Evaluation of reference genes and expression of key genes involved in the isoprenoid metabolic pathway of rice leaves after infection by the Southern rice black-streaked dwarf virus

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Abstract

The Southern rice black-streaked dwarf virus (SRBSDV), a novel *Fijivirus*, poses a major threat to rice production in East Asia. Analysis of the gene expression of SRBSDV-infected rice plants may reveal the molecular basis of interactions between the virus, its vector and rice plants. Reliable reference genes are required for accurate qRT-PCR analysis. However, no reliable, valid reference genes for examining gene expression in SRBSDV-infected rice plants have so far been identified. We examined the expression of eight candidate reference genes in the leaves of SRBSDV-infected, and healthy, rice plants at different points in time after virus inoculation. We used four dedicated algorithms, geNorm, BestKeeper, NormFinder and RefFinder, to evaluate the performance of these candidate genes. The RefFinder results indicate that *18S*, *EF1α* and *UBQ10* are suitable reference genes. In addition, we used these three reference genes to analyze the expression of key genes involved in the isoprenoid metabolic pathway in rice leaves after infection by SRBSDV. The results of this analysis reveal that SRBSDV may suppress the production of the rice plant volatiles that attract natural enemies of its vector *Sogatella furcifera*, thereby increasing the likelihood of pathogen transmission.

Keywords Southern rice black-streaked dwarf virus · Reference genes · qRT-PCR · Normalization · Isoprenoid metabolic pathway

Introduction

The quantitative reverse transcription polymerase chain reaction (qRT-PCR) is the most sensitive, precise and reproducible method for quantifying the mRNA transcript levels of individual genes. However, normalization with reference genes is essential to minimize inconsistencies in protocols and variation in the quality and stability of RNA and enzyme

efficiency [1–4]. For example, numerous studies have demonstrated that the expression levels of some reference genes can vary in different developmental stages and experimental conditions [5–8]. There is, therefore, an urgent need to identify reference genes suitable for specific developmental stages and experimental conditions. Indeed, more than one reference gene is usually required to normalize qRT-PCR data [9, 10].

In 2008, a devastating disease, the Southern rice black-streaked dwarf virus (SRBSDV), was reported in rice (*Oryza sativa*) [11]. The rice plants infected with SRBSDV showing dwarfism, dark leaves, twisting of leaf tips, excessive tillering, small waxy swellings on the stalks and leaf-backs, up-growing rootlets on stem nodes, and seriously diseased plants may die of withering [12]. Diseased plants often showing small spikes and barren grains or none at all, causing serious loss in rice production [12, 13]. The white-backed planthopper (WBPH, *Sogatella furcifera*) is the only known vector of SRBSDV. There has been ongoing research on the effects of SRBSDV on rice and the response of rice to SRBSDV and its vectors [14–19]. No one has yet,

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however, identified suitable reference genes for investigating gene expression in SRBSDV-infected rice plants.

Infection of host cells by a virus causes certain metabolic pathways to be over- or under-expressed resulting in both physiological and phenotypic changes in the host [20–22]. Viruses can mediate the host selection behavior of vectors by altering host plant volatiles thereby attracting potential vectors to infected plants [23]. For example, non-viruliferous *S. furcifera* are more attracted by SRBSDV-infected rice plants than healthy rice plants [24]. Moreover, researchers have revealed that SRBSDV suppresses plant defenses against *S. furcifera* [15, 16, 19, 25]. Isoprenoids are important components of plant volatiles. However, there has been no research on the mRNA transcript levels of the key genes involved in the isoprenoid metabolic pathway in SRBSDV-infected rice plants.

In this paper, we present the results of experiments designed to identify suitable reference genes in the leaves of SRBSDV-infected, and healthy, rice plants. In addition, we compare the mRNA transcript levels of key genes involved in the isoprenoid metabolic pathway in SRBSDV-infected rice plants with those in healthy rice plants.

