Virus Research 208 (2015) 146–155

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Quantification of southern rice black streaked dwarf virus and rice black streaked dwarf virus in the organs of their vector and nonvector insect over time

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ARTICLE INFO

Article history:
Received 9 March 2015
Received in revised form 15 June 2015
Accepted 16 June 2015
Available online 23 June 2015

Keywords:
SRBSDV
RBSDV
RT-qPCR
Copy number
Threshold
Dissemination
Incompetency

ABSTRACT

Southern rice black streaked dwarf virus (SRBSDV) and rice black streaked dwarf virus (RBSDV) are serious rice-infecting reoviruses, which are transmitted by different planthoppers in a persistent propagative manner. In this study, we quantitatively compared the spatial distribution of SRBSDV and RBSDV contents over time in their vector and nonvector insects using real time-PCR. Genome equivalent copies (GEC) were assessed every 2 days from 0 to 14 days after a 3-days acquisition access period (AAP) on infected plants. Results revealed 293.2 ± 21.6 to 404.1 ± 46.4 SRBSDV GEC/ng total RNA in whole body of white-backed planthopper (WBPH, *Sogatella furcifera*) at day 0 and 12 and 513.5 ± 88.4 to 816.8 ± 110.7 RBSDV GEC/ng total RNA in the whole body of small brown planthopper (SBPH, *Laodelphax striatellus*) at day 0 and 14, respectively, after 3-days AAP. Highest GEC of both viruses were found in the gut of their respective vectors. Although SRBSDV was detected in the gut of SBPH, it did not spread into the hemolymph or other organs. After an 8-day latent period, the transmission efficiency of SRBSDV and RBSDV by their respective vectors was significantly positively correlated with GEC in the salivary gland ($r^2 = 0.7808$, $P = 0.0036$ and $r^2 = 0.9351$, $P < 0.0001$, respectively, at $\alpha = 0.05$). Together, these results confirm that accumulation of >200 SRBSDV or RBSDV GEC/ng total RNA in the gut of vector, indicated threshold for further spread and the virus content in the salivary gland was significantly correlated with transmission efficiency by their respective vectors.

1. Introduction

Southern rice black streaked dwarf virus (SRBSDV), a tentative member of genus *Fijivirus*, family *Reoviridae*, was first recorded in 2001 in Yangxi County, China (Zhou et al., 2008), and has spread to southern China, Vietnam, and Japan via long-distance migration of the white-backed planthopper (WBPH, *Sogatella furcifera* Horvath) (Zhou et al., 2010, 2012; Xu et al., 2012; Li et al., 2012; Hoang et al., 2011; Cheng et al., 2013; He et al., 2013; Matsukura et al., 2013). Another reovirus, rice black streaked dwarf virus (RBSDV) was first discovered in 1941 in Japan, in the 1960s in China and Korea (Hibino, 1996; Chen and Zhang, 2005; Lee et al., 2005), but re-emerged in 1996 and then rapidly spread to the most of regions where *Oryza sativa* ssp. *japonica* is grown in China (Wang et al., 2009; Zhang et al., 2013a). During recent years, both viruses have caused considerable losses in rice yield.

SRBSDV is effectively transmitted by WBPH, but not by the small brown planthopper (SBPH, *Laodelphax striatellus* Fallen) (Zhou et al., 2008, 2013; Mar et al., 2014). Upon ingestion by WBPH feeding on a rice plant infected with SRBSDV, the virus initially infects and replicates in the epithelial cells of the insect's midgut, then spreads to extensively in the midgut and other tissues (Jia et al., 2012a). The virus finally infects the salivary glands where it replicates and will later be introduced into the rice plants during insect feeding (Jia et al., 2012a). SRBSDV is acquired by WPBH in only 5 min, whereas latent periods range from 6 to 14 days (Pu et al., 2012). On the other hand, SRBSDV can be acquired by SBPH, but is not transmitted to plants perhaps because the virus is not disseminated into the hemocoel or the salivary glands (Pu et al., 2012; Jia et al., 2012b).

