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Investigation of viruses infecting rice in southern China using a multiplex RT-PCR assay

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ABSTRACT

Rice black streaked dwarf virus (RBSDV), southern rice black streaked dwarf virus (SRBSDV), rice ragged stunt virus (RRSV) and rice gall dwarf virus (RGDV) are common pathogens that affect rice in eastern and southeastern Asia. They are transmitted by the white-backed planthopper, brown planthopper, leafhopper and small brown planthopper, respectively, and require different control strategies. Although rice plants develop characteristic symptoms when infected by each virus, infected plants may also exhibit similar symptoms, which can hinder accurate symptom-based diagnoses. Thus, a one-step multiplex reverse transcription-PCR assay was developed for the simultaneous detection of the four viruses. The amplification products in singly or co-infected plants were easily distinguished during agarose gel electrophoresis. The optimized multiplex reverse transcription-PCR method was used to analyze 582 field samples collected from southern China between 2013 and 2015. RGDV was the most common virus, detected in ~36.1% of the samples, while RRSV, SRBSDV, and RBSDV were detected in ~34.0%, ~30.1%, and ~0.5% of the samples, respectively. There were also differences in the geographical distributions of the four viruses. RRSV mostly occurred in southern Hainan, RGDV was mainly distributed in southwestern and northeastern Guangdong, SRBSDV was detected across southern China, and RBSDV was detected only in the northern parts of southern China.

1. Introduction

Viral diseases constitute a serious threat to rice production in southeastern Asia, where rice black-streaked dwarf virus (RBSDV), southern rice black-streaked dwarf virus (SRBSDV), rice ragged stunt virus (RRSV), and rice gall dwarf virus (RGDV) are common, with serious outbreaks having occurred recently (Anh et al., 2011; Ren et al., 2014; Zhang et al., 2008; Zhou et al., 2013). SRBSDV and RBSDV belong to the genus *Fijivirus* of the family *Reoviridae*. RBSDV was first reported in Japan and is transmitted by the small brown planthopper, *Laodelphax striatellus* (Shikata and Kitagawa, 1977). Recently, there have been RBSDV outbreaks in Jiangsu, Zhejiang, and Shandong provinces in China, resulting in serious crop losses (Ren et al., 2014; Zhang et al., 2001). SRBSDV was first discovered in southern China in 2001 and was identified as a tentative new species in 2008 (Zhou et al., 2008). This virus is transmitted by the white-backed planthopper, *Sogatella furcifera*, which is a long-distance migratory pest (Zhou et al., 2008). Outbreaks of SRBSDV have recently occurred in southern China, northern Vietnam, and Japan (Anh et al., 2011; Matsukura et al., 2013; Zhou et al., 2013). Rice plants infected by SRBSDV are smaller than normal, with dark green leaves and small spikes on the stems or undersides of leaves. These symptoms are similar to those of plants infected by RBSDV (Zhang et al., 2013). RRSV and RGDV belong to the genera *Oryzavirus* and *Phytoreovirus*, respectively, of the family *Reoviridae*. The main vector for RRSV is the brown planthopper, *Nilaparvata lugens* (Ling et al., 1978). RRSV-infected rice plants have dark green ragged leaves, and they exhibit stunting, twisting of new leaf tips, and linear vein swelling on the surfaces of leaves and leaf sheaths (Ghosh and John, 1980). RRSV was discovered in the 1960s, first in Indonesia and the Philippines, then in Thailand, Vietnam, and southern China during the late 1980s and 1990s (Ling et al., 1978). Until recently, RRSV-associated disease outbreaks had become less frequent; however, the virus has again been detected in rice fields in southern China, indicating that RRSV is becoming more common (Wang et al.,

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2014). RGDV is mainly transmitted by the leafhopper vectors, *Recila dorsalis* and *Nephotettix cincticeps* (Omura et al., 1980). It was first detected in Thailand in 1979 and later caused serious damage in Guangdong and Guangxi provinces in China (Deng et al., 2007; Fan et al., 1983; Omura et al., 1980; Zhang et al., 2008). Infected rice plants exhibit nearly spherical small nubs on the under surfaces of leaves and sheaths.

SRBSDV, RRSV, RGDV, and RBSDV can occur together in southeastern Asia, especially in the rice fields of southern China. Although each virus produces unique symptoms, the early ones, such as reduced plant height and dark green leaves, are very similar. This can result in inaccurate viral diagnoses. A rapid diagnosis is important for efficient disease prevention and control at the initial infection stage, and it is necessary to establish such detection methods for these four viruses. Each virus contains a dsRNA genome. Those of SRBSDV, RRSV, and RBSDV contain 10 segments, while that of RGDV contains 12 segments (Hibino, 1996). The sequence homology levels of the four viruses are different. SRBSDV and RBSDV are the most closely related, with their most highly conserved components (segments 1, 2, and 10) being 78.5%–79.2% identical (Wang et al., 2010). The genomes of RRSV and RGDV are significantly different from those of SRBSDV and RBSDV. Thus, the development of a method to simultaneously detect these viruses based on their genome sequence differences may be possible.

In the present paper, SRBSDV, RBSDV, RRSV, and RGDV, which commonly occur in southern China, were simultaneously detected for the first time. The data presented may prove useful in determining the conditions required for, and the general distribution of, rice viruses in southern China.

