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Quantitative analysis of Rice stripe virus in a transovarial transmission cycle during the development and reproduction of its vector, Laodelphax striatellus

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Virus Genes

Quantitative analysis of Rice stripe virus in a transovarial transmission cycle during the development and reproduction of its vector, Laodelphax striatellus

--Manuscript Draft--

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Abstract:	The amount of Rice stripe virus (RSV) maintained through transovarial transmission was analyzed during the development and reproduction of its vector, Laodelphax striatellus. Reverse transcription-quantitative PCR analysis was used to quantify RNA expressed from the RSV coat protein (CP) gene as an estimate of RSV content in nymphs and adults of L. striatellus at various developmental stages. The 18S ribosome RNA gene of L. striatellus was chosen as the reference for calculating RSV CP expression by using the comparative Ct method. Based on the CP transcript levels, the amount of RSV did not differ significantly throughout the nymphal stage or between adult females of different ages; however, RSV content tended to increase slightly as males became older. The average RSV content in males was 1.30 to 2.49 times that in females. The amount of RSV in L. striatellus adults was compared between generations. The RSV content of female adults did not differ significantly between the parent and progeny populations three of three different females. L. striatellus grown to adults on a susceptible cultivar and five RSV-resistant cultivars were compared to analyze whether the amount of RSV varied among cultivars. Although the amount of RSV in L. striatellus adults differed significantly among the six rice cultivars evaluated, the difference seemed independent of whether resistance genes were present. In addition, the percentage of viruliferous insects was similar among cultivars.

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Dear Dr. A. Lorena Passarelli,

Manuscript reference #VIRU-D-17-00138

Please find attached a revised version of our manuscript “Quantitative analysis of Rice stripe virus in a transovarial transmission cycle during the development and reproduction of its vector, Laodelphax striatellus”, which we would like to resubmit for publication in Virus Genes.

Your comments and those of the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. Below are our point-by-point responses to each of the comments.

Revisions in the text are shown using the tracked changes function. We hope that the revisions in the manuscript and our accompanying responses will ensure that our manuscript is now suitable for publication in Virus Genes.

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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Responses to the comments of Reviewer #1
The sentences in green are the reviewer and editor's comments.

Reviewer #1:

Aims/Objectives of the research. The last paragraph of the introduction states two objectives or "questions" that are answered in the manuscript. First (1), what is the behavior of RSV concentration in L. striatellus during various developmental stages of the insect. Second (2), concentration of RSV was analyzed in L. striatellus grown on different rice cultivars (seems the question is: Does RSV content in its insect vector change depending on the rice cultivar on which the insect feeds?). The rationale for such question is not stated or guided in the introduction. It is helpful to the readers if authors provide the rationale and previous data (references) to support their hypothesis/question in the introduction.

As the reviewer commented, the aim of this study is to analyze the amount of RSV in *L. striatellus* during its development and reproduction. We also analyzed the amount of RSV and the percentage of transovarial transmission to identify if resistant cultivars influence them. The introduction is revised to describe these points more clearly.

Selection of the reference gene. A good reference gene should be expressed consistently through different tissues and life stages of the organism of interest. Therefore, an assay to select the most appropriate gene, should include samples from different life stages (ages) and/or tissues. Several samples (replication) are necessary for the appropriate statistical comparisons and to measure the degree of variation. However, in Materials and Methods, I understood that a single sample (1 sample) consisting of a pool of RNA extracted from 20 adults was used. Later on, in the results section the authors show data and information that suggests there were replicates and samples representing different growth stages. This is confusing, the experimental procedure and design should be clearly stated. I consider that an approach using a single pooled sample is not appropriated to select a reference gene.

To choose the reference gene for relative quantification, Ct values of α 1-tubulin, β-actin, 5.8S rRNA, and 18S rRNA genes were determined for RNA of 20 viruliferous L. striatellus at different growth stages, and the stability of their expression was statistically analyzed using NormFinder and BestKeeper. The sentence was revised to describe it more clearly. To calculate the relative quantities of gene expression by using the comparative CT method, a reference sample, which normalize Ct values in each qPCR reaction (each plate), was used. The reference sample was a mixture of RNA extracted from 20 viruliferous L. striatellus adults (different samples from those used in Bestkeeper and Normfinder analyses). The sentence was revised to describe it more clearly.

Terminology. Just an observation how I am costumed to read abbreviations regarding real-time PCR. But I have seen different usages as well. Generally, "RT-PCR" states for retrotranscription followed by end point PCR reaction. Real-time PCR is quantitative PCR and is denoted as "qPCR". Thus, retrotranscription real time PCR is "RT-qPCR".

There seems to be a lot of words expressing a quantification method of RNA by real-time PCR, among them, "reverse transcription-quantitative PCR (RT-qPCR)" is the most commonly used in scientific articles. Therefore, "real-time RT-PCR" was replace with "RT-qPCR" in the manuscript.

Page 2, Lines 19 to 26. These two sentences in the abstract are somehow confusing. Without reading the complete manuscript, those two sentences are not clear. An abstract should stand alone.

The sentences were rewritten to be more clearly understandable.

Page 7, lines 4 to 6. I do not understand what is meant by "…5-fold serial dilutions of L. straitellus RNA (from 500ng/µl to 0.16ng/µl) was quantified …". The point is that samples were diluted to a final concentration of 0.16ng/µl to test by real-time PCR or serial dilution series (several dilutions per sample) were tested?

One RNA sample was diluted from 500ng/µl to 0.16ng/µl, and used for RT-qPCR analysis in order to calculate the efficiency of PCR for each primer set. The sentence was revised to describe it more clearly.

Page 8, Lines 22 to 31. Analysis of RSV content in different generations of L. striatellus. This section of material and methods requires more detail and specificity in writing. It seems the three male-female pairs were placed in a single cage. Later on, it is mentioned that progenies were designated G1a, G1b and G1c. Therefore, I assumed that each male-female pair was kept apart. For example, in line 24, change "was" to "were", it refers to three pairs. Also eliminate "a" and change "cage" to "cages". It will read; … were placed in separate cages …. The information on how many 7-day-old adult females were collected per each male-female pair is included in figure 2, but I consider it is helpful to have it clearly stated in Mat&Met, as well.

As the reviewer suggested, the male and female pairs were separately placed in a cage to obtain progenies, and the amount of RSV in the progeny populations were analyzed. The sentence was revised to describe it more clearly.

Page 10, Lines 48 to 50. It is stated that a comparison of RSV content in parents versus progeny was not different. However, de description to how was done this comparison, number of individuals, etc.. is not clear in Mat&Met.

The sentence was revised to describe it more clearly.

About the rice resistant cultivars. Does, the rice plants exhibit real resistance: no virus replication or systemic infection? Rather, is it a tolerance phenotype; low virus titer, attenuated symptoms, restricted systemic movement of the virus, etc…? If tolerance is the situation, it would be interesting to measure the virus titer in the plants on which the insects fed. It would have been a nice data to compare against the virus content in the insects fed on each rice cultivar.

The resistant cultivars used in the study are infected with RSV under high infection pressure as explained in the text. The titre of RSV in the cultivars was not measured because they were changed every week to prevent RSV infection. The sentence was revised to describe it more clearly.

The Manuscript VIRU-D-17-00138 entitled "**Quantitative analysis of *Rice stripe virus* in a transovarial transmission cycle during the development and reproduction of its vector, *Laodelphax striatellus***" with Dr. Okuda as contact author reports the changes, or lack thereof, of viral loads within the insect vector of a persistent-propagative and transovarially transmitted

plant virus. Furthermore, this manuscript reports about viral load differences within the insect vector when they developed on different rice cultivars. Overall, the manuscript is well written, clear and concise. However, my main concern with this manuscript is the fact that the nymphs and adults used for the different experiments conducted here were transferred to new seedlings every 7 days to "prevent" their acquisition of RSV from RSV-infected seedlings. Isn't this too much time to truly avoid RSV inoculation into the plants by potentially viruliferous insects? Followed by replication within the rice plants and the subsequent acquisition by these nymphs and adults?

This requires at least some clarification by the authors referencing previous work stating that 7 days is enough to prevent acquisition of RSV from RSV-infected rice seedling by *L. striatellus* nymphs and adults. Nonetheless, other procedures for experimentation, identification of a stable and reliable reference gene for determining the relative amount of RSV CP transcripts in the *L. striatellus* body, and statistical analyses used here are appropriate for the biological assays conducted.

In this study, the nymphs and adults were transferred to new seedlings every 7 days to prevent their acquisition of RSV from RSV-infected seedlings. Normally, although inoculation access period for RSV transmission to rice seedlings is less than 24 hours, it takes at least 6 days to showed typical disease symptom on the inoculated plants. Actually, we observed few seedlings showed clear symptoms when we changed them. According to the previous report, *L. striatellus* do not acquire RSV from rice plants showing no symptoms. In addition, about a half of tested insects in the population analyzed in our experiments were non-viruliferous, and RSV could not be detected by RT-qPCR. If RSV was acquired from RSV-infected rice seedlings, small amount of RSV should be detected by RT-qPCR. Thus, we asuure that the acquisition from infected rice seedlings was very limited, and all viruliferous insects obtained in the experiments are transovarial transmission.

In Figure 3 on the bottom panel (for males) the bar for the Koshihikari rice cultivar is missing the letter from the Tukey's multiple comparisons test.

As described in the text, only two viruliferous male adults were obtained from Koshihikari, so that they were excluded from statistical analysis. The annotation was added also in the figure legend.

Minor corrections

In line 36 on page 6, it should say "cylindrical cage (diameter, 9 cm) where they remained together".

The sentence was revised according to the suggestion.

In line 38 on page 6, it should say “were separately tested to determine if they were viruliferous”.

The sentence was revised according to the suggestion.

