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Analysis of rice stripe virus whole-gene expression in rice and in the small brown planthopper by real-time quantitative PCR

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Summary. – Rice stripe virus (RSV) is a typical member of the *Tenuivirus* genus, which is transmitted mainly by the small brown planthopper (SBPH) in a persistent, circulative-propagative manner. At present, expression levels of viral genes in rice and in SBPH remain unclear. In this study, we investigated RSV gene expression in RSV-infected rice tissues and in viruliferous SBPH using a sensitive and reliable real-time quantitative PCR (Q-PCR) method. The results revealed that NS3 gene exhibited the highest abundant expression in both, rice plants and SBPH. Disease-specific protein (SP) gene was the only gene with highest expression in rice, but not in SBPH. The expression levels of other genes were lower than SP and approximately equal to each other in both, in rice and SBPH. RNA-dependent RNA polymerase (RdRp) in SBPH was present in a notably low level. Furthermore, our results also provided a basis for further investigation on how RSV coordinates its own gene expression in the interactions with its plant and insect hosts.

Keywords: rice stripe virus; viral gene expression; real-time Q-PCR; rice plants; SBPH

Introduction

Rice stripe virus (RSV, the genus *Tenuivirus*, causes rice stripe disease) is one of the most serious rice diseases in subtropical and temperate regions (Toriyama, 1986). RSV was reported to cause severe disease in rice fields in China in recent years (Wang *et al*., 2004). The small brown planthopper (*Metazoa, Arthropoda, Insecta, Hemiptera, Delphacidae*, *Laodelphax striatellus* Fallén) (SBPH) is main vector species of RSV. RSV relies on SBPH for its horizontal transmission in a persistent, circulative-propagative manner. The virus can replicate in the SBPH's ovary and is transmitted transovarially (vertically) to offspring by eggs (Toriyama, 1986; Falk and Tsai, 1998). Even at a lower density, viruliferous SBPH could lead to significant yield losses by virus infection (Hibino, 1996). RSV has two hosts, rice plants and SBPH, therefore RSV is not only a plant virus, but also an insect virus. To reveal the mechanism how RSV interacts with its two hosts and how it is transmitted specifically by SBPH is crucial for disease prevention and control in practice. At present, the molecular mechanisms of RSV-host interaction remain unclear. Gene expression analysis of RSV in rice and in SBPH is significant for understanding the interaction relations between RSV and its two hosts.

RSV is a single stranded RNA virus with four segmented genomes which contain seven ORFs, and use a negative and ambisense coding strategy for replication and infection in hosts (Ramirez and Haenni, 1994). RNA1 encodes a putative protein of 337 kDa in a negative sense, which acts as an RNA-dependent RNA polymerase (RdRp) (Toriyama *et al*., 1994). RNA2, RNA3, and RNA4 use ambisense coding strategy and each RNA segment contains two ORFs, one at

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Abbreviations: C_T = threshold of cycle; NCP = nucleocapsid protein; Q-PCR = quantitative PCR; RdRp = RNA-dependent RNA polymerase; RSV = rice stripe virus; SBPH = small brown planthopper; siRNA = small interference RNA; SP = disease-specific protein

the 5′-end of viral RNA (vRNA) and the other at the 5′-end of viral complementary RNA (vcRNA). RNA2 encodes NS2 protein, a silencing suppressor with other unknown functions (22.8 kDa) and NSvc2, a putative membrane glycoprotein (94 kDa) (Takahashi *et al*., 1993; Du *et al*., 2011). RNA3 encodes NS3, a suppressor of gene silencing (23.9 kDa) and a nucleocapsid protein (NCP or CP, 35 kDa) (Kakutani *et al*., 1991; Zhu *et al*., 1991; Xiong *et al*., 2009). RNA4 encodes a nonstructural disease-specific protein (SP, 20.5 kDa) and a movement protein NSvc4 (32.5 kDa) (Toriyama, 1986; Kakutani *et al*., 1990; Zhu *et al*., 1992; Xiong *et al*., 2008).