Materials and methods

Rice plants, *S. furcifera*, and SRBSDV materials

The seeds of the rice cultivar ‘Fengyuanyou 299’ used in this study were purchased from Hunan Longping Seed Industry Co., Ltd. A number of 4-leaf stage rice seedlings were individually transplanted into the pots (9 cm in diameter and 15 cm in height). Seedlings were cultured in insect-proof cages in a greenhouse at 24–32 °C. *S. furcifera* were collected from rice plants in Changsha, Hunan Province, China and reared on healthy rice plants for more than three generations in an illuminated incubator (26 ± 1 °C, 85% relative humidity (RH), 16 h of light and 8 h of darkness). SRBSDV-infected rice plants were kindly provided by Prof. Guohui Zhou (South China Agricultural University, Guangzhou, China).

Collection of healthy, and SRBSDV-infected, rice plants leaves

Third instar *S. furcifera* nymphs were identified and collected under a stereoscopic microscope. These nymphs were then randomly assigned to two groups, one fed on SRBSDV-infected rice plants to obtain viruliferous *S. furcifera*, and the other on healthy rice plants to obtain uninfected *S. furcifera*. Fifteen groups of 10 viruliferous *S. furcifera* were transferred to individual early-tillering rice plants. Five groups of 10 healthy *S. furcifera* were also transferred to

individual early-tillering rice plants. All insects were subsequently removed after feeding on rice plants for 3 days. 0.2 g of the leaves of each rice plant were collected 0, 15, 25, 35 and 45 days (different disease stages) after insects had been removed and stored at –80 °C. The presence of SRBSDV in the rice plants inoculated by viruliferous *S. furcifera* was detected by RT-PCR (reverse-transcription polymerase chain reaction) 45 days after insects had been removed as described previously [24]. Five samples of SRBSDV-infected rice plants were then used in subsequent experiments.

RNA extraction and cDNA synthesis

Total RNA was isolated using the MiniBEST Universal RNA Extraction Kit (TaKaRa, Tokyo, Japan) according to the manufacturer’s instruction. A NanoDrop spectrophotometer (Thermo Scientific NanoDrop 1000, Wilmington, USA) was used to measure the concentration and purity of total RNA. 125 ng of total RNA was used to synthesize first-strand cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa), according to the manufacturer’s instructions.

Candidate genes

Eight candidate reference genes (*UBQ10*, *ACTIN*, *GAPDH*, *UBQ5*, *EF1α*, *25S*, *18S*, *β-TUB*) were selected and their sequences downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) [26–28]. The sequences of 10 key genes (*CMS*, *DMD*, *FPPS*, *GGPPS*, *SPS3*, *HDS*, *IPI*, *MTC*, *PHS*, *SQS*) involved in the isoprenoid metabolic pathway were downloaded from the National Rice Data Centre of China (<http://www.ricedata.cn/gene/index.htm>). The names of these 18 genes are listed in Table 1.

Quantitative real-time PCR

qRT-PCR experiments were carried out using the TB Green Premix Ex Taq™ II (TaKaRa) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The qRT-PCR primers of the 18 genes were designed using information on the National Center for Biotechnology Information profile server (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). The efficiency of qRT-PCR primers was determined using a fivefold dilution series of healthy rice cDNA. The primers and their amplification efficiencies are shown in Table 1. The qRT-PCR protocol was as follows: preheating for 30 s at 95 °C; 40 cycles of 5 s at 95 °C (denaturation), 30 s at 60 °C (annealing); followed by melting curve analysis (HRM rate = 0.5 °C) after the amplification step.

