sugarcane viruses by one-step RT-PCR

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**Abstract**

Sugarcane mosaic disease (SMD) caused by the Sugarcane mosaic virus (SCMV), Sorghum mosaic virus (SrMV) and Sugarcane streak mosaic virus (SCSMV) and sugarcane yellow leaf disease (SYLD) caused by the Sugarcane yellow leaf virus (SCYLV) are the two most prevalent and economically important viral diseases of sugarcane. In this study, a one-step quadruplex reverse transcription (RT)-PCR method that employed virus-specific primers was developed for the simultaneous detection and differentiation of SCMV, SrMV, SCSMV and SCYLV. Several sets of primers for each target virus were evaluated for their sensitivity and specificity by simplex and quadruplex RT-PCR. The optimum primer combinations and concentrations, RT temperature and time, and PCR annealing temperature and extension time were determined for the quadruplex RT-PCR. The assay was then validated using sugarcane samples affected with SMD and/or SYLD collected from sugarcane breeding fields and farmers’ fields in southern China.

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1. Introduction

Sugarcane mosaic disease (SMD) and sugarcane yellow leaf disease (SYLD) are two important viral diseases that threaten sugarcane production worldwide. Three viruses, Sugarcane mosaic virus (SCMV, genus Potyvirus, family Potyviridae), Sorghum mosaic virus (SrMV, genus Potyvirus and Potyvirus) and Sugarcane streak mosaic virus (SCSMV, unassigned genus, family Potyviridae) have been identified as causative agents of SMD to date. Among these viruses, SCMV is distributed worldwide (Oertel et al., 1997; Handley et al., 1998; Seifers et al., 2000; Gemechu et al., 2006), while SrMV is primarily found in the United States, China and Vietnam (Yang and Mirkov, 1997; Grisham and Pan, 2007; Chen and Chen, 2002; Ha et al., 2008), and SCSMV is limited to Pakistan, India, Bangladesh, Sri Lanka, Thailand and Vietnam (Hall et al., 1998; Hema et al., 1999; Chatenet et al., 2005). Generally, Sugarcane yellow leaf virus (SCYLV, genus Polerovirus, family Luteoviridae) is recognized as the pathogen responsible for SYLD, although it has been reported that phytoplasma is associated with the disease in some areas of Africa (Cronje et al., 1998). SCYLV was first described in Hawaii in 1988 (Schencck, 2001), and was found to be responsible for serious losses in Brazil in the early 1990s (Vega et al., 1997). Since then, SCYLV has been found in other areas in which sugarcane is grown throughout the world (Moonan and Mirkov, 2002; Rassaby et al., 2003; Xu et al., 2005; Ahmad et al., 2006; Viswanathan et al., 2008).

As a vegetatively propagated crop, sugarcane is prone to viral infection via seed cane. Viral screening plays an important role for controlling the disease during germplasm exchange and seed cane production. Therefore, it is valuable to establish a rapid, convenient and reliable method of viral detection. Serological and molecular methods including the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)-based techniques have been developed for the detection of sugarcane viruses over the past two decades. Reverse transcription (RT)-PCR was developed for SCMV detection in 1994 by Smith and Van De Velde, and later improved by Alegria et al. (2003) and Xu et al. (2008). Additionally, a RT-PCR-restriction fragment length polymorphism (RFLP) for SCMV strain discrimination was reported in 1997 by Yang and Mirkov (1997). RT-PCR-RFLP, RT-PCR and immunocaptured (IC)-RT-PCR were used for the detection of SrMV (Yang and Mirkov, 1997; Qiu et al., 2007; Xu et al., 2008). Several methods have been utilized for the detection of SCSMV, including direct antigen coating (DAC)-ELISA and IC-RT-PCR (Hema et al., 2003). Among these, IC-RT-PCR was demonstrated to be the best method due to its higher sensitivity. Nucleic acid sequence-based amplification (NASBA), RT-PCR and a real-time RT-PCR assay were reported to be suitable methods of detecting SCYLV detection (Goncalves et al., 2002; Korimbocus et al., 2002; Viswanathan et al., 2008). Multiplex RT-PCR is a PCR-based method that allows simultaneous detection and/or identification of at least two viruses in one test and is desirable for routine diagnosis of a large number of samples for different...
targets (Bertolini et al., 2001; Dovas et al., 2002; Sanchez et al., 2005; Gambino and Gribaudo, 2006; Bright et al., 2007; Mahua and Joseph, 2008). In this study, a method for the simultaneous detection of SCMV, SrMV, SCSMV and SCYLV using one-step quadruplex RT-PCR was developed and evaluated in sugarcane field samples.