Real-time quantitative PCR (Q-PCR) is a very sensitive, accurate and reproducible technique that can be used to detect small amounts of RNA molecules. Additionally, this technology has been proved to be efficient for analysis of virus gene expression, for example, Eepstein-Barr virus (Pan *et al*., 2005) and pseudorabies virus (Tombacz *et al*., 2009). Zhang *et al*. (2008a) reported one-step real time RT-PCR methods for quantifying RSV-CP gene in rice tissues and in SBPH. In this study, we used real time Q-PCR method to analyze RSV gene expression in RSV-infected rice tissues and in viruliferous SBPH for the first time.

Materials and Methods

Virus sources and insect vector. The RSV isolate used in the experiment was obtained from rice plants showing typical stripe symptoms in Jiangsu Province, China. The isolate was identified as RSV by indirect ELISA using monoclonal antibodies against RSV and RT-PCR using specific primers, and then inoculated into the susceptible rice cultivar Wuyujing No.3 by virus-free SBPH to increase virus concentration. Rice plants were later tested for RSV by indirect ELISA, and leaves were collected from RSV-positive plants displaying typical stripe symptoms and stored at -70°C. Monoclonal antibodies (3B9) against RSV were prepared and conserved by Zhejiang University and author's laboratory (Wang *et al*., 2004).

SBPHs used in this study were collected from Jiangsu Province, China, and has been maintained in the laboratory for nearly 6 years. High-viruliferous populations were screened and reared in glass beakers as stock population, and the proportion of viruliferous SBPH was above 90%. Rice plants (cultivar Wuyujing No.3) as SBPH's diet were grown in soil at 25°C with a photoperiod of 16 hrs/8 hrs (light/dark) in a growth incubator. After insects were introduced into a glass beaker which contained rice seedlings (2–3 cm high), the beaker was enclosed with a piece of nylon mesh. The planthoppers were transferred to fresh seedlings every 10–14 days to assure sufficient nutrition.

RNA extraction. Total RNA from 1 g of RSV-infected rice leaf tissue and 20 SBPH individuals was extracted following the standard protocol of TRIzol reagent (Invitrogen, USA). The concentration and quality of each RNA sample was determined with an Eppendorf Biophotometer plus (Eppendorf, Germany). Only the RNA samples with an *A*260/*A*280 ratio (an indication of protein contamination) of 1.9–2.1 and an *A*260/*A*230 ratio (an indication of reagent contamination) greater than 2.0 were used for the analysis. The integrity of RNA samples was assessed by agarose gel electrophoresis.

Primer design and cDNA synthesis. In order to investigate the expression level of seven RSV genes in rice plants and in SBPH, valid Q-PCR primers for each RSV gene were needed. For Q-PCR primers design, seven ORFs were amplified via RT-PCR using primer pairs designed according to RSV isolate T sequence information (GenBank No. RdRp, D31879; NS2, and NSvc2, D13176; NS3, and CP, X53563; SP and NSvc4, D10979). The amplified products were sequenced, thus thre information concerning the complete sequence of RSV isolate Jiangsu in rice and in SBPH population was obtained and used for primer design in the experiment. 18S rRNA gene (GenBank Acc. No. AK059783) was selected as rice internal control, while β-actin gene (GenBank Acc. No. AY192151) was used as SBPH internal control. Their expressions were the most stable in respective samples. Q-PCR primer pairs were designed by using the Primer3 (http://frodo.wi.mit.edu/primer3/) to comply with the criteria suitable for SYBR Green-based Q-PCR assay (Table 1). First strand cDNA was synthesized with oligo(dT) primer by using 1st strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocols.