Table 1 The qRT-PCR primers used in this study

Gene symbol	Gene name	Gene ID	Primer sequence (5'–3')	E (%)	R ²
<i>UBQ10</i>	Ubiquitin 10	AK101547.1	F: CAGCCCCTCGTCTGCGATAA R: TCCTTCTGGCCCATTCCGAC	99.1	0.993
<i>ACTIN</i>	Actin	AB047313.1	F: CAGCCCCTCGTCTGCGATAA R: TCCTTCTGGCCCATTCCGAC	104.2	0.988
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	GQ848049.1	F: CAGCCCCTCGTCTGCGATAA R: TCCTTCTGGCCCATTCCGAC	92.8	0.985
<i>UBQ5</i>	Ubiquitin 5	AK061988.1	F: GCGGGAGAAACCACAGGTAA R: GTGATCGTCTCCCCGTCAG	90.5	0.984
<i>EF1α</i>	Eukaryotic elongation factor 1-alpha	GQ848073.1	F: CAGCCCCTCGTCTGCGATAA R: TCCTTCTGGCCCATTCCGAC	106.0	0.993
<i>25S</i>	25S ribosomal RNA	M11585.1	F: AAGGCCGAAGAGGAGAAAGGT R: CGTCCCTTAGGATCGGCTTAC	96.1	0.993
<i>18S</i>	18S ribosomal RNA	AF069218.1	F: ATGGTGGTGACGGGTGAC R: CAGACACTAAAGCGCCCGGTA	93.8	0.991
<i>β-TUB</i>	β-Tubin	D30716.1	F: CTGCCTAAGGTTCCCTGGTC R: ACGGGGAAAGGGGATAAGGT	108.8	0.994
<i>CMS</i>	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol synthase	Os01g0887100	F: CTGCTGTTCTTGGTGTGCCTG R: CCTCATCACCTGTGGAGTTTGC	97.6	0.988
<i>DMD</i>	Diphosphomevalonate decarboxylase	Os02g0107200	F: GAAGCGTGCTCAAGATGTCG R: ACCAAGCCAGCAACCGAAG	97.0	0.987
<i>FPPS</i>	Farnesyl diphosphate synthase 1	Os01g0703400	F: GCCAGATCCTGAATGTGTTGC R: CTTGATGTGCGCAATCAGC	96.7	0.986
<i>GGPPS</i>	Geranylgeranyl diphosphate synthase 1	Os07g0580900	F: GGTGGCTCTGATGAGCAGATT R: TCTTGTCACCTCGCCAAGTCC	90.4	0.992
<i>SPS</i>	Solanesyl diphosphate synthase 3	Os12g0271700	F: ATCTCGGTCTCTCCTTCCAGG R: GCAAAGCGAAAATGACCGGG	102.0	0.988
<i>HDS</i>	1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	Os02g0603800	F: TGATCCTTGCCGGAGATTGG R: GGTGCAAGACCCCTCTGTAG	94.3	0.993
<i>IPI</i>	Isopentenyl diphosphate isomerase	Os07g0546000	F: ATGCTGCTCAGAGGAAGCTC R: AAAGCATCCGACCAAGAGGG	92.8	0.999
<i>MTC</i>	Terpene synthase	Os10g0489500	F: AGCTCACAAAGATCGCCTCC R: ATGACCTCATGCAGCTAGGC	91.8	0.987
<i>PHS</i>	Phytoene synthase	Os06g0729000	F: GACCAAATGCCTCGCACATC R: ATCAAGCATGTCGTAGGGGC	97.8	0.988
<i>SQS</i>	Squalene synthase	Os03g0805100	F: AAGTTTCGCCCTGTCTCCACG R: TTCCTGCTCCCATAGCCTAGT	92.6	0.992

Data analysis

The stability of expression of each candidate reference gene was ranked using three different types of Microsoft Excel-based software: geNorm [29], Normfinder [30] and BestKeeper [31]. In addition, RefFinder [32] was used to rank the overall stability of all candidate reference genes. The relative expression levels of the key genes involved in the isoprenoid metabolic pathway in rice leaves infected by SRBSDV were quantified using the $2^{-\Delta\Delta C_t}$ method [33]. The statistical significance of differences in mean expression levels between healthy and SRBSDV-infected rice plants was assessed with Student's *t*-test in the SPSS program for windows (SPSS Inc., Chicago, USA).

Results

Performance of qRT-PCR primers

A standard curve was generated for each primer pair using the fivefold dilution series of healthy rice cDNA. The amplification efficiency (E) and correlation coefficient (R²) of each primer pair were obtained from the standard curve and are shown in Table 1. The correlation coefficients for all primer pairs ranged from 0.984 to 0.994. All primers had acceptable amplification efficiencies of between 90.4 and 108.8.