RBSDV is also transmitted by two other planthoppers (*Unkanodes sapporona* Matsumura and *Unkanodes albifascia* Matsumura) in addition to SBPH (Ling, 1972). Infection and the dissemination route of RBSDV in SBPH are similar to those of SRBSDV in WPBH

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http://dx.doi.org/10.1016/j.virusres.2015.06.015
0168-1702/

(Jia et al., 2014). When RBSDV was acquired by SBPH feeding on the infected plants, 32% of the individual insects transmitted the virus to a new plant after a latent period of 4–35 days (in most cases 7–21 days) (Ling, 1972). Although the main details of SRBSDV and RBSDV transmission by their respective vectors are well known, no information is available on the threshold viral titer required for dissemination of the virus into various organs of vector insects and finally transmission into a plant.

Virus threshold has been demonstrated as a key factor for several arboviruses in their vectors (Hardy, 1988). A high virus producer females of *Culex tarsalis* Coquillett could not transmit western equine encephalomyelitis virus (WEEV) after taking in a low virus dose of 10^3 PFU/0.1 mL (Mahmood et al., 2006). Todd et al. (2010) found that maize fine streak virus (MFSV) titer in black-faced leafhopper (*Graminella nigrifrons* Forbes) and transmission were increased with longer post-first access to diseased periods (PADPs), but no transmission occurred regardless of the titer when the PADP was shorter than 4 weeks. Similarly, Rotenberg et al. (2009) found that as the titer of tomato spotted wilt virus (TSWV) increased in individual western flower thrips, the transmission efficiency also increased.

RT-PCR is considered the most effective tool for quantifying the amount of RNA present in a sample (Pfaffl and Hageleit, 2001; Wong and Medrano, 2005). At present, two methods of quantification are used. (1) Absolute quantification is used to determine the actual amount of RNA present in a sample in relation to a standard curve. (2) Relative quantification is used to compare the relative amount of RNA between two samples by using an internal control (Bustin, 2000; Orlando et al., 1998). Both methods of RT-PCR have been widely used to detect viruses in plants and insect vectors (Lett et al., 2002; Varga and James, 2005; Zhang et al., 2010, 2013b; Watanabe and Bressan, 2013; Wang et al., 2014a,b). In the present study, we sought to quantify two viruses over time in the various organs of their respective vector and nonvector insects using quantitative RT-PCR (RT-qPCR) and conventional RT-PCR. The results will contribute to our understanding of the transmission mechanism of SRBSDV and RBSDV by their respective vector insects.

2. Materials and methods

2.1. Insect populations and virus sources

Populations of WBPH, SBPH and the zigzag-striped leafhopper (ZLH, *Recilia dorsalis* Motschulsky) were maintained on susceptible rice (*O. sativa* ssp. *japonica*) cultivar (cv.) Huaidao 5 in a climate chamber at 27 °C with a photoperiod of 16 h light and 8 h dark and transferred at various times to newly grown seedlings in glass jars (Huo et al., 2014). Rice plants infected with SRBSDV were collected from Fujian, and RBSDV-infected plants were collected from Kaifeng, Henan, China. The collected rice samples were confirmed for virus infection by conventional RT-PCR (Zhang et al., 2008). Newly hatched nymphs of WBPH and SBPH were allowed to feed on the respective infected plants to acquire and transmit the virus to cv. Huaidao 5, which was then used as the virus source plant.