In line 46 on page 9, it should say “showed acceptable efficiencies” instead of similar high efficiencies as 88.4%, 89.7%, and 89.3% efficiencies for α1-tubulin, 5.8S rRNA, and 18S rRNA, respectively, are not really similar to the 99.8% efficiency of the RSV CP primer pair.

The sentence was revised according to the suggestion.

In the **Author Contributions** section, it is not specified who exactly conducted the experiments reported in this manuscript.

The Author Contributions section was revised to show who conducted the experiments.

In line 20 on page 16, the authors have two #6 references. Correct to have one #5 and one #6.

The sentence was revised according to the suggestion.

In line 22 on page 18 within Table 1, the authors have SBPH-Tub-Rv2 twice while it should have been SBPH-Tub-Fw2 for the forward primer in the pair.

The sentence was revised according to the suggestion.

Ultimately, I recommend this manuscript be accepted with significant revisions (*i.e.* until the authors provide clear and compelling evidence of the proven fact that transferring *L. striatellus* nymphs and adults to new seedlings every 7 days really prevent acquisition of RSV from RSV-infected seedlings to differentiate that from transovarial transmission) before publication in Virus Genes.

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Quantitative analysis of *Rice stripe virus* in a transovarial transmission cycle during the development and reproduction of its vector, *Laodelphax striatellus*

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Abstract

The amount of *Rice stripe virus* (RSV) maintained through transovarial transmission was analyzed during the development and reproduction of its vector, *Laodelphax striatellus*. Reverse transcription–quantitative PCR analysis was used to quantify RNA expressed from the RSV coat protein (CP) gene as an estimate of RSV content in nymphs and adults of *L. striatellus* at various developmental stages. The 18S ribosome RNA gene of *L. striatellus* was chosen as the reference for calculating RSV CP expression by using the comparative Ct method. Based on the CP transcript levels, the amount of RSV did not differ significantly throughout the nymphal stage or between adult females of different ages; however, RSV content tended to increase slightly as males became older. The average RSV content in males was 1.30 to 2.49 times that in females. The amount of RSV in *L. striatellus* adults was compared between generations. The RSV content of female adults did not differ significantly between the parent and progeny populations three of three different females. *L. striatellus* grown to adults on a susceptible cultivar and five RSV-resistant cultivars were compared to analyze whether the amount of RSV varied among cultivars. Although the amount of RSV in *L. striatellus* adults differed significantly among the six rice cultivars evaluated, the difference seemed independent of whether resistance genes were present. In addition, the percentage of viruliferous insects was similar among cultivars.

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Keywords: small brown planthopper, Tenuivirus, rice stripe disease

Introduction

Rice stripe virus (RSV) belonging to the genus *Tenuivirus* [1] causes chlorotic stripes, mottling, and necrotic streaks on the leaves of rice (*Oriza sativa*). Plants severely infected with RSV often show panicle sterility, resulting in lower yield [2]. RSV occurs in many Asian countries, including Japan, Korea, and China. In Japan, rice stripe disease devastated rice production from 1960 to 1985 [3]. Although the disease gradually subsided from 1985 to 2004, its incidence began to increase again in 2005. In 2015, more than 137,000 hectares of rice fields, accounting for approximately 9% of the total cultivated area in Japan, were affected by RSV [4]. Therefore, effective control measures are urgently needed.

Virus particles of RSV contains four single-stranded RNA molecules (RNA1 through RNA4), and their seven genes are encoded in negative or ambisense orientation [5, 6]. RSV is persistently transmitted by *Laodelphax striatellus* and other planthoppers [2]. The virus propagates in the insect vectors and is transmitted from female adults to their progeny at the egg stage. Under experimental conditions, the percentage of eggs exhibiting transovarial transmission was estimated to be greater than 90% [7]. Thus, transovarial transmission of RSV is considered the main reason for the high viruliferous rate in nature, which makes rice stripe disease difficult to control. Understanding how RSV is maintained in *L. striatellus* during its development and reproduction is one of the keys to developing effective control measures against this disease.

Rice cultivars resistant to viruses are widely used to minimize the damage due to virus infection [8]. Breeding programs to introduce a resistance gene (*Stvb-i*) from an Indica-type rice cultivar Modan, which shows high resistance to RSV [9], successfully yielded resistant cultivars, which effectively control RSV disease in some areas. However, because RSV is transmitted transovarially, the percentage of viruliferous insects decreases slowly even in areas where resistant cultivars are grown. Therefore, susceptible cultivars grown in the same area as resistant ones may be at increased risk of infection due to the decreased emphasis on vector control, consequently increasing the number of vectors in an area. If a resistant cultivar directly decreases transovarial transmission or the amount of RSV in *L. striatellus*, cultivating resistant cultivars would be useful in

suppressing the incidence of rice stripe disease in a region. Some cultivars show resistance to *L. striatellus* due to antixenosis or tolerance to the insect [10]. However, the host factors that affect transovarial transmission or the amount of RSV remain unclear. In the current study, the RSV levels in *L. striatellus* adults grown on different rice cultivars were analyzed.

Recent developments in molecular techniques have made it possible to quantify viral RNA in their hosts [11]. Because of its sensitivity, reverse transcription–quantitative PCR (RT-qPCR) analysis is one of the most widely used methods of RNA quantification. Zhang et al. [12] developed a RT-qPCR–based method for quantifying expression of RSV coat protein (CP) RNA in rice tissues and *L. striatellus*. However, although transovarial transmission plays an important role in the life cycle of RSV, whether the amount of RSV maintained in *L. striatellus* via transovarial transmission changes during various developmental stages has not yet been well characterized. In this study, the amount of RSV maintained via transovarial transmission was analyzed during the development and reproduction of its vector, *L. striatellus*.

Materials and methods

Insect population

Adults of *L. striatellus* were collected from rice plants showing typical symptoms of rice stripe disease in Ibaraki Prefecture, Japan. They were maintained in an insect cage (340 mm × 260 mm × 340 mm, Sanshin Industrial, Kanagawa, Japan) containing rice seedlings under controlled conditions of temperature (25 °C) and photoperiod (16 h light, 8 h dark). To increase the percentage of viruliferous insects, male–female pairs were transferred to rice seedlings in a cylindrical cage (diameter, 9 cm) where they remained together for 5 days to lay eggs, after which females were separately tested to determine whether they were viruliferous by using a simplified enzyme-linked immunosorbent assay (ELISA) [13, 14]. Descendants of the viruliferous females were collected, maintained, and used for this study.

Preparation of RNA templates from insects

Each adult or nymph of *L. striatellus* was collected into a disposable homogenizing tube containing zirconia beads (Biomasher IV, Nippi, Tokyo, Japan), homogenized with 250 µl of ISOGEN II (Nippon Gene, Toyama, Japan) using a Multibeads Shocker (Yasui Kikai, Tokyo, Japan), and kept at –80 °C until RNA extraction. RNA was extracted according to the manufacturer's recommended procedure, except 1 µl of glycogen (20 mg/ml) was added during the nucleic acid precipitation step, and dissolved in distilled water. The concentration of RNA in the prepared solutions was measured using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA).

Quantification of RSV in *L. striatellus*

RT-qPCR analysis was used to quantify RNA expressed from the RSV coat protein (CP) gene as an estimate of the RSV content in *L. striatellus.* The primer set CP-F and CP-R [15] was used to detect RSV CP RNA. RT-qPCR analysis was performed using RNA Direct SYBR qPCR Mix (Toyobo, Tokyo, Japan) and an MX3000P system (Agilent Technologies, Santa Clara, CA, USA). After reverse transcription at 61 °C for 20 min and denaturation at 95 °C for 30 s, the PCR conditions consisted of 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 74 °C for 30 s. The intensity of SYBR Green I fluorescence (wavelength, 497 nm) was measured at the end of each cycle. The cycle threshold (Ct), in which the fluorescent signal reaches a threshold value, was determined using software provided with the MX3000P. When the Ct of RSV CP transcripts for a sample exceeded 30, the insect was regarded as non-viruliferous and was excluded from the analysis. Duplicate reactions were performed for each sample, and the reaction was repeated when the difference between the two Ct values exceeded 0.5. After PCR analysis was completed, amplification specificity was validated using melting curve analysis, which consisted of denaturation at 95 °C for 60 s and annealing at 55 °C at 30 s, followed by continuous measurement of fluorescence intensity at increasing temperatures of 0.1 °C per second until the temperature reached 90 °C.

To choose an appropriate reference gene for determining the relative amount of RSV CP transcripts in the *L. striatellus* body, α1-tubulin, β-actin, 5.8S rRNA, and 18S rRNA genes (GenBank accession numbers AY508717, AY192151, AB625609, and AB085211,

respectively) were selected. Primers for the actin gene were published previously [16]; primers corresponding to the other genes were designed using a Geneious software (Biomatters, Auckland, New Zealand) (Table 1). First, the Ct values of RT-qPCR using these primer sets were obtained from 5-fold serial dilutions of the RNA of 20 viruliferous *L. striatellus* (from 500 ng/µl to 0.16 ng/µl) to calculate amplification efficiencies. Second, to choose the most appropriate gene for reference, the Ct values of these four genes were obtained from each RNA of 20 viruliferous *L. striatellus* at different growth stages (four isolates each of 2nd-, 4th-, and 5th-instar nymphs and 1–4- and 7–12-day-old adults), and the stability of their expression was statistically analyzed by using NormFinder [17] and BestKeeper [18]. Genes with lower stability values in the Normfinder analysis and higher coefficient of correlation in the BestKeeper analysis was regarded more stable. Then, the Ct values of RSV CP and the reference gene were obtained for each *L. striatellus* RNA sample. The relative quantities of RSV CP transcripts were calculated according to the comparative C_T method [19]. The mixture of RNA extracted from 20 viruliferous *L. striatellus* adults was used as the reference sample for the comparative C_T method in each reaction.