Real-time Q-PCR assay. Q-PCR analysis was independently performed three times with different RNA preparations for each sample and conducted on ABI 7500 Real-Time PCR system (Applied Biosystems, USA) following the manufacturer′s instructions. Each reaction was performed in a 25-µl mixture, which contained 0.2 µmol/l of each primer, 1 µl of template cDNA, 12.5 µl of 2×SYBR Green PCR Master Mix (Applied Biosystems, USA, including AmpliTaq Gold DNA polymerase with buffer, dNTP, SYBR Green I dye and Rox dye), and 10.5 µl ddH_2O. The amplification program proceeded at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min. After the cycling protocol, the melting curves were obtained to eliminate the production of non-specific products. The temperature of PCR products was raised from 60°C to 95°C in 0.1°C/sec increment. The amplified products were analyzed by agarose gel electrophoresis and sequenced to confirm that they were target genes. The C_T (threshold of cycle) value of each detected gene was determined by automated threshold analysis on ABI System. The C_T value of each RSV gene was normalized to $C_{T(\text{internal control})}$ to obtain $\Delta C_{T(\text{RSV gene})}$ ($= C_{T(\text{RSV gene})} - C_{T(\text{internal control})}$). The relative expression quantity of RSV genes was indicated with $\Delta\Delta C_T$ ($= \Delta C_{T(\text{RSV gene})} - \Delta C_{T(\text{RSV CP})}$) according to $2^{-\Delta\Delta Ct}$ algorithm.

Results

Analysis of gene expression level of RSV in rice plants

For gene expression analysis, the information concerning viral gene expression level in RSV-infected rice plants was

Table 1. Q-PCR primers used in the experiment

Primer	Sequence (5′→3′)	Amplicon length	Target gene
RdRP-F	ATAGGCAGTCCAGAATCAGGGTAT	217bp	RSV RdRp
RdRP-R	CGTTCAGTAGTATGAGGAGTGTCCAA		
NS2-F	GGCATTACTCCTTTTCAATGATCA	201bp	RSV NS2
NS2-R	GTTGCTATGGCGAGAGCATGT		
NSvc2-F	TTTCCCTGACACCCATTCTTG	213bp	RSV NSvc2
NSvc2-R	TGGAGAGGCAGCTTGTAATCG		
NS3-F	ATTTGATCATCCTCTGCTTTTGG	159bp	RSV NS3
NS3-R	CCATGAAGAAGCCTCAGAAACTG		
CP-F	TGCAGAAGGCAATCAATGACAT	150bp	RSV CP
CP-R	TGTCACCACCTTTGTCCTTCAA		
SP-F	CCTGTTAGGAGGTGAAGATGATGA	181bp	RSV SP
SP-R	GCTCTCAGCCTTAGCCATCTTG		
NSvc4-F	TGGAATAACTACCCTCCGCATAA	229bp	RSV NSvc4
NSvc4-R	CCTTTCAATTCCCCAGAACCA		
18S rRNA-F	ATGGTGGTGACGGGTGAC	159bp	Rice 18S rRNA
18S rRNA-R	CAGACACTAAAGCGCCCGGTA		
Actin-F	TCTTGAGATTGGACTTGGC	131bp	SBPH β-actin
Actin-R	GTAGCACAGTTTCACCTTG		

collected. In all repeated experiments, the C_T value of internal control 18S rRNA was very stable. The relative mRNA expression quantity of RSV genes was calculated according to $2^{-\Delta\Delta Ct}$ algorithm. The resulting $2^{-\Delta\Delta Ct}$ values for each RSV gene were used to plot. As demonstrated in Fig. 1a, it was noticeable that the NS3 gene exhibited the most abundant expression level and it was 5.9-fold higher than CP gene. SP had 2.9-fold higher expression level than CP. Five other genes, including RdRp, NS2, NSvc2, CP, and NSvc4, were present in low levels and approximately equivalent to each other. They were arranged as NSvc4, NS2, CP, RdRp, and NSvc2 in descending order of expression quantity.