Expression levels of candidate reference genes

The expression levels of candidate reference genes were quantified using Cq (quantification cycle) values in the leaves of SRBSDV-infected and healthy rice plants. These assays are summarized as Box-Whisker plots in Fig. 1, the box represents the 25th and 75th percentile range and the median is indicated by a horizontal lines across the box. Average Cq values were within 8.93 and 34.72 cycles. *25S* had the lowest Cq values and β -*TUB* the highest.

Stability of candidate reference genes

For the SRBSDV-infected rice plants, geNorm ranks the candidate reference genes based on the stability measure (M value) which is defined as the mean variation of a given gene in relation to all the others tested. The results of geNorm showed that *18S* and *EF1 α* had the most stable expression with an M value of 0.683 and *ACTIN* was the least stable with an M value of 1.503 (Table 2). The geNorm manual also determines the minimal number of reference genes required for normalization using a pairwise variation, it suggests that when the pairwise variation ($V_n/n+1$) > 0.15, more than $n+1$ genes should be used as internal reference genes. All $V_n/n+1$ values were > 0.15 (Fig. 2A), suggesting

Fig. 1 Expression profiles of the 8 candidate reference genes in leaves of **A** SRBSDV-infected rice plant and **B** healthy rice plant. The expression level of candidate reference genes was given as quantification cycle number (Cq-value) and analyzed by using box which represents the 25th and 75th percentiles, the median is indicated by a horizontal lines across the box

Table 2 Ranking orders of eight candidate reference genes according to their transcription stability

Treatments	Rank	geNorm		NormFinder		BestKeeper	
		Gene name	Stability value	Gene name	Stability value	Gene name	SD
SRBSDV-infected rice	1	<i>18S</i> <i>EF1α</i>	0.683	<i>18S</i>	0.289	<i>25S</i>	0.276
	2	<i>UBQ10</i>	0.808	<i>EF1α</i>	0.357	<i>UBQ10</i>	0.825
	3	<i>GAPDH</i>	0.925	<i>UBQ10</i>	0.468	<i>18S</i>	0.985
	4	β - <i>TUB</i>	1.078	β - <i>TUB</i>	0.595	β - <i>TUB</i>	1.253
	5	<i>UBQ5</i>	1.258	<i>GAPDH</i>	0.627	<i>EF1α</i>	1.265
	6	<i>25S</i>	1.392	<i>UBQ5</i>	0.662	<i>UBQ5</i>	1.539
	7	<i>ACTIN</i>	1.503	<i>ACTIN</i>	0.725	<i>ACTIN</i>	1.563
	8			<i>25S</i>	0.778	<i>GAPDH</i>	1.634
Healthy rice	1	<i>18S</i> <i>EF1α</i>	0.699	<i>18S</i>	0.366	<i>25S</i>	0.231
	2	<i>UBQ10</i>	0.990	<i>EF1α</i>	0.378	<i>UBQ10</i>	0.951
	3	β - <i>TUB</i>	1.140	<i>UBQ10</i>	0.417	<i>ACTIN</i>	1.184
	4	<i>25S</i>	1.299	β - <i>TUB</i>	0.590	<i>18S</i>	1.188
	5	<i>GAPDH</i>	1.414	<i>ACTIN</i>	0.595	<i>GAPDH</i>	1.300
	6	<i>ACTIN</i>	1.508	<i>UBQ5</i>	0.605	β - <i>TUB</i>	1.375
	7	<i>UBQ5</i>	1.607	<i>GAPDH</i>	0.607	<i>EF1α</i>	1.403
	8			<i>25S</i>	0.654	<i>UBQ5</i>	1.619

Fig. 2 Determination of optimal number of reference genes using geNorm. Pairwise variation (V_n/V_{n+1}) analysis of candidate reference genes in the leaves of **A** SRBSDV-infected rice plants and **B** healthy rice plants

Fig. 3 Stability rankings of the 8 candidate reference genes as calculated by RefFinder. A lower geomean of ranking value indicates more stable expression. **A** SRBSDV-infected rice plants, **B** healthy rice plants

that at least 3 genes should be used as control genes. The NormFinder results suggest that *18S* is the best reference gene (Table 2), whereas the BestKeeper results suggest that *25S* is the most suitable (Table 2). The overall RefFinder stability ranking of the candidate reference genes, from most to least stable, was: *18S* > *EF1a* > *UBQ10* > *25S* > β -*TUB* > *GAPDH* > *UBQ5* > *ACTIN* (Fig. 3A).