Analysis of RSV content in *L. striatellus* nymphs and adults

Seedlings of rice (cv. Koshihikari) at the first to second true-leaf stage were put in the insect cage with the viruliferous *L. striatellus* population for 2 days. Insects were then removed, and the seedlings were placed under a cylindrical cage covered with nylon mesh and kept in the growth cabinet (25 °C; 16 h light: 8 h dark photoperiod). Nymphs were collected at 2, 9, 13, and 16 days after first-instar nymphs emerged; 0-, 7-, 14-, and 21-day-old adults were also collected. The nymphs and adults were transferred to new seedlings every 7 days to prevent their acquisition of RSV from RSV-infected seedlings. RNA was extracted from each insect, and the amount of RSV CP RNA was measured by RT-qPCR.

Analysis of RSV content in different generations of *L. striatellus*

Male–female pairs of the viruliferous population (designated as G0) were separately placed in individual cages containing rice seedlings (cv. Koshihikari) to obtain progeny. The adult females were removed after 7 days and were assessed by RT-qPCR to determine

whether they were viruliferous. The progeny of three different viruliferous females (designated as G1a, G1b, and G1c) were grown to adults as described above and collected at 7 days after emerging. The number of samples from G1a, G1b and G1c were 24, 15, and 16, respectively. RNA was extracted from each sample, and the amount of RSV was measured by RT-qPCR analysis.

Analysis of the RSV content in *L. striatellus* on different rice cultivars

The susceptible cultivar Koshihikari and the RSV-resistant cultivars Asahino-yume (*stv-bi*), Koshihikari Kinchushi SBL1 (*stv-a* and *stv-b*), and near-isogenic lines of Koshihikari—NIL-STV2 (*stv-b*), NIL-STV11 (*stv-a*), and NIL-STV2/STV11 (*stv-a* and *stv-b*) [20]—were used to assess whether RSV content in *L. striatellus* varies among cultivars. Seedlings (first to second true-leaf stage) of these plants were placed in the same insect cage with a viruliferous *L. striatellus* population for 2 days. After insects were removed, the seedlings of each cultivar were placed under individual cylindrical cages with nylon mesh, and the progenies were grown to the adult stage as described above. Insects were transferred to new seedlings of the same cultivar every 7 days to prevent their acquisition of RSV from seedlings. RNA was extracted from approximately 7-day-old adults, and the amount of RSV was measured by RT-qPCR analysis.

Statistical analysis

Statistical analyses were performed using Prism 7 for Mac OS X (Graph Pad Software, La Jolla, CA, USA). The percentage of viruliferous insects was analyzed using the chi-square test. The amount of RSV in *L. striatellus* nymphs was compared between growth stages by using one-way ANOVA followed by Tukey's multiple-comparisons test. The amount of RSV in *L. striatellus* adults at different ages was compared by two-way ANOVA to determine whether an interaction between sex and age was present, followed by Tukey's multiple-comparisons test, in which data from males and females were analyzed separately. The RSV content was compared between males and females at the same age by using two-sided Student's *t*-test and between female parents and their progenies by using one-way ANOVA followed by Dunnett's multiple-comparisons test. The amount of RSV grown on different cultivars was analyzed using one-way ANOVA

followed by Tukey's multiple-comparisons test. Data were regarded significantly different when $P < 0.05$.

Results

Validation of RT-qPCR primers

RT-qPCR analysis of 5-fold serial dilutions of viruliferous *L. striatellus* RNA revealed increases in fluorescence intensity specific to RSV CP at 0.16 ng/μl to 500 ng/μl; the signal intensity of non-viruliferous samples increased very slowly or did not increase (data not shown). A calibration curve indicating the regression coefficient between the Ct and the quantity of the diluted cDNA demonstrated the high (99.8%) efficiency of the PCR analysis. The Ct and corresponding calibration curves for four housekeeping genes of *L. striatellus*—α1-tubulin, β-actin, 5.8S rRNA, and 18S rRNA—showed acceptable efficiencies (88.4%, 95.79%, 89.7%, and 89.3%, respectively). The Ct of RT-qPCR analysis for the housekeeping genes was analyzed for stability by using RNA of viruliferous *L. striatellus* (n = 20) at different growth stages. Results of Bestkeeper and Normfinder analyses indicated that 18S rRNA was the most suitable gene for standardizing expression levels throughout the development of viruliferous *L. striatellus* (Table 2). Therefore, the expression of 18S rRNA was used as a reference in further analyses.

Quantification of RSV throughout the development of *L. striatellus*

RSV CP transcript levels, as a measure of RSV content, in 2-, 9-, 13-, and 16-day-old nymphs and 0-, 7-, 14-, and 21-day-old adults of *L. striatellus* were detected by using RT-qPCR analysis. The percentage of viruliferous insects did not differ significantly between nymphal stages, adult stages, or males and females (chi-square test, $P = 0.34$) (Table 3). The RSV content did not differ significantly throughout the growth of nymphs (Tukey's multiple comparisons test, $P > 0.05$) (**Fig. 1**). Two-way ANOVA failed to reveal any significant interaction between sex and age in adults ($P = 0.085$), but significant effects of sex ($P < 0.0001$) and age ($P = 0.011$) were present. The RSV content was significantly higher in males than females at 7 and 21 days (Student's *t*-test, $P < 0.0001$ and $P = 0.012$) but not at the other time points. The average amount of RSV in male adults

at 0, 7, 14, and 21 days was 1.30, 2.49, 1.40, and 2.09 times, respectively, that of their age-matched female counterparts.

In female adults, the RSV content did not differ between age groups (Tukey's multiple comparisons test, $P > 0.05$). In male adults, the RSV content at 21 days was significantly higher than that at 0 days (Dunnett's multiple comparisons test, $P = 0.01$) and demonstrated a statistical trend toward increasing RSV content with age ($P = 0.017$).

Comparison of RSV content between parents and their progenies of *L. striatellus*

The RSV content of *L. striatellus* female adults and their female progenies was compared (**Fig. 2**). The RSV content of the viruliferous females in the parent population (G0) ranged from 0.21 to 2.1 when the mean value of viruliferous females was set as 1, compared with 0.42 to 2.1 in the G1a, 0.66 to 2.4 in G1b, and 0.36 to 1.4 in G1c. The efficiencies of the transovarial transmission were 83%, 73%, and 94%, respectively. RSV content did not differ significantly between parents and their progenies (Dunnett's multiple-comparisons test, $P > 0.05$).

Quantification of RSV content in *L. striatellus* adults grown on different hosts

RSV CP RNA in *L. striatellus* adults grown on six rice cultivars was detected by using RT-qPCR (**Fig. 3**). The percentage of viruliferous insects did not differ among cultivars (chi-square test, $P = 0.27$ for males and $P = 0.66$ for females) (Table 4). The mean RSV content of female *L. striatellus* adults differed slightly between cultivars and ranged from 0.64 to 1.74 when the average value of parent females was set as 1. The RSV content of adult females grown on NIL-STV11 was significantly higher than that of those grown on Koshihikari, Koshihikari Kinchushi SBL1, NIL-STV2/STV11, or Asahino-yume (Tukey's multiple comparisons test, $P = 0.02$, 0.008, 0.006, and 0.005, respectively).

The mean RSV content in male *L. striatellus* adults also varied among cultivars, ranging from 1.0 to 3.52 relative to the average value of parent female adults. Because only two viruliferous male adults were obtained from Koshihikari, they were excluded from statistical analysis. Male adults grown on NIL-STV11 showed the highest RSV content, which was significantly higher than those grown on Koshihikari Kinchushi SBL1 or Asahino-yume (Tukey's multiple-comparisons test, $P = 0.004$ and 0.006, respectively). The average amount of RSV in males was 1.42 to 3.27 times that of females grown on

the same cultivars.

Discussion

In this study, the amount of RSV maintained transovarially was analyzed throughout the development of its vector, *L. striatellus*. RT-qPCR analysis was used to quantify RSV CP RNA, as a measure of RSV content. Selecting an appropriate reference gene is essential for quantifying RNA by using RT-qPCR analysis [21]. According to the results of NormFinder and BestKeeper analyses, the expression of the 18S rRNA gene was the most consistent among the candidates tested. The 18S rRNA gene has been reported as one of the most stable genes in the planthopper *Delphacodes kuscheli* [22]. In addition, the expression level of the 18S rRNA gene is sufficiently high that it is useful when the RNA yield is low, such as when evaluating tiny insects or early-stage nymphs. The nymphs and adults were transferred to new seedlings every 7 days to prevent their acquisition of RSV from RSV-infected seedlings. Although the inoculation access period for RSV transmission to rice seedlings is typically less than 24 hours, it takes at least 6 days for the inoculated plants to showed characteristic disease symptoms [7]. In fact, few seedlings had clear disease symptoms at the 7-day point when we transferred the insects to fresh seedlings. *L. striatellus* does not acquire RSV from rice plants that show no disease symptoms [7]. In addition, about half of the tested insects in the populations we analyzed were non-viruliferous, and RT-qPCR analysis was unable to detect any RSV in these non-viruliferous samples. If RSV was acquired from RSV-infected rice seedlings, then RT-qPCR analysis should detect even a small amount of RSV. Thus, the acquisition of RSV from infected rice seedlings was likely very limited, and all viruliferous insects obtained in the experiments were due to transovarial transmission.

The relative amount of RSV maintained in *L. striatellus* via transovarial transmission remained consistent throughout the nymphal stage. The amount of total RNA in insects increases throughout the nymphal stages as body size increases. This similarity in the relative RSV content throughout the nymphal stage indicates that RSV multiplies in synchrony with the multiplication of the host cells. After emergence, the relative RSV content of female adults remained consistent regardless of their age; however the RSV content in male adults showed a tendency to increase as they became older. Overall these data indicate that RSV replication stopped or plateaued after the vector became an adult.