Analysis of relative mRNA expression quantity of RSV genes in SBPH

Information concerning gene expression level of RSV in viruliferous SBPH was collected. In parallel experiments, the C_T value of β-actin was stable. The $2^{-\Delta\Delta Ct}$ values for each RSV gene were calculated and plotted. The relative expression quantity of RSV genes in SBPH was shown in Fig. 1b. Similarly to the results in rice, NS3 exhibited the most abundant expression level and it was 8.3-fold higher than CP gene. Also, comparably to the results in rice, expression quantity of SP gene was only 1.7-fold higher than that of CP. SP expression level was more or less similar to the expression of all other genes (NS2, NSvc2, CP, and NSvc4). SP, NS2, NSvc4, CP, and NSvc2 were arranged from high to low abundance. RdRp was present in a noticeably low level in SBPH and only 0.2-fold the quantity of CP.

Discussion

In recent years, RSV had caused severe disease outbreaks in rice fields in China. The functions of some RSV genes in infection process are still unknown. Analyzing the differences in expression quantity of RSV genes in rice and SBPH is of a great significance for further investigation of the viral gene functions and the mechanisms how RSV coordinates its own gene expression during virus-plant and virus-insect interactions. Currently, the expression level of RSV genes in rice plants and in SBPH is still not fully understood.

In this study, the gene expression level of RSV genes in rice plants and SBPH was investigated by using Q-PCR. NS3 gene exhibited the highest expression quantity in both, rice plants and SBPH. Zhang *et al*. (2010) searched EST library for RSV gene sequences of viruliferous SBPH, and discovered that NS3 was the most abundant transcript. As a suppressor of gene silencing, NS3 can significantly reduce the accumulation of siRNA in plant cells, and can bind 21-nt single-strand siRNA, siRNA duplexes, and long single-strand RNA (Xiong *et al*., 2009). The dominant expression of NS3 gene suggests that after invading the host cells, RSV must suppress the immune responses of plant and insect host. In future study, it would be interesting to identify the host cellular targets that are suppressed by NS3. However, NS2 is also a silencing suppressor, but it did not exhibit higher expression. NS2 might suppress RNA silencing by targeting proteins involved in silencing amplification (Du *et al*., 2011). Therefore, the functional coordination of NS3 and NS2 is important and worthy of further study. Furthermore,