2. Materials and methods

2.1. Plant materials and RNA extractions

Rice plants singly infected by SRBSDV, RRSV, RGDV, or RBSDV were obtained in 2013 from Guangzhou, Guangdong and Henan provinces in China. The samples were identified by reverse transcription (RT)-PCR, and then stored at −80 °C. We also collected 582 symptomatic rice samples between 2013 and 2015 from Guangdong, Guangxi, and Hainan provinces, China. The samples were stored at −80 °C.

RNAiso Plus (TaKaRa, Dalian, China) was used to extract rice total RNA. Approximately 200 mg of leaf tissue from an infected rice plant or a healthy control was ground to a fine powder in liquid nitrogen, mixed with 1 ml RNAiso Plus, and then transferred to a 2.0 ml centrifuge tube. Samples were vortexed and centrifuged at 12,000 rpm for 15 min at 4 °C. Upper phases were transferred to new 1.5 ml centrifuge tubes. Isometric precooled isopropyl alcohol was added, and samples were incubated at −20 °C for 20 min. Supernatants were discarded after samples were centrifuged at 12,000 rpm for 10 min at 4 °C. Precooled alcohol (75%) was added, and the samples were centrifuged at 7500 rpm for 5 min at 4 °C. Supernatants were discarded, and the RNA samples were dried at room temperature. The RNA samples were dissolved in RNase-free double-distilled H_2O. Total RNA samples were stored at −20 °C or used immediately.

2.2. Designing and screening virus-specific primers

To develop an efficient multiplex RT-PCR system, gene sequences from the four viruses and a rice *actin* gene sequence were retrieved from the GenBank database of the National Center for Biotechnology Information. The *coat protein* genes of RBSDV, SRBSDV, RGDV, and RRSV were the most conserved genes (Deng et al., 2007; Pattayawat et al., 2004; Wang et al., 2010), and multiple sequence alignments were performed using Clustal W2 (Larkin et al., 2007). Sequence-specific primers were then designed based on the aligned sequences. Primers specific for the internal control, the rice *actin* gene, were also designed. Each primer set was analyzed, using the program Oligo, for the presence of secondary structures and the possibility of 3′ terminal pairings (Liu et al., 2014; http://www.basic.northwestern.edu/biotools/oligocalc.html).

2.3. Specificity and sensitivity of uniplex and multiplex RT-PCR

To evaluate the specificity and sensitivity of our method, uniplex and multiplex RT-PCR experiments were completed using the primers specific for the four viruses and *actin*. Total RNA purified from rice plants singly infected with RGDV, RRSV, SRBSDV, and RBSDV were mixed and used as templates. To determine the specificity of the primer pairs, multiplex RT-PCR was performed using the One Step RT-PCR Kit (TaKaRa) after modifying the manufacturer's recommended procedure. Total RNA from healthy rice plants was used as a control. The multiplex RT-PCR was performed in a final volume of 20 μl, which contained 10.0 μl 2 × One Step buffer, primers (0.3, 0.4, 0.3, 0.25, and 0.15 μM of RBSDV-, SRBSDV-, *actin*-, RGDV-, and RRSV-specific primers, respectively), 0.5 μl PrimeScript One Step Enzyme Mix (5 U/μl), and 1 μl total RNA (100 ng–1 μg). Water was added to the final volume. The uniplex RT-PCR was completed in a final volume of 15 μl, which consisted of 7.5 μl 2 × One Step buffer, 0.5 μl PrimeScript One Step Enzyme Mix (5 U/μl), 1 μl forward and reverse primers (10 μM for each), and 1 μl total RNA (100 ng–1 μg). Water was added to the final volume. The RT-PCR program was as follows: 50 °C for 30 min; 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 52–58 °C for 30 s, and 72 °C for 40 s; followed by 72 °C for 10 min. The PCR products were analyzed by 1.2% agarose gel electrophoresis and staining with 5 μl/100 ml GoldView (Leagene, Beijing, China). Gel images were recorded using a UV illumination imaging system.

2.4. Field sample analyses

From 2013 to 2015, 582 rice samples that exhibited stunted growth were collected in Guangdong, Guangxi, and Hainan provinces in southern China. At least 10 to 25 rice fields were surveyed in each province. To determine the incidences of the four viruses, multiplex RT-PCR was performed. Selected PCR products were excised from agarose gels and sequenced. The resulting sequences were aligned with those from the National Center for Biotechnology Information database.

3. Results

3.1. Uniplex and multiplex RT-PCR specificities

With mixed RNA templates, the uniplex RT-PCR produced a single band without any primer-dimers. The total RNA from plants infected with the four viruses (RGDV, RRSV, SRBSDV, and RBSDV) was mixed and used for the multiplex RT-PCR to identify the most appropriate primers (Table 1). Distinct amplified products were observed in the 1.2% agarose gel (Fig. 1), and the size of each amplicon was as follows: 245 bp for RGDV, 397 bp for RRSV, 548 bp for the *actin* gene, 682 bp for SRBSDV, and 904 bp for RBSDV. No amplification product was generated in the negative control.

3.2. Optimization of multiplex RT-PCR conditions and analysis of assay sensitivity

Initial experiments revealed that the amplification efficiencies