The stability of the 8 candidate reference genes was also analyzed in the leaves of healthy rice plants. *EF1a* and *18S* were the most stable reference genes with a geNorm M value of 0.699 and *UBQ5* was the least stable (Table 2). All V_n/V_{n+1} values were > 0.15, suggesting that more than 2 reference genes are required (Fig. 2B). The NormFinder results suggest that *18S* is the most stable gene (Table 2) whereas BestKeeper found *25S* to be the most suitable (Table 2). The RefFinder ranking, from most to least stable, was: *18S* > *UBQ10* > *EF1a* > *25S* > β -*TUB* > *ACTIN* > *GAPDH* > *UBQ5* (Fig. 3B).

Transcript levels of key enzymes genes involved in isoprenoid metabolic pathway

Based on the RefFinder ranking, *18S*, *EF1a* and *UBQ10* were selected to normalize different expression levels of the

key genes involved in the isoprenoid metabolic pathway in SRBSDV-infected, and healthy, rice plants.

Compared with healthy rice plants, *SQS*, *SPS* and *FPPS* were significantly ($t = 3.241$, $df = 3$, $p = 0.048$; $t = 3.207$, $df = 4$, $p = 0.049$ and $t = 27.059$, $df = 4$, $p < 0.01$, respectively) up-regulated in the leaves of SRBSDV-infected rice plants at 0 day post inoculation, whereas *IPI* was significantly down-regulated ($t = 6.017$, $df = 3$, $p < 0.01$) (Fig. 4). At 15 days post inoculation, *SPS*, *PHS* and *MTC* were significantly up-regulated ($t = 4.929$, $df = 4$, $p < 0.01$; $t = 2.918$, $df = 4$, $p = 0.043$ and $t = 3.191$, $df = 4$, $p = 0.033$, respectively) and *HDS* was significantly down-regulated ($t = 3.621$, $df = 4$, $p = 0.022$) (Fig. 4). At 25 days post inoculation, *SQS* and *MTC* were significantly up-regulated ($t = 4.042$, $df = 4$, $p = 0.016$ and $t = 6.614$, $df = 4$, $p < 0.01$, respectively) and *HDS* and *DMD* were significantly down-regulated ($t = 2.925$, $df = 4$, $p = 0.043$ and $t = 6.800$, $df = 4$, $p < 0.01$, respectively) (Fig. 4). There were no significantly up-regulated genes 35 days after inoculation but *SQS*, *FPPS*, *HDS*, *GGPPS*, *DMD* and *CMS* were significantly down-regulated ($t = 4.024$, $df = 4$, $p = 0.016$; $t = 3.355$, $df = 4$, $p = 0.028$; $t = 3.563$, $df = 4$, $p = 0.024$; $t = 3.818$, $df = 4$, $p = 0.019$; $t = 5.054$, $df = 4$, $p < 0.01$ and $t = 2.784$, $df = 4$,

Fig. 4 Expression levels of the key enzymes genes involved in isoprenoid metabolic pathway in the leaves of rice plants. Mean ($\pm$ SE) expression level is based on 5 biological replicates, and asterisk above bars indicate significant differences (t -test: * $p < 0.05$, ** $p < 0.01$)

$p = 0.050$, respectively) (Fig. 4). There were also no significantly up-regulated genes 45 days after inoculation but *SQS*, *FPPS*, *IPI*, *HDS*, *DMD* and *CMS* were significantly down-regulated ($t = 13.110$, $df = 4$, $p < 0.01$; $t = 6.711$, $df = 4$, $p < 0.01$; $t = 7.815$, $df = 4$, $p < 0.01$; $t = 6.727$, $df = 4$, $p < 0.01$; $t = 14.373$, $df = 4$, $p < 0.01$ and $t = 3.211$, $df = 4$, $p = 0.033$, respectively) (Fig. 4).