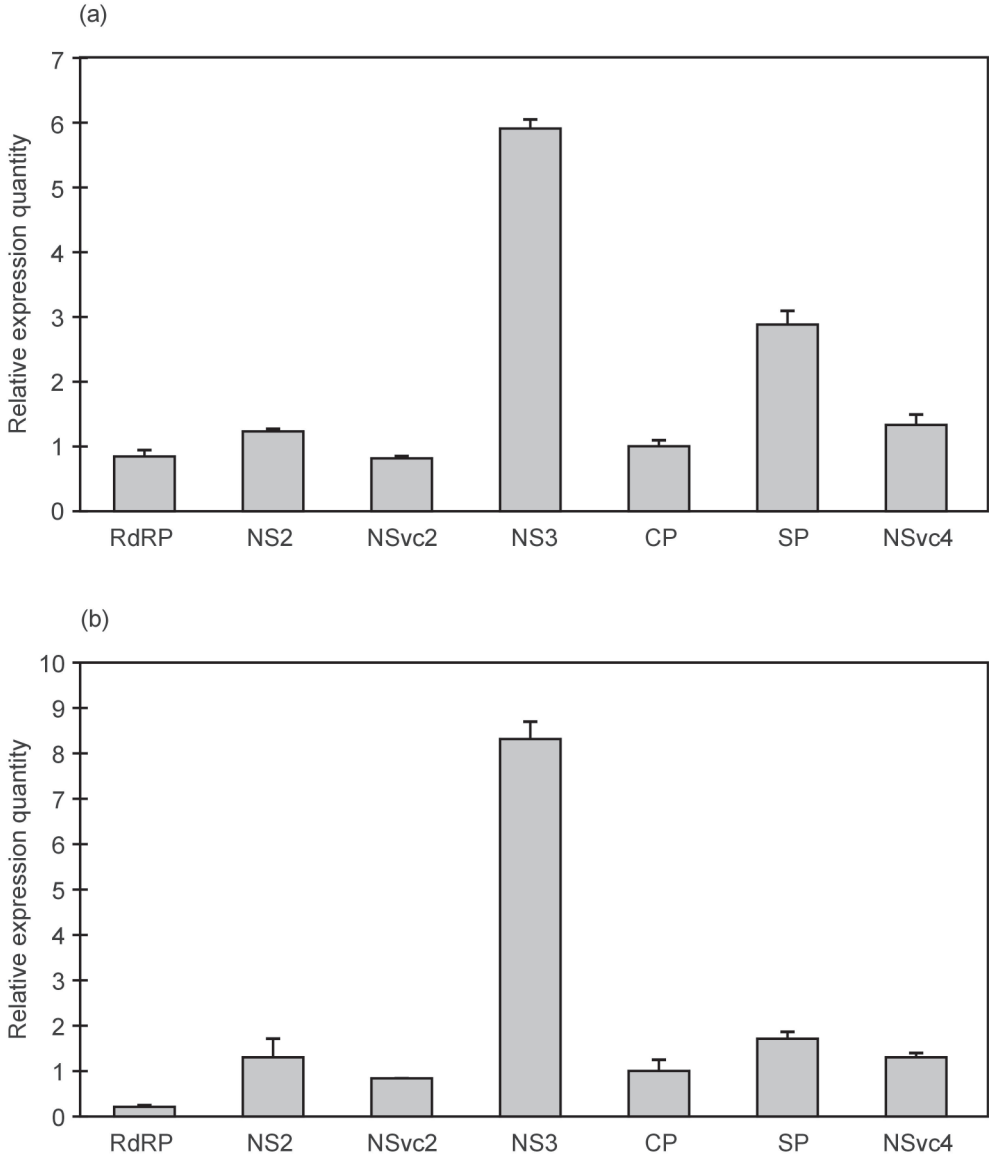

Fig. 1

Relative gene expression of RSV in rice plants and in SBPH

The $2^{-\Delta\Delta Ct}$ values for each RSV gene in RSV-infected rice plants and in SBPH were obtained and plotted. The histograms show relative mRNA expression quantity of RSV genes in rice plants (a) and in SBPH (b).

because of its dominant expression, NS3 gene could be used as a more appropriate target gene than CP gene in disease diagnostics and molecular biology studies of RSV. SP was present in a higher abundance in rice, but not in SBPH. The higher expression quantity of SP gene in rice plants was consistent with previous reports and they also proved that the disease-specific protein was abundant and its accumulation was closely related to symptoms in host plants (Toriyama, 1986; Lin *et al*., 1998). However, the low expression level of SP gene in the insect might be the reason for absence of particularly important viral functions in SBPH. Higher abundance of RdRp in rice, compared to its low level, in SBPH may be due to the more intensive replication and assembly of RSV in rice plants than in the insect. Zhang *et al.* (2010) failed to identify transcript of movement protein NSvc4 in transcriptome of viruliferous SBPH. They also suggested that the movement protein plays important role during virus spreading to neighboring cells through the plasmodesmata (Xiong *et al*., 2008), and thus it is downregulated to a very low level in the insect. Our results indicated that expression

level of NSvc4 gene was 1.3-fold higher than CP gene in the insect; however, this was not in accordance with Zhang′s (Zhang *et al*., 2010) suggestions. Zhang *et al*. (2008b) also detected NSvc4 protein in viruliferous SBPH with western-blot. Therefore, we think that movement protein might have an important function in the interplay between RSV and SBPH. For the future, it would be interesting to investigate NSvc4's functions in the insect.

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