2. Materials and methods

2.1. Plant materials and RNA extraction

Single infections of SCMV, SrMV, SCSMV and SCYLV and mixed infections of SCMV and SrMV, SrMV and SCYLV, and SCSMV and SCYLV in leaf samples of sugarcane plants were obtained from the germplasm preservation fields located in the Guangzhou Sugarcane Industry Research Institute at Guangzhou, Guangdong, China. In addition, leaf samples from three sugarcane plants grown in fields of the sugarcane breeding program and farms in southern China were used to validate the multiplex RT-PCR.

Leaf tissues (50 mg) from healthy and infected sugarcane plants were used for total RNA extraction using an HQ&Q RNA Reagent Kit (U-Gene Biotechnology Co., Ltd., Hefei, China) according to the manufacturer’s instructions. The pellet was resuspended in 50 μl of DEPC-treated water and stored at −20°C until use.

2.2. Design of virus-specific primers

At least three sets of primers were designed and evaluated for each virus based on available sequences of SCMV, SrMV, SCSMV and SCYLV in GenBank using the PrimerSelect program of DNAStar 5.01. After being designed, the primers were synthesized by Invitrogen Biotech. Co., Ltd., (Shanghai, China). For SCMV and SrMV, primer pairs were designed from different regions because the genomic sequences of the two viruses are approximately 70–71% homologous. For SrMV and SCSMV, primer pairs were designed to anneal to conserved regions according to the alignment of multiple strains/isolates. These primer pairs were designed to generate PCR products of different sizes for each virus that would be discernible by agarose gel electrophoresis. Additionally, Primer PREMIER 5.0 was used to ensure that these primers had similar annealing melting temperatures (Tm), did not form secondary structures and did not form primer–dimers during PCR assays. Potential interactions among primers were also analyzed using ClustalW (http://www.ebi.ac.uk/clustalw/index.htm).

2.3. Specificity and compatibility of primer pairs

Uniplex one-step RT-PCR was conducted using each primer pair to evaluate its specificity and to determine the most common cycling conditions using a Prime Script One-Step RNA PCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The initial RT-PCR was performed by adding 1 μl of the extract to 29 μl of a PCR master mix containing a final concentration of 1× One-Step RNA PCR buffer, 3.3 μM of each primer set and 1.2 μl of One-Step Enzyme Mix. Gradient RT-PCR was conducted under the following conditions: one cycle at 50°C for 30 min and 94°C for 2 min; 35 cycles of 94°C for 30 s, 50, 52, 54, 56 or 58°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 5 min. PCR products were electrophoresed on a 1.2% agarose gel and then observed under UV illumination after staining with ethidium bromide.

To evaluate the compatibility of primer pairs, different combinations of primers were tested using a mixture of single infection extracts of SCMV, SrMV, SCSMV and SCYLV by RT-PCR. The concentrations of each primer pair were altered in increments of 60 nM depending on band intensity, to determine the optimal primer concentrations required to yield the balanced virus-specific fragments. A mixture of equal volumes of four single infection extracts was used as the template for the RT-PCR.

2.4. Evaluation of field sugarcane samples using one-step quadruplex RT-PCR

The quadruplex RT-PCR was evaluated using 202 leaf samples from asymptomatic and symptomatic sugarcane plants including cultivated hybrid sugarcane (Saccharum inter-specific hybrid) and noble sugarcane (Saccharum officinarum).

2.5. Confirmation of RT-PCR products by sequencing

To confirm the identity of the amplified DNA fragments, the RT-PCR bands obtained from the respective viruses of select samples were excised from the agarose gels after electrophoresis and then purified using a gel midi Purification Kit (TianGen Biotech. Co., Ltd., Tianjing, China). The purified amplicons were then directly sequenced from both directions with the virus-specific primers using an ABI3730 DNA sequencer (Invitrogen Biotech. Co., Ltd., Shanghai, China). At least five independent amplicons specific for each virus in the selected samples were sequenced, and the nucleotide sequences obtained were then verified by a BLAST search of GenBank (http://www.ncbi.nlm.nih.gov).