RSV reportedly multiplied after its injection into healthy *L. striatellus* adults [23]. Therefore, there may be a limit to the amount of RSV content that a single *L. striatellus* cell can accommodate, and cells might be nearly saturated with RSV when the virus is passed to insect progeny through transovarial transmission.

The relative amount of RSV in male adults was about two times that in females, and the difference became more pronounced with age. In contrast, the transmission of RSV to plants by *L. striatellus* female adults is more efficient than that by males [7]. This apparent contradiction may reflect differences in body size or feeding behavior between sexes. Compared with males, female adults tend to prolong sucking, to obtain sufficient nutrition for laying eggs; this feeding behavior might increase opportunities for virus transmission.

The efficiency of transovarial transmission of RSV in *L striatellus* is estimated to exceed 90% under experimental conditions [7]. In the current study, the rate of transovarial transmission was similar (73% to 94%) among the three populations of viruliferous female adults evaluated. In addition, our RT-qPCR assay did not detect RSV in any of the non-viruliferous progenies born from viruliferous females, even though the theoretical lower limit of RSV detection of the method we used was 10^5 times lower than the average amount we obtained. This result suggests that either no RSV particles were transferred to the eggs of non-viruliferous insects or that RSV was completely excluded at a very early stage of development. Li et al. [24] reported that the VP1 protein of Himetobi P virus (HiPV) may facilitate the accumulation of RSV in *L. striatellus*. Further analysis of the distribution of HiPV to eggs might reveal the mechanism of transovarial transmission of RSV.

The resistance gene *Stv-bi*, which originates from the Indian cultivar Modan [25], has been introduced into many of the rice cultivars currently commercially available in Japan. Two molecular markers for resistance genes, *stv-a* and *stv-b*, which originate from an upland variety of rice [26], were identified recently and are now being used for breeding resistant cultivars, such as an isogenic line of Koshihikari [27]. Although the precise mechanisms by which these genes convey resistance have not been identified, our current results show that these resistant cultivars have no effect on the percentage of viruliferous insects, at least within the span of a single generation. Our preliminary experiments have

indicated that the efficiency of transovarial transmission does not differ between *L. striatellus* populations grown on Koshihikari and Asahino-yume (*Stv-bi*) over at least four generations (Okuda, unpublished data). Other experiments have shown that the percentage of RSV-viruliferous *L. striatellus* decreased in paddy fields where cultivars resistant to RSV were grown [28]. Our current results suggest that this decrease merely reflects that (1) the percentage of transovarial transmission is approximately 90%, and (2) the likelihood of acquiring RSV from resistant cultivars is lower than that from susceptible ones because of the lower number of diseased plants. However, because resistant cultivars carrying *Stv-bi* succumb to RSV infection under high inoculation pressure [29], cultivating these cultivars in areas where the incidence of RSV is very high should be accompanied by appropriate control measures against *L. striatellus*, such as insecticides.

Although the RSV content in *L. striatellus* differed among rice cultivars, the difference seemed unrelated to the resistance genes present. In particular, the RSV content in *L. striatellus* grown on NIL-STV2/STV11, which carries both *stv-a* and *stv-b*, was the highest among the six cultivars evaluated, whereas Koshihikari Kinchushi SBL1, which also carries both *stv-a* and *stv-b*, did not differ from Koshihikari in this regard. Differences in nutritional status among plants might affect the metabolism of vector insects. The influence of the amount of RSV in *L. striatellus* on the efficiency of its transmission to plants has yet to be determined. Symbiotic microorganisms in insects have recently been reported to influence the amount of insect-borne plant viruses transmitted and the efficiency of this transmission [30, 31]. Although more research is needed to reveal the mechanisms by which RSV accumulates in *L. striatellus*, the findings of the current study might facilitate the selection of new resistant varieties that decrease the amount of RSV in the vectors.

This study focused on RSV maintained in *L. striatellus* via transovarial transmission. This process is a major reason for the devastating effect of this virus in the field. However, because the efficiency of transovarial transmission is not 100%, *L. striatellus* must somehow acquire RSV from infected plants to maintain a constant viruliferous rate in the field. Little is known about the molecular mechanisms through which *L. striatellus* acquires RSV from virus-infected rice plants. Further study is needed to reveal how RSV is maintained in *L. striatellus* populations.

AUTHOR CONTRIBUTIONS

Mitsuru Okuda designed the experiments, conducted all experiments, analyzed the data, prepared figures and tables, and drafted the paper; Takuya Shiba cooperated in designing the experiments, performed statistical analysis of the data, and reviewed drafts of the paper; Masahiro Hirae collected and maintained *L. striatellus* populations, cooperated in designing the experiments, and reviewed drafts of the paper.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

Figure legends

Figure 1.

RSV content in 2-, 9-, 13-, and 16-day-old nymphs, and 0-, 7-, 14-, and 21-day-old adults of *L. striatellus*, measured as the relative RSV CP transcript level compared to the 18S transcript level. Sample numbers are shown in Table 3. Each value was calculated by the comparative Ct method. Data are shown as the mean and the 95% confidence interval of the means, where that of 2-day-old nymphs is set as 1. Asterisks indicate significant differences ($P < 0.05$) among nymphs (Tukey's multiple comparisons test) or between males and females at the same age (two-sided Student's t-test); ns, not significant. F, female; M, male.

Figure 2.

RSV content in female parents of *L. striatellus* (G0) and their female progenies (G1a, G1b and G1c), measured as the relative RSV CP transcript level compared to the 18S transcript level. Each value was calculated by the comparative Ct method. Data are shown as the mean and the 95% confidence interval of the means, where that of G0 is set as 1. The percentages of viruliferous insects (no. positive/ no. evaluated) are shown above each bar.

Figure 3.

RSV content in *L. striatellus* parents and their progenies grown on rice cultivars Koshihikari; Koshihikari Kinchushi SBL1 (SBL1); near-isogenic lines of Koshihikari, NIL-STV2 (STV2), NIL-STV11 (STV11), and NIL-STV2/STV11 (STV2/STV11); and Asahino-yume, measured as the relative RSV CP transcript level compared to the 18S transcript level. Each value was calculated by the comparative Ct method. Data are shown as the mean and the 95% confidence interval of the means, where that of the female parents is set as 1. Different letters above each bar represent significant differences (Tukey's multiple comparisons test, $P < 0.05$). Because only two viruliferous male adults were obtained from Koshihikari, they were excluded from statistical analysis.

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Tables

Table 1. List of primers used in RT-qPCR analysis

Target gene	Primer	Sequence (5′ to 3′)	Size of amplicon (bp)
5.8S rRNA	Ls5.8s-482F	TGGTGGATCACTTGGCTCG	137
	Ls5.8s-618R	AGACATGGCCCTCGGGATA	
18S rRNA	SBPH-18S-Fw2	ACGCGCGCTACACTGAAGGA	91
	SBPH-18S-Rv2	AGCCCCAATCCCAAGCACGA	
β-actin	SBPH-actin-F	CCGGTATTGTGCTCGACTCC	244
	SBPH-actin-R	GCTGTGGCCATTTCCTGTTC	
α1-tubulin	SBPH-Tub-Fw2	AGCCACCTACCGTTGTGCCA	150
	SBPH-Tub-Rv2	ACCAGTGCACGAAAGCACGC	
RSV CP	CP-F	TGCAGAAGGCAATCAATGACAT	150
	CP-R	TGTCACCACCTTTGTCCTTCAA	

Table 2. Stability values of candidate reference genes across developmental stages of *L. striatellus.*

Gene	Coefficient of correlation[a)]	Stability value[b)]
18s rRNA	0.972	0.171
β-actin	0.985	0.397
5.8s rRNA	0.863	0.340
α1-tubulin	0.751	0.391

[a)] Values were calculated by using Bestkeeper. Higher values indicate greater similarity in expression levels between developmental stages.

[b)] Values were calculated by using NormFinder. Lower values indicate greater similarity in expression levels between developmental stages.

Table 3. Percentage of viruliferous insects at each developmental stage

Stage	Age	Sex	Total no. of samples	No. of RSV-positive samples	Viruliferous rate
Nymph	2 days	–	40	22	55.0%
	9 days	–	40	18	45.0%
	13 days	–	40	11	27.5%
	16 days	–	40	20	50.0%
Adult	0 days	F	24	14	58.3%
		M	24	13	54.2%
	7 days	F	24	15	62.5%
		M	24	13	54.2%
	14 days	F	24	10	41.7%
		M	24	12	50.0%
	21 days	F	20	11	55.0%
		M	24	13	54.2%

F, female; M, male

Table 4. Percentage of viruliferous insects for parent population and their progenies grown on six rice cultivars

Generation	Host	Sex	Total no. of samples	No. of RSV-positive samples	Viruliferous rate
Parents	Koshihikari	F	20	8	40%
		M	20	8	40%
Progenies	Koshihikari	F	28	7	25%
		M	16	2	13%
	Asahino-yume	F	28	10	36%
		M	16	7	44%
	NIL-STV2	F	16	9	56%
		M	16	7	44%
	NIL-STV11	F	16	6	38%
		M	16	7	44%
	NIL-STV2/STV11	F	16	7	44%
		M	16	7	44%
	SBL	F	28	11	39%
		M	16	10	63%