Discussion

Reliable reference genes play a vital role in gene expression profiling. The fundamental assumption is that reference gene expression is consistent regardless of tissue type and experimental conditions. There is, however, no universal reference gene suitable for all species or experimental conditions [34]. Although many studies have assessed reference genes in different tissues and development stages of rice plants, and after subjecting plants to different kinds of stress [28, 35–41], to the best of our knowledge, no one has attempted to identify stable reference genes in the leaves of SRBSDV-infected rice plants.

We assessed the stability of 8 reference genes using four statistical models. According to the RefFinder results, *18S*, *UBQ10* and *EF1 α* were the best reference genes for the leaves of SRBSDV-infected rice plant at different times after inoculation. The infection of SRBSDV can cause profound metabolic changes in rice plants and affecting the expression of different genes [16, 19, 42]. Therefore, our results represent the critical first step in investigating changes in gene expression in the leaves of SRBSDV-infected rice plants.

18S was ranked the most stable gene by geNorm, and the third most stable by NormFinder and BestKeeper in SRBSDV-infected rice plants. *18S* has also been identified as a reliable reference gene during the growth stages of rice [43]. The genes encoding *elongation factor 1- α* and *ubiquitin* have also been considered reliable reference genes under different experimental conditions [44]. We also found *UBQ10* and *EF1 α* to be reliable reference genes in the leaves of SRBSDV-infected rice plants. *EF1 α* has been identified as a stable reference gene in rice subject to low temperatures [36], and *UBQ10* has been recommended as a reliable reference gene in rice plant leaves subject to salt stress [28]. *GAPDH* is commonly regarded as the “classical” reference gene [45], and has been suggested as a reliable reference gene in rice seedlings suffering from iron toxicity [35]. However, we found the expression of *GAPDH* to be quite variable. This variation in results demonstrates the importance of identifying suitable reference genes for specific experimental conditions.

The optimal number of reference genes required to normalize gene expression levels was analyzed using pairwise variation ($V_n/n + 1$) values in geNorm. All pairwise variation values were > 0.15 which suggests that a minimum of 3 reference genes are required [46]. Since rice seedlings grew from the tillering stage to the booting stage over the 45 day experimental period, the observed large changes in gene expression are not unexpected.

Most of the genes involved in the isoprenoid metabolic pathway were up-regulated 0 day after viruliferous *S. furcifera* nymphs were removed from rice plants (Fig. 4). However, nymphs had already fed on plants for 3 days before they were removed and viruliferous *S. furcifera* spend more

time salivating and ingesting phloem sap than virus-free *S. furcifera* [47]. Plant growth, nutrient composition, and secondary compounds can all be affected by viral infection [15, 48, 49]. Secondary compounds are important signals linking plants, pests and natural enemies [50]. Compared to healthy rice plants, the expression of *SQS*, *PHS*, *FPPS*, *IPI*, *HDS*, *GGPPS*, *DMD* and *CMS* was suppressed in SRBSDV-infected rice plants 35 and 45 days after virus inoculation (Fig. 4). Moreover, the mRNA transcript levels of *MTC*, *FPPS* and *SQS*, which catalyze the synthesis of monoterpenes and sesquiterpenes, had rapidly declined 35 and 45 days after virus inoculation (Fig. 4). SRBSDV titres are high in rice 35–50 days after inoculation [16]. Therefore, we suspect that SRBSDV suppressed the chemical defenses of rice against *S. furcifera*, thereby enhancing pathogen transmission. Predators of pest insects are also attracted by some terpenoids, such as monoterpenes and sesquiterpenes [51].

In addition, the expression of *PHS* in SRBSDV-infected rice plants was lower than in healthy rice plants. Since the down-regulation of *PHS* has been found to result in a dwarf phenotype of *Oncidium hybrid* [52] and the viral titre was high 25, 35 and 45 days after virus inoculation, we think that the dwarf phenotype of SRBSDV-infected rice could be related to the down-regulation of *PHS*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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