3. Results

3.1. Primer specificity and compatibility

Among the primer pairs tested using uniplex RT-PCR, one pair of SCYLV primers, SCYLV-F/SCYLV-R, and two pairs of SCMV, SrMV and SCSMV primers, respectively, amplified DNA fragments with the expected sizes from infected plants (data not shown). Direct sequencing of these RT-PCR products confirmed their viral origin. The optimum annealing temperatures for amplification of SCMV,
SrMV, SCSMV and SCYLV were determined to 55, 55, 52 and 58 °C, respectively.

Different combinations of these primer pairs were then tested by RT-PCR. Four pairs of primers, one for each virus, amplified DNA fragments with the expected sizes from extracts containing single infections and different mixtures of 2–4 viruses (Fig. 1). The primer pairs SCMV-F1/R1, SrMV-F/R, SCSMV-F/R and SCYLV-F/R simultaneously amplified DNA fragments of 720, 860, 1160 and 512 bp, respectively, and each of the RT-PCR bands produced using these primers was observed clearly following gel electrophoresis. No significant primer pair interactions or primer–dimer formation were observed. Additionally, efficient amplification of the respective viruses was obtained using a combination of the SCMV-F1/SCMV-R1, SrMV-F/SrMV-R and SCYLV-F/SCYLV-R primer (Fig. 1, lanes 7, 9 and 12). Relatively weak products were generated by the SCSMV-F/SCSMV-R primer set, however, the amplified fragment was obtained from extracts of samples of plants infected by a single virus and from mixtures containing SCSMV (Fig. 1, lanes 3, 6, 8, 10, 11, and 13–15). The sequences, their location within the viral genome and the expected sizes of the fragments amplified by these primers are listed in Table 1.

### 3.2. Optimization of quadruplex RT-PCR

The optimization tests revealed that the best cycling conditions for the quadruplex RT-PCR were one cycle of 50 °C for 30 min and 95 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min.

Notable variation in band intensity was observed for each of the amplified DNA fragments in different mixtures of four viruses.
when equal concentrations the four primer pairs were used in the RT-PCR, indicating that these primer pairs had different amplification efficiencies under various PCR conditions (Fig. 1, lanes 5–14). To balance the amplification efficiency, combinations of primers concentrations of 60, 120, 180 and 240 nM were tested in the quadruplex RT-PCR using an extract mixture containing various concentrations of the four viruses as the template. The results indicated that the primer mixture containing 180 nM of SCMV-F/SCSMV-R1, 120 nM of SrMV-F/SrMV-R, 240 nM of SCSV/F/SCSMV-R and 60 nM of SCYLV-F/SCYLV-R generated a similar yield of amplicons (Fig. 1, lane 15). This primer combination allowed empirical detection of the four target viruses.

3.3. Sensitivity of uniplex and quadruplex RT-PCR

The detection limits of the uniplex and quadruplex RT-PCR were determined by testing a series of 10-fold dilutions of a mixture of single infection extracts. In uniplex assays, virus-specific bands for SCMV, SrMV, SCSMV and SCYLV were obtained by RT-PCR amplification after the samples were diluted up to $10^{-7}$, $10^{-8}$, $10^{-4}$ and $10^{-3}$, respectively (Fig. 2A–D). In quadruplex assay, all viruses were detected after the mixed extracts of the four viruses were diluted up to $10^{-3}$ however, only SCMV (720 bp) and SrMV (860 bp) were amplified when the sample was diluted by $10^{-4}$ (Fig. 2E).