F, female; M, male

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Figure 1

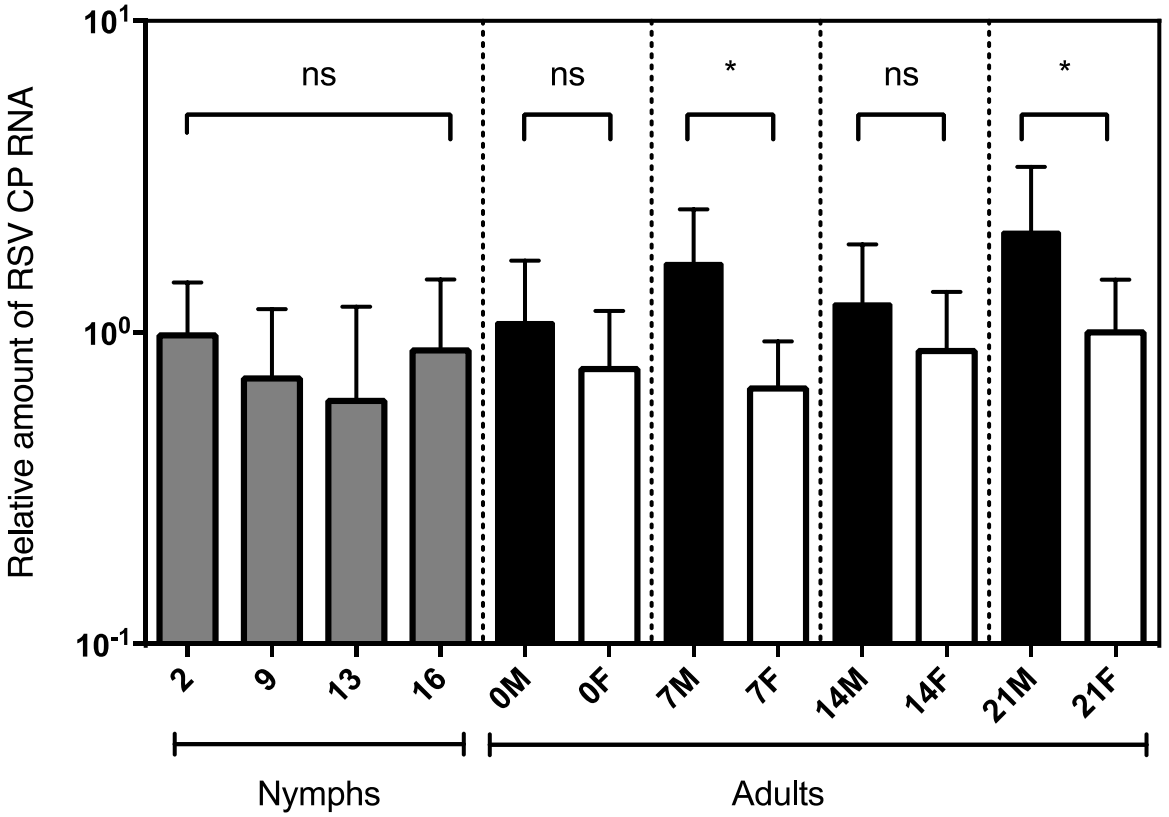


Figure 2

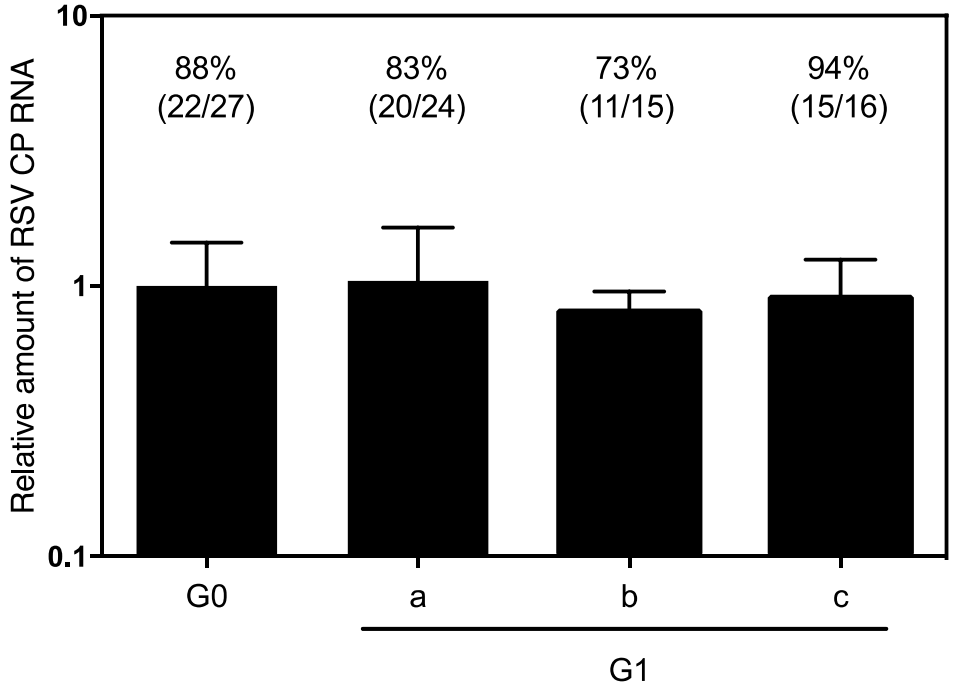
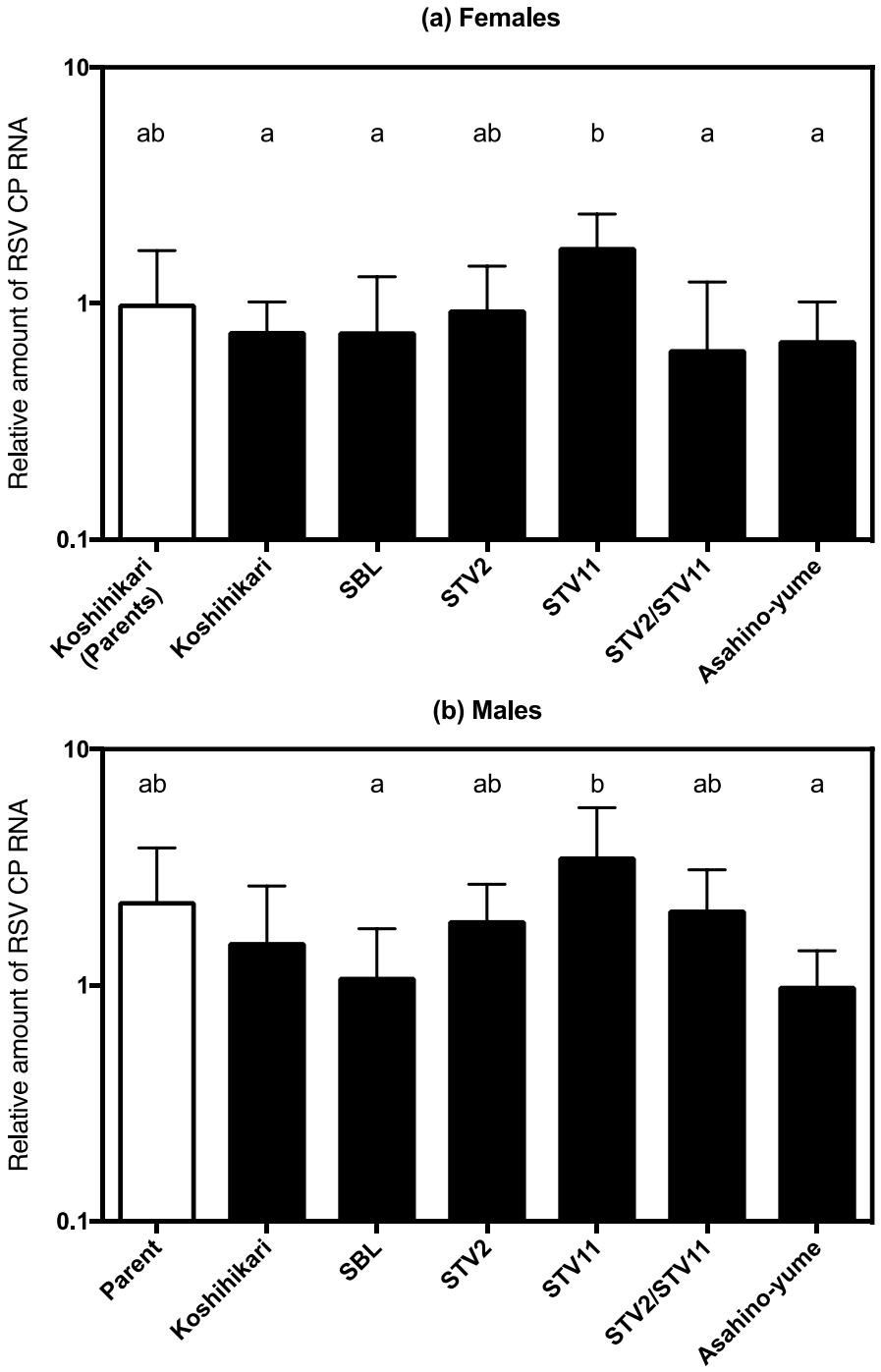

Relative amount of RSV CP RNA
10
1
0.1
88%
(22/27)
83%
(20/24)
73%
(11/15)
94%
(15/16)
G0
a
b
c
G1

Figure 3


(a) Females
Relative amount of RSV CP RNA
10
1
0.1
ab
a
a
ab
b
a
a
Koshihikari (Parents)
Koshihikari
SBL
STV2
STV11
STV2/STV11
Asahino-yume
(b) Males
Relative amount of RSV CP RNA
10
1
0.1
ab
a
ab
b
ab
a
Parent
Koshihikari
SBL
STV2
STV11
STV2/STV11
Asahino-yume

Revised Manuscript with tracked changes

Quantitative analysis of *Rice stripe virus* in a transovarial transmission cycle during the development and reproduction of its vector, *Laodelphax striatellus*

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Abstract

The amount of *Rice stripe virus* (RSV) maintained through transovarial transmission was analyzed during the development and reproduction of its vector, *Laodelphax striatellus*. Reverse transcription–quantitative PCR ~~Real-time reverse transcription-polymerase chain reaction (RT-PCR)~~ analysis was used to quantify RNA expressed from the RSV coat protein (CP) gene as an estimate of RSV content in nymphs and adults of *L. striatellus* at various developmental stages. The 18S ribosome RNA gene of *L. striatellus* was chosen as the reference for calculating RSV CP expression ~~from the RSV CP gene~~ by using the comparative Ct method~~was normalized to that of the 18S ribosome RNA gene of *L. striatellus*~~. Based on the CP transcript levels, the amount of RSV did not differ significantly throughout the nymphal stage or between adult females of different ages; however, RSV content tended to increase slightly as males became older. The average RSV content in males was 1.30 to 2.49 times that in females. The amount of RSV in *L. striatellus* adults was compared between generations. The RSV content of female adults did not differ significantly between the parent~~s~~ and progeny populations three of three different females. *L. striatellus* grown to adults on a susceptible cultivar and five RSV-resistant cultivars were compared to analyze whether the amount of RSV varied among cultivars. Although the amount of RSV in *L. striatellus* adults differed significantly among the six rice cultivars evaluated, the difference seemed independent of whether resistance genes were present. In addition, the percentage of viruliferous insects was similar among cultivars.