2.2. Virus acquisition, sampling of insects and RNA extraction

Third instar nymphs of WBPH, SBPH and ZLH were starved for 2 h, then allowed a 3-day acquisition access period (AAP) on approximately 50-day-old plants at 35 days post-inoculation with either SRBSDV or RBSDV, before they were transferred to healthy rice seedlings. Samples of whole insects or body tissues of the respective insect were collected every 2 days from 0 to 14 days after the initial AAP. Whole insects were quickly frozen in liquid nitrogen, and the internal organs (gut, salivary gland, fat body) were excised separately in a drop of diethylpyrocarbonate (DEPC)-treated water using forceps and a stereomicroscope. The drop of the hemolymph released after decapitation of the insect was also collected using a fine pipet tip. Total RNA was extracted from a cohort of 10 whole insects bodies and/or dissected tissues using TRIzol reagent (Invitrogen Trading, Shanghai, Co.) and the manufacturer's instructions, then stored at −80 °C. The RNA samples were separated electrophoretically in an agarose gel to evaluate integrity by visualizing under ultraviolet light, then the concentration was measured using a NANODROP-2000 spectrometer (Thermo Fisher Scientific, Roskilde, Denmark).

2.3. Primer design

Because the genomes of SRBSDV and RBSDV are relatively the same, primer design is a major consideration for accurate detection and quantification. The similarity between genomic segments was assessed by alignment of sequences using Vector NTI sequence analysis software (Invitrogen, CA). Whole viral genomic sequences of SRBSDV isolate Hainan and RBSDV isolate Zhejiang were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/). The S4 genomic segment of both viruses showed less identity and was selected for designing primers. The nucleotide sequence of SRBSDV was downloaded from the GenBank database (accession FN563992.1). Primer pair SRBSDV-S4F (5′TAGCGAACACAAGCCAGAAG3′) and SRBSDV-S4R (5′TTTCCATCCAACCATACGAT3′) was designed to amplify the expected 109-bp fragment in a RT-qPCR. A second primer pair, SRBSDV-S4F (5′CGAAACCAGCACTCTACCGAAC3′) and SRBSDV-S4R (5′AATGAGGAGACTCCGCTCCATG3′), was designed from GenBank accession EU523360.1 to amplify a 620-bp fragment from S4 genomic segment using conventional RT-PCR. Similarly, the nucleotide sequence of the S4 genomic segment of RBSDV was downloaded from the GenBank database (accession AJ409146.1). Another primer pair, RBSDV-S4F (5′CATCAAAAAAGCCGGAAGCT3′) and RBSDV-S4R (5′CAACCATGATCCCTGTAAGAATAAAA3′), was designed to amplify this 140-bp fragment in a RT-qPCR, and primer pair RBSDV-S4F (5′TACTCGCCATGCTTGGTCTA3′) and RBSDV-S4R (5′CAACGAAATCAACGCTCACT3′) was developed using GenBank accession AJ293984.1 to amplify a 969-bp fragment from the S4 genomic segment in a conventional RT-PCR. Actin gene of WBPH and SBPH was amplified using universal degenerated primers designed by Wu et al. (2013) ACT-1 (5′CCYGAYGGYCARGTRATCACMATTGG3′) (Y = C,T; R = A,G; M = A,C) and ACT-2 5′-GAKATCCACATCTGYTGGAARGTG3′) (K = G,T; Y = C,T; R = A,G) with expected length of 347-bp in conventional RT-PCR as internal control. In case of ZLH, 195-bp fragment of Co1 gene (accession LN681350.1) was amplified as internal control using primers ZLH-Co1F (5′′CCTGATATAGCATTTCCCCG3′) and ZLH-Co1R (5′TCCTGCAAGATGGAGTGAAA3′) in conventional RT-PCR. The primer sets for RBSDV or SRBSDV designed for RT-qPCR were also used to produce cDNA fragments.

2.4. Preparation of recombinant plasmid DNA for standard curve

The 109-bp and 140-bp fragments from S4 of SRBSDV and RBSDV, respectively, were cloned in pGEM-T Easy Vector System 1 (Promega, Madison, WI, USA) according to the manufacturer's instructions and used to transform the JM109 component in *Escherichia coli* cells. The cells containing the recombinant plasmid were selected using ampicillin (50 mg/mL), and the presence of the plasmid was verified by PCR and subsequent sequencing of the PCR products. The recombinant plasmid DNA was extracted using an Axyprep™ Plasmid Miniprep Kit (Axygen Biosciences). The concentration of extracted plasmid DNA was