3.4. Field sample detection

All four target viruses, SCMV, SrMV, SCSMV and/or SCYLV, were detected in 202 field samples collected throughout the entire growing season (Fig. 3, partial data). Specifically, SCMV was detected in 20 samples (9.9%), SrMV in 41 samples (20.3%), SCSMV in 5 samples (2.5%) and SCYLV in 64 samples (31.7%). Most positive samples were infected with a single virus, but some were infected with two viruses. Six of double infected samples (3.0%) were infected with SCMV and SCYLV, while eighteen (8.9%) were infected with SrMV and SCYLV, three (1.5%) with SCMV and SrMV and two (0.9%) with SCSMV and SCYLV. The SCSMV-infected plants were only found in germplasm accessions from various breeding and commercial fields in southern China. The results indicated that, although mixed infections were detected, most samples from commercial fields were infected with one virus (SCMV, SrMV or SCYLV), which confirms the results of a previously conducted study (Xu et al., 2008). However, SCSMV was only detected in germplasm accessions introduced to the sugarcane breeding fields from India. Additionally, several plants, including two accessions from India that were introduced to the sugarcane breeding fields from India. The detection of SCSMV in Southern China indicates that inclusion of all known viruses in quarantine indexing procedures during plant germplasm exchange is important to avoid the introduction of exotic pathogens.

The detection of multiple infections of the four viruses in one reaction from one sample using the quadruplex RT-PCR method developed here can greatly reduce the cost and labour involved when large scale studies are conducted. Additionally, this specific and sensitive assay could greatly facilitate sugarcane virus testing in epidemiological studies for spatial and temporal distributions of these viruses in the field, in breeding programs for resistance and in certificate and quarantine programs for virus-free plants.

4. Discussion

Herein, the development of a one-step quadruplex RT-PCR for the effective detection of SCMV, SrMV, SCSMV and SCYLV in sugarcane is described. This assay allowed simultaneous detection and differentiation of four important sugarcane viruses for the first time. In addition, the assay developed here was successfully used to detect multiple infections of these viruses in field samples collected during different growing seasons.

Specific amplification of target viruses depends on the design and selection of primers. The genomic sequences of different isolates of some viruses such as SCMV and SCSMV are diverse, therefore, primers must be designed to anneal to the conserved regions of each virus during multiplex RT-PCR to enable detection of a wide range of isolates. Because the primer pairs used in this study were designed to match all available sequences of each virus in GenBank, they have the potential to detect most, if not all variants of the target viruses.

Primer interaction is a crucial factor involved in the successful development of multiplex RT-PCR (Wei et al., 2008). Although primer analyses were conducted using computer software to avoid the formation of secondary structures, primer-dimers and the interactions between different primers, empirical testing was required to identify primers that were virus-specific and amplified the targets efficiently during multiplex RT-PCR. Accordingly, parameters such as the compatibility of the primer pairs, concentrations of the primer pairs and reagents and the cycling conditions were evaluated in this study to optimize the quadruplex RT-PCR. The results revealed that the primers SCSV/F/SCSMV-R worked well in uniplex RT-PCR; but that the expected fragment of 1160 bp was not detected during quadruplex RT-PCR unless the concentration of the primer pair was 125 nM. This may have been due to the interaction between the SCMV primer pair and other primer pairs or the relatively large size of the amplified DNA fragment. The final concentration of SCSV/F/SCSMV-R was increased to 240 nM so that the quantity of the target product could be detected during multiple RT-PCR. The shorter fragments of SCYLV (512 bp), SCMV (720 bp) and SrMV (860 bp) could be amplified effectively using the lower primer concentrations.

The quadruplex RT-PCR was used to investigate sugarcane samples from various breeding and commercial fields in southern China. The results indicated that, although mixed infections were detected, most samples from commercial fields were infected with one virus (SCMV, SrMV or SCYLV), which confirms the results of a previously conducted study (Xu et al., 2008). However, SCSMV was only detected in germplasm accessions introduced to the sugarcane breeding fields from India. Additionally, several plants, including two accessions from India that were introduced to the sugarcane breeding fields were infected with all four viruses. The detection of SCSMV in Southern China indicates that inclusion of all known viruses in quarantine indexing procedures during plant germplasm exchange is important to avoid the introduction of exotic pathogens.

The detection of multiple infections of the four viruses in one reaction from one sample using the quadruplex RT-PCR method developed here can greatly reduce the cost and labour involved when large scale studies are conducted. Additionally, this specific and sensitive assay could greatly facilitate sugarcane virus testing in epidemiological studies for spatial and temporal distributions of these viruses in the field, in breeding programs for resistance and in certificate and quarantine programs for virus-free plants.

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