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Keywords: small brown planthopper, Tenuivirus, rice stripe disease

Introduction

Rice stripe virus (RSV) belonging to the genus *Tenuivirus* [1] causes chlorotic stripes, mottling, and necrotic streaks on the leaves of rice (*Oriza sativa*). Plants severely infected with RSV often show panicle sterility, resulting in lower yield [2]. RSV occurs in many Asian countries, including Japan, Korea, and China. In Japan, rice stripe disease devastated rice production from 1960 to 1985 [3]. Although the disease gradually subsided from 1985 to 2004, its incidence began to increase again in 2005. In 2015, more than 137,000 hectares of rice fields, accounting for approximately 9% of the total cultivated area in Japan, were affected by RSV [4]. Therefore, effective control measures are urgently needed.

–Virus particles of RSV contains four single-stranded RNA molecules (RNA1 through RNA4), and their seven genes are encoded in negative or ambisense orientation [5, 6]. RSV is persistently transmitted by *Laodelphax striatellus* and other planthoppers [2]. The virus propagates in the insect vectors and is transmitted from female adults to their progeny at the egg stage. Under experimental conditions, the percentage of eggs exhibiting transovarial transmission was estimated to be greater than 90% [7]. Thus, transovarial transmission of RSV is considered the main reason for the high viruliferous rate in nature, which makes rice stripe disease difficult to control. Understanding how RSV is maintained in *L. striatellus* during its development and reproduction is one of the keys to developing effective control measures against this disease.

Rice cultivars resistant to viruses are widely used to minimize the damage due to virus infection [8]. Breeding programs to introduce a resistance gene (*Stvb-i*) from an Indica-type rice cultivar Modan, which shows high resistance to RSV [9], successfully yielded resistant cultivars, which effectively control RSV disease in some areas. However, because RSV is transmitted transovarially, ~~decreasing~~ the percentage of viruliferous insects ~~is~~ decreases ~~difficult~~ slowly even in areas where resistant cultivars are grown. Therefore, susceptible cultivars grown in the same area as resistant ones may be at increased risk of infection due to the decreased emphasis on vector control, consequently increasing the number of vectors in an area. If a resistant cultivar directly decreases transovarial transmission or the amount of RSV in *L. striatellus*, cultivating resistant

cultivars would be useful in suppressing the incidence of rice stripe disease in a region. Some cultivars show resistance to *L. striatellus* due to antixenosis or tolerance to the insect [10]. However, the host factors that affect transovarial transmission or the amount of RSV remain unclear. In the current study, the RSV levels in *L. striatellus* adults grown on different rice cultivars were analyzed.

Recent developments in molecular techniques have made it possible to quantify viral RNA in their hosts [~~10~~11]. Because of its sensitivity, reverse transcription–quantitative PCR (RT-qPCR)~~real-time reverse transcription–polymerase chain reaction (RT-PCR)~~ analysis is one of the most widely used methods of RNA quantification. Zhang et al. [~~11~~12] developed a ~~real-time~~ RT-qPCR–based method for quantifying expression of RSV coat protein (CP) RNA in rice tissues and *L. striatellus*. However, although transovarial transmission plays an important role in the life cycle of RSV, whether the amount of RSV maintained in *L. striatellus* via transovarial transmission changes during various developmental stages has not yet been well characterized. In this study, the amount of RSV maintained via transovarial transmission was analyzed during the development and reproduction of its vector, *L. striatellus*.

~~In addition, t~~The RSV levels in *L. striatellus* ~~adults grown on different rice cultivars were analyzed.~~

Materials and methods

Insect population

Adults of *L. striatellus* were collected from rice plants showing typical symptoms of rice stripe disease in Ibaraki Prefecture, Japan. They were maintained in an insect cage (340 mm × 260 mm × 340 mm, Sanshin Industrial, Kanagawa, Japan) containing rice seedlings under controlled conditions of temperature (25 °C) and photoperiod (16 h light, 8 h dark). To increase the percentage of viruliferous insects, male–female pairs were transferred to rice seedlings in a cylindrical cage (diameter, 9 cm) ~~and were~~where they remained together for 5 days to lay eggs, ~~then~~ after which females were separately tested

to determine ~~if~~ whether they were viruliferous by using a simplified enzyme-linked immunosorbent assay (ELISA) [~~12~~13, ~~13~~]14]. Descendants of the viruliferous females were collected, maintained, and used for this study.

Preparation of RNA templates from insects

Each adult or nymph of *L. striatellus* was collected into a disposable homogenizing tube containing zirconia beads (Biomasher IV, Nippi, Tokyo, Japan), homogenized with 250 µl of ISOGEN II (Nippon Gene, Toyama, Japan) using a Multibeads Shocker (Yasui Kikai, Tokyo, Japan), and kept at –80 °C until RNA extraction. RNA was extracted according to the manufacturer's recommended procedure, except 1 µl of glycogen (20 mg/ml) was added during the nucleic acid precipitation step, and dissolved in distilled water. The concentration of RNA in the prepared solutions was measured using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA).

Quantification of RSV in *L. striatellus*

RT-qPCR analysis was used to quantify RNA expressed from the RSV coat protein (CP) gene as an estimate of the RSV content in *L. striatellus*. The primer set CP-F and CP-R [~~14~~][15] was used to detect RSV CP RNA. ~~The amount of RSV in 5-fold serial dilutions of *L. striatellus* RNA (from 500 ng/µl to 0.16 ng/µl) was quantified by using real-time RT-PCR with the specific primer set CP-F and CP-R for RSV CP RNA [14].~~ RT-qPCR~~Real-time PCR~~ analysis was performed using RNA Direct SYBR qPCR Mix (Toyobo, Tokyo, Japan) and an MX3000P system (Agilent Technologies, Santa Clara, CA, USA). After reverse transcription at 61 °C for 20 min and denaturation at 95 °C for 30 s, the PCR conditions consisted of 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 74 °C for 30 s. The intensity of SYBR Green I fluorescence (wavelength, 497 nm) was measured at the end of each cycle. The cycle threshold (Ct), in which the fluorescent signal reaches a threshold value, was determined using ~~a~~ software provided with the MX3000P. When the Ct of RSV CP transcripts for a sample exceeded 30, the insect was regarded as non-viruliferous and was excluded from the analysis. Duplicate reactions were performed for each sample, and the reaction was repeated when the difference between the two Ct values exceeded 0.5. After PCR analysis was completed, amplification specificity was validated using melting curve analysis, which consisted of

denaturation at 95 °C for 60 s and annealing at 55 °C at 30 s, followed by continuous measurement of fluorescence intensity at increasing temperatures of 0.1 °C per second until the temperature reached 90 °C.

To choose an appropriate reference gene for determining the relative amount of RSV CP transcripts in the *L. striatellus* body, α1-tubulin, *β*-actin, 5.8S rRNA, and 18S rRNA genes (GenBank accession numbers AY508717, AY192151, AB625609, and AB085211, respectively) were selected. Primers for the actin gene were published previously ~~[15]~~[16]; primers corresponding to the other genes were designed using a Geneious software (Biomatters, Auckland, New Zealand) (Table 1). First, the Ct values of RT-qPCR using these primer sets were obtained from 5-fold serial dilutions of the RNA of 20 viruliferous *L. striatellus* (from 500 ng/μl to 0.16 ng/μl) to calculate amplification efficiencies. Second, ~~To~~ to choose the most appropriate gene for reference, the Ct values of these four genes were obtained from each RNA of 20 viruliferous *L. striatellus* at different growth stages (four isolates each of 2nd-, 4th-, and 5th-instar nymphs and 1–4- and 7–12-day-old adults), and the stability of ~~mRNA~~ their expression was statistically analyzed by using NormFinder ~~[16]~~[17] and BestKeeper ~~[17]~~[18].~~–~~ Genes with lower stability values in the Normfinder analysis~~,~~ and higher coefficient of correlation in the BestKeeper analysis was regarded more stable. Then, the Ct values of RSV CP and the reference gene were obtained for each *L. striatellus* RNA sample. The relative quantities of RSV CP transcripts were calculated according to the comparative C_T method ~~[18]~~[19]. ~~A~~ The mixture of RNA extracted from 20 viruliferous *L. striatellus* adults was used as the reference sample for the comparative C_T method in~~to standardize~~ each reaction.

Analysis of RSV content in *L. striatellus* nymphs and adults

Seedlings of rice (cv. Koshihikari) at the first to second true-leaf stage were put in the insect cage with the viruliferous *L. striatellus* population for 2 days. Insects were then removed, and the seedlings were placed under a cylindrical cage covered with nylon mesh and kept in the growth cabinet (25 °C; 16 h light: 8 h dark photoperiod). Nymphs were collected at 2, 9, 13, and 16 days after first-instar nymphs emerged; 0-, 7-, 14-, and 21-day-old adults were also collected. The nymphs and adults were transferred to new seedlings every 7 days to prevent their acquisition of RSV from RSV-infected seedlings.

RNA was extracted from each insect, and the amount of RSV CP RNA was measured by ~~real-time~~ RT-qPCR.

Analysis of RSV content in different generations of *L. striatellus*

Male–female pairs of the viruliferous population (designated as G0) ~~was~~ were separately placed in individual ~~a separate~~ cages containing rice seedlings (cv. Koshihikari) to obtain progeny. The adult females were removed after 7 days and were assessed by RT-qPCR to determine whether they were viruliferous. ~~The progeny population of t~~The progeny of three different viruliferous females (designated as G1a, G1b, and G1c) were grown to adults as described above and ~~were~~ collected at 7 days after emerging. The number of samples from G1a, G1b and G1c were 24, 15, and 16, respectively. RNA was extracted from each sample, ~~The~~ and the amount of RSV was measured by ~~real-time RT-PCR~~RT-qPCR analysis.

Analysis of the RSV content in *L. striatellus* on different rice cultivars

The susceptible cultivar Koshihikari and the RSV-resistant cultivars Asahino-yume (*stv-bi*), Koshihikari Kinchushi SBL1 (*stv-a* and *stv-b*), and near-isogenic lines of Koshihikari—NIL-STV2 (*stv-b*), NIL-STV11 (*stv-a*), and NIL-STV2/STV11 (*stv-a* and *stv-b*) ~~[19]~~[20]—were used to assess whether RSV content in *L. striatellus* varies among cultivars. Seedlings (first to second true-leaf stage) of these plants were placed in the same insect cage with a viruliferous *L. striatellus* population for 2 days. After insects were removed, the seedlings of each cultivar were placed under individual cylindrical cages with nylon mesh, and the progenies were grown to the adult stage as described above. Insects were transferred to new seedlings of the same cultivar every 7 days to prevent their acquisition of RSV from seedlings. RNA was extracted from approximately 7-day-old adults, and the amount of RSV was measured by ~~real-time RT-PCR~~RT-qPCR analysis.

Statistical analysis

Statistical analyses were performed using Prism 7 for Mac OS X (Graph Pad Software, La Jolla, CA, USA). The percentage of viruliferous insects was analyzed using

the chi-square test. The amount of RSV in *L. striatellus* nymphs was compared between growth stages by using one-way ANOVA followed by Tukey's multiple-comparisons test. The amount of RSV in *L. striatellus* adults at different ages was compared by two-way ANOVA to determine whether an interaction between sex and age was present, followed by Tukey's multiple-comparisons test, in which data from males and females were analyzed separately. The RSV content was compared between males and females at the same age by using two-sided Student's *t*-test and between female parents and their progenies by using one-way ANOVA followed by Dunnett's multiple-comparisons test. The amount of RSV grown on different cultivars was analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. Data were regarded significantly different when $P < 0.05$.

Results

Validation of ~~real-time RT-PCR~~RT-qPCR primers

~~Real-time RT-PCR~~RT-qPCR analysis of 5-fold serial dilutions of viruliferous *L. striatellus* RNA revealed increases in fluorescence intensity specific to RSV CP at 0.16 ng/µl to 500 ng/µl; the signal intensity of non-viruliferous samples increased very slowly or did not increase (data not shown). A calibration curve indicating the regression coefficient between the Ct and the quantity of the diluted cDNA demonstrated the high (99.8%) efficiency of the PCR analysis. The Ct and corresponding calibration curves for four housekeeping genes of *L. striatellus*—α1-tubulin, *β*-actin, 5.8S rRNA, and 18S rRNA—showed ~~similar high~~acceptable efficiencies (88.4%, 95.79%, 89.7%, and 89.3%, respectively). The Ct of RT-qPCR~~real-time PCR~~ analysis for the housekeeping genes was analyzed for stability by using RNA of viruliferous *L. striatellus* (n = 20) at different growth stages. Results of Bestkeeper and Normfinder analyses indicated that 18S rRNA was the most suitable gene for standardizing expression levels throughout the development of viruliferous *L. striatellus* (Table 2). Therefore, the expression of 18S rRNA was used as a reference in further analyses.

Quantification of RSV throughout the development of *L. striatellus*

RSV CP transcript levels, as a measure of RSV content, in 2-, 9-, 13-, and 16-day-

old nymphs and 0-, 7-, 14-, and 21-day-old adults of *L. striatellus* were detected by using RT-qPCR~~real-time PCR~~ analysis. The percentage of viruliferous insects did not differ significantly between nymphal stages, adult stages, or males and females (chi-square test, $P = 0.34$) (Table 3). The RSV content did not differ significantly throughout the growth of nymphs (Tukey's multiple comparisons test, $P > 0.05$) (**Fig. 1**). Two-way ANOVA failed to reveal any significant interaction between sex and age in adults ($P = 0.085$), but significant effects of sex ($P < 0.0001$) and age ($P = 0.011$) were present. The RSV content was significantly higher in males than females at 7 and 21 days (Student's *t*-test, $P < 0.0001$ and $P = 0.012$) but not at the other time points. The average amount of RSV in male adults at 0, 7, 14, and 21 days was 1.30, 2.49, 1.40, and 2.09 times, respectively, that of their age-matched female counterparts.

In female adults, the RSV content did not differ between age groups (Tukey's multiple comparisons test, $P > 0.05$). In male adults, the RSV content at 21 days was significantly higher than that at 0 days (Dunnett's multiple comparisons test, $P = 0.01$) and demonstrated a statistical trend toward increasing RSV content with age ($P = 0.017$).

Comparison of RSV content between parents and their progenies of *L. striatellus*

The RSV content of *L. striatellus* female adults and their female progenies was compared (**Fig. 2**). The RSV content of the viruliferous females in the parent population (G0) ranged from 0.21 to 2.1 when the mean value of viruliferous females was set as 1, compared with 0.42 to 2.1 in the G1a, 0.66 to 2.4 in G1b, and 0.36 to 1.4 in G1c. The efficiencies of the transovarial transmission were 83%, 73%, and 94%, respectively. RSV content did not differ significantly between parents and their progenies (Dunnett's multiple-comparisons test, $P > 0.05$).

Quantification of RSV content in *L. striatellus* adults grown on different hosts

RSV CP RNA in *L. striatellus* adults grown on six rice cultivars was detected by using ~~real-time RT-PCR~~RT-qPCR (**Fig. 3**). The percentage of viruliferous insects did not differ among cultivars (chi-square test, $P = 0.27$ for males and $P = 0.66$ for females) (Table 4). The mean RSV content of female *L. striatellus* adults differed slightly between cultivars and ranged from 0.64 to 1.74 when the average value of parent females was set as 1. The RSV content of adult females grown on NIL-STV11 was significantly higher

than that of those grown on Koshihikari, Koshihikari Kinchushi SBL1, NIL-STV2/STV11, or Asahino-yume (Tukey's multiple comparisons test, P = 0.02, 0.008, 0.006, and 0.005, respectively).

The mean RSV content in male *L. striatellus* adults also varied among cultivars, ranging from 1.0 to 3.52 relative to the average value of parent female adults. Because only two viruliferous male adults were obtained from Koshihikari, they were excluded from statistical analysis. Male adults grown on NIL-STV11 showed the highest RSV content, which was significantly higher than those grown on Koshihikari Kinchushi SBL1, or Asahino-yume (Tukey's multiple-comparisons test, P = 0.004 and 0.006, respectively). The average amount of RSV in males was 1.42 to 3.27 times that of females grown on the same cultivars.

Discussion

In this study, the amount of RSV maintained transovarially was analyzed throughout the development of its vector, *L. striatellus*. Real-time RT-PCRRT-qPCR analysis was used to quantify RSV CP RNA, as a measure of RSV content. Selecting an appropriate reference gene is essential for quantifying RNA by using real-time RT-PCRRT-qPCR analysis [20]21]. According to the results of NormFinder and BestKeeper analyses, the expression of the 18S rRNA gene was the most consistent among the candidates tested. The 18S rRNA gene has been reported as one of the most stable genes in the planthopper *Delphacodes kuscheli* [2122]. In addition, the expression level of the 18S rRNA gene is sufficiently high that it is useful when the RNA yield is low, such as when evaluating tiny insects or early-stage nymphs.

The nymphs and adults were transferred to new seedlings every 7 days to prevent their acquisition of RSV from RSV-infected seedlings. Although the inoculation access period for RSV transmission to rice seedlings is typically less than 24 hours, it takes at least 6 days for the inoculated plants to showed characteristic disease symptoms [7]. In fact, few seedlings had clear disease symptoms at the 7-day point when we transferred the insects to fresh seedlings. *L. striatellus* does not acquire RSV from rice plants that show no disease symptoms [7]. In addition, about half of the tested insects in the populations we analyzed were non-viruliferous, and RT-qPCR analysis was unable to detect any RSV in these non-viruliferous samples. If RSV was acquired from RSV-

infected rice seedlings, then RT-qPCR analysis should detect even a small amount of RSV. Thus, the acquisition of RSV from infected rice seedlings was likely very limited, and all viruliferous insects obtained in the experiments were due to transovarial transmission.

The relative amount of RSV maintained in *L. striatellus* via transovarial transmission remained consistent throughout the nymphal stage. The amount of total RNA in insects increases throughout the nymphal stages as body size increases. This similarity in the relative RSV content throughout the nymphal stage indicates that RSV multiplies in synchrony with the multiplication of the host cells. After emergence, the relative RSV content of female adults remained consistent regardless of their age; however the RSV content in male adults showed a tendency to increase as they became older. Overall these data indicate that RSV replication stopped or plateaued after the vector became an adult. RSV reportedly multiplied after its injection into healthy *L. striatellus* adults [~~22~~23]. Therefore, there may be a limit to the amount of RSV content that a single *L. striatellus* cell can accommodate, and cells might be nearly saturated with RSV when the virus is passed to insect progeny through transovarial transmission.

The relative amount of RSV in male adults was about two times that in females, and the difference became more pronounced with age. In contrast, the transmission of RSV to plants by *L. striatellus* female adults is more efficient than that by males [7]. This apparent contradiction may reflect differences in body size or feeding behavior between sexes. Compared with males, female adults tend to prolong sucking, to obtain sufficient nutrition for laying eggs; this feeding behavior might increase opportunities for virus transmission.

The efficiency of transovarial transmission of RSV in *L striatellus* is estimated to exceed 90% under experimental conditions [7]. In the current study, the rate of transovarial transmission was similar (73% to 94%) among the three populations of viruliferous female adults evaluated. In addition, our ~~real-time RT-PCR~~RT-qPCR assay did not detect RSV in any of the non-viruliferous progenies born from viruliferous females, even though the theoretical lower limit of RSV detection of the method we used was ~~less than~~ 10^{-5} times lower than the average amount we ~~detected~~obtained. This result suggests that either no RSV particles were transferred to the eggs of non-viruliferous insects or that RSV was completely excluded at a very early stage of development. Li et

al. [~~23~~24] reported that the VP1 protein of Himetobi P virus (HiPV) may facilitate the accumulation of RSV in *L. striatellus*. Further analysis of the distribution of HiPV to eggs might reveal the mechanism of transovarial transmission of RSV.

The resistance gene *Stv-bi*, which originates from the Indian cultivar Modan [~~24~~25], has been introduced into many of the rice cultivars currently commercially available in Japan. Two molecular markers for resistance genes, *stv-a* and *stv-b*, which originate from an upland variety of rice [~~25~~26], were identified recently and are now being used for breeding resistant cultivars, such as an isogenic line of Koshihikari [~~26~~27]. Although the precise mechanisms by which these genes convey resistance have not been identified, our current results show that these resistant cultivars have no effect on the percentage of viruliferous insects, at least within the span of a single generation. Our preliminary experiments have indicated that the efficiency of transovarial transmission does not differ between *L. striatellus* populations grown on Koshihikari and Asahino-yume (*Stv-bi*) over at least four generations (Okuda, unpublished data). Other experiments have shown that the percentage of RSV-viruliferous *L. striatellus* decreased in paddy fields where cultivars resistant to RSV were grown [28]. Our current results suggest that this decrease merely reflects that (1) the percentage of transovarial transmission is approximately 90%, and (2) the likelihood of acquiring RSV from resistant cultivars is lower than that from susceptible ones because of the lower number of diseased plants. However, because resistant cultivars carrying *Stv-bi* succumb to RSV infection under high inoculation pressure [~~27~~[29], cultivating these cultivars in areas where the incidence of RSV is very high should be accompanied by appropriate control measures against *L. striatellus*, such as insecticides.

Although the RSV content in *L. striatellus* differed among rice cultivars, the difference seemed unrelated to the resistance genes present. In particular, the RSV content in *L. striatellus* grown on NIL-STV2/STV11, which carries both *stv-a* and *stv-b*, was the highest among the six cultivars evaluated, whereas Koshihikari Kinchushi SBL1, which also carries both *stv-a* and *stv-b*, did not differ from Koshihikari in this regard. Differences in nutritional status among plants might affect the metabolism of vector insects. The influence of the amount of RSV in *L. striatellus* on the efficiency of its transmission to plants has yet to be determined. Symbiotic microorganisms in insects have recently been

reported to influence the amount of insect-borne plant viruses transmitted and the efficiency of this transmission ~~[28~~[30, ~~29]~~31]. Although more research is needed to reveal the mechanisms by which RSV accumulates in *L. striatellus*, the findings of the current study might facilitate the selection of new resistant varieties that decrease the amount of RSV in the vectors.

This study focused on RSV maintained in *L. striatellus* via transovarial transmission. This process is a major reason for the devastating effect of this virus in the field. However, because the efficiency of transovarial transmission is not 100%, *L. striatellus* must somehow acquire RSV from infected plants to maintain a constant viruliferous rate in the field. Little is known about the molecular mechanisms through which *L. striatellus* acquires RSV from virus-infected rice plants. Further study is needed to reveal how RSV is maintained in *L. striatellus* populations.

AUTHOR CONTRIBUTIONS

Mitsuru Okuda designed the experiments, conducted all experiments, analyzed the data, prepared figures and tables, and drafted the paper; Takuya Shiba cooperated in designing the experiments, performed statistical analysis of the data, and reviewed drafts of the paper; Masahiro Hirae collected and maintained *L. striatellus* populations, cooperated in designing the experiments, and reviewed drafts of the paper.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

Figure legends

Figure 1.

RSV content in 2-, 9-, 13-, and 16-day-old nymphs, and 0-, 7-, 14-, and 21-day-old adults of *L. striatellus*, measured as the relative RSV CP transcript level compared to the 18S transcript level. Sample numbers are shown in Table 3. Each value was calculated by the comparative Ct method. Data are shown as the mean and the 95% confidence interval of the means, where that of 2-day-old nymphs is set as 1. Asterisks indicate significant differences ($P < 0.05$) among nymphs (Tukey's multiple comparisons test) or between males and females at the same age (two-sided Student's t-test); ns, not significant. F, female; M, male.

Figure 2.

RSV content in female parents of *L. striatellus* (G0) and their female progenies (G1a, G1b and G1c), measured as the relative RSV CP transcript level compared to the 18S transcript level. Each value was calculated by the comparative Ct method. Data are shown as the mean and the 95% confidence interval of the means, where that of G0 is set as 1. The percentages of viruliferous insects (no. positive/ no. evaluated) are shown above each bar.

Figure 3.

RSV content in *L. striatellus* parents and their progenies grown on rice cultivars Koshihikari; Koshihikari Kinchushi SBL1 (SBL1); near-isogenic lines of Koshihikari, NIL-STV2 (STV2), NIL-STV11 (STV11), and NIL-STV2/STV11 (STV2/STV11); and Asahino-yume, measured as the relative RSV CP transcript level compared to the 18S transcript level. Each value was calculated by the comparative Ct method. Data are shown as the mean and the 95% confidence interval of the means, where that of the female parents is set as 1. Different letters above each bar represent significant differences (Tukey's multiple comparisons test, $P < 0.05$). Because only two viruliferous male adults were obtained from Koshihikari, they were excluded from statistical analysis.

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Tables

Table 1. List of primers used in ~~real-time RT-PCR~~RT-qPCR analysis

Target gene	Primer	Sequence (5′ to 3′)	Size of amplicon (bp)
5.8S rRNA	Ls5.8s-482F	TGGTGGATCACTTGGCTCG	137
	Ls5.8s-618R	AGACATGGCCCTCGGGATA	
18S rRNA	SBPH-18S-Fw2	ACGCGCGCTACACTGAAGGA	91
	SBPH-18S-Rv2	AGCCCCAATCCCAAGCACGA	
β-actin	SBPH-actin-F	CCGGTATTGTGCTCGACTCC	244
	SBPH-actin-R	GCTGTGGCCATTTCCTGTTC	
α1-tubulin	SBPH-Tub-~~Rv2~~Fw2	AGCCACCTACCGTTGTGCCA	150
	SBPH-Tub-Rv2	ACCAGTGCACGAAAGCACGC	
RSV CP	CP-F	TGCAGAAGGCAATCAATGACAT	150
	CP-R	TGTCACCACCTTTGTCCTTCAA	

Table 2. Stability values of candidate reference genes across developmental stages of *L. striatellus.*

Gene	Coefficient of correlation[a)]	Stability value[b)]
18s rRNA	0.972	0.171
β-actin	0.985	0.397
5.8s rRNA	0.863	0.340
α1-tubulin	0.751	0.391

[a)] Values were calculated by using Bestkeeper. Higher values indicate greater similarity in expression levels between developmental stages.

[b)] Values were calculated by using NormFinder. Lower values indicate greater similarity in expression levels between developmental stages.

Table 3. Percentage of viruliferous insects at each developmental stage

Stage	Age	Sex[a)]	Total no. of samples	No. of RSV-positive samples	Viruliferous rate
Nymph	2 days	–	40	22	55.0%
	9 days	–	40	18	45.0%
	13 days	–	40	11	27.5%
	16 days	–	40	20	50.0%
Adult	0 days	F	24	14	58.3%
		M	24	13	54.2%
	7 days	F	24	15	62.5%
		M	24	13	54.2%
	14 days	F	24	10	41.7%
		M	24	12	50.0%
	21 days	F	20	11	55.0%
		M	24	13	54.2%

a) F, female; M, male

Table 4. Percentage of viruliferous insects for parent population and their progenies grown on six rice cultivars

Generation	Host	Sex[a)]	Total no. of samples	No. of RSV-positive samples	Viruliferous rate
Parents	Koshihikari	F	20	8	40%
		M	20	8	40%
Progenies	Koshihikari	F	28	7	25%
		M	16	2	13%
	Asahino-yume	F	28	10	36%
		M	16	7	44%
	NIL-STV2	F	16	9	56%
		M	16	7	44%
	NIL-STV11	F	16	6	38%
		M	16	7	44%
	NIL-STV2/STV11	F	16	7	44%
		M	16	7	44%
	SBL	F	28	11	39%
		M	16	10	63%

a) F, female; M, male