Rice Stripe Virus Infection Alters mRNA Levels of Sphingolipid-Metabolizing Enzymes and Sphingolipids Content in *Laodelphax striatellus*

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Abstract

Sphingolipids and their metabolites have been implicated in viral infection and replication in mammal cells but how their metabolizing enzymes in the host are regulated by viruses remains largely unknown. Here we report the identification of 12 sphingolipid genes and their regulation by Rice stripe virus in the small brown planthopper (*Laodelphax striatellus* Fallén), a serious pest of rice throughout eastern Asia. According to protein sequence similarity, we identified 12 sphingolipid enzyme genes in *L. striatellus*. By comparing their mRNA levels in viruliferous versus nonviruliferous *L. striatellus* at different life stages by qPCR, we found that RSV infection upregulated six genes (LsCGT1, LsNAGA1, LsSGPP, LsSMPD4, LsSMS, and LsSPT) in most stages of *L. striatellus*. Especially, four genes (LsCGT1, LsSMPD2, LsNAGA1, and LsSMS) and another three genes (LsNAGA1, LsSGPP, and LsSMS) were significantly upregulated in viruliferous third-instar and fourth-instar nymphs, respectively. HPLC-MS/MS results showed that RSV infection increased the levels of various ceramides, such as Cer18:0, Cer20:0, and Cer22:0 species, in third and fourth instar *L. striatellus* nymphs. Together, these results demonstrate that RSV infection alters the transcript levels of various sphingolipid enzymes and the contents of sphingolipids in *L. striatellus*, indicating that sphingolipids may be important for RSV infection or replication in *L. striatellus*.

Key words: ceramide, sphingomyelin synthase, rice stripe virus, serine palmitoyltransferase, HPLC-MS/MS

Sphingolipids are a family of lipids which play important roles in maintaining the integrity and the function of eukaryotic membranes (Hanmun and Obeid 2008) and mediating various biological processes, such as cell proliferation, differentiation, and apoptosis (Phan et al. 2007). More recently, sphingolipids and their metabolizing enzymes have also been implicated in regulating the interactions between bacterial or viral pathogens and plants or animals (Heung et al. 2006; Gulbins and Petrache 2013). For instance, several studies showed that the enrichment of ceramides could support cell fusion and enhance the entry of viruses (Schneider-Schauleis and Schneider-Schaules 2015). Ceramides are mainly generated by the desaturation of dihydroceramide and breakdown of sphingomyelins. Inhibition of Serine palmitoyltransferase (SPT), the first-step enzyme in ceramide biosynthesis (Kong et al. 2015), suppressed the replication of hepatitis C virus (HCV) in mammalian cells (Umehara et al. 2006; Katsume et al. 2013). Sphingomyelinase (SMase), which is responsible for the degradation of sphingomyelin and synthesis of ceramide, was activated by several viruses such as measles virus, rhinovirus (RV), and dengue virus (Schneider-Schauleis and Schneider-Schaules 2015). Sphingomyelin synthase (SMS) was also required for HIV-mediated membrane fusion by co-localizing with HIV receptor in the plasma membrane (Hayashi et al. 2014).

S1P, which is synthesized from sphingosine by the action of sphingosine kinases, has been shown to be a crucial mediator in the pathogenesis of viral-associated disease (Monick et al. 2004). Inhibition of SK blocked S1P synthesis, resulting in accelerated cell death after respiratory syncytial virus infection (Monick et al. 2004). Overexpression of SK increased the replication of MV in HEK cells (Vijayan et al. 2014). Glycosphingolipids, which have great influence on membrane fluidity, curvature, and organization, can impact HIV trans-infection in dendritic cells by modulating virus infectivity or cell membrane (Puryear and Gummuluru 2013). Increased alpha-N-acetylgalactosaminidase (NAGA) activity has been associated with viral infection (Bradstreet et al. 2012).

To date, numerous researchers have elucidated the interactions between virus infection and sphingolipids in mammals, especially in...
human cells. Despite the biochemical pathways of sphingolipid synthesis are well established (Fig. 1) and most enzymes have been identified and cloned in mammals (Levy and Futerman 2010), little has been understood on sphingolipid in insects, especially less in virus infected insects. Rice stripe virus, which is mainly transmitted by the *Laodelphax striatellus* (small brown planthopper), is one of the most economically devastating rice diseases that mainly occur in temperate and subtropical areas (Zhu et al. 2005). RSV is vertically transmitted from parents to offspring by eggs for 40 generations (Huo et al. 2014). Reports show *L. striatellus* nymphs transmitted RSV more efficiently than adults (Deng et al. 2013). As the previous study shows, the mRNA level of neutral ceramidase is elevated after *L. striatellus* infected rice stripe virus (Zhou et al. 2013). In plant, neutral ceramidase is found up-regulated in the stripe rust virus infected wheat (Yu et al. 2010). Given that sphingolipid enzymes may play an important role in virus infection, investigating their expression profiles in RSV-free and RSV-infected *L. striatellus* may provide novel insights into interactions between *L. striatellus* and RSV. RSV-specific gene-coat protein (CP) has already been quantified in RSV-infected *L. striatellus* via qPCR method (Li et al. 2012), but the relationship between the CP expression levels and sphingolipids or their metabolizing enzymes in different life stages of *L. striatellus* has not been reported. In this study, we used qRT-PCR technology to analyze the expression level of different sphingolipid enzymes and RSV CP gene in different developmental life stages of RSV-free and RSV-infected *L. striatellus*. Since virus infection may affect transcriptional level of sphingolipid enzymes and the content of sphingolipids (Machesky et al. 2008), the contents of several sphingolipids, including ceramides, dihydroceramides, sphingosines, sphingomyelins, and sphingasines, were investigated using high performance liquid chromatography-tandem mass spectrometry. We for the first time demonstrate that RSV infection significantly alters sphingolipids and their metabolizing enzymes in *L. striatellus*.

**Materials and Methods**

**Insects**

The RSV-free and RSV-infected *L. striatellus* colonies were reared on XiuShui rice seedling in separated cages at 25 ± 1 °C, 70% relative humidity and 16:8 light:dark photoperiod. The viruliferous *L. striatellus* colony was purified by rearing the offspring of a single female. After detecting the RSV offspring by reverse transcription

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Fig. 1. The pathway of Sphingolipid metabolism. The enzymes analyzed in this study are in red and full names were listed in Table 1. KDS, 3-keto-dihydrosphingosine reductase; DES, dihydroceramide desaturase; C1PP, C1P phosphatase; Csase, ceramidase; SK, sphingosine kinase; SPL, sphingosine-1-phosphate.
PCR with the coat protein (CP) gene primers, the non-viruliferous *L. striatellus* was screened. The viruliferous rate of viruliferous *L. striatellus* colony was maintained at around 90% and the high-viruliferous rate was screened. The viruliferous rate of *L. striatellus* was reared in glass beakers (size: 500 mL) as stock population.

**Identification of Sphingolipid Enzyme Genes**

We first used the algorithm Basic Local Alignment Search Tool (BLAST) to search the sequences of 10 known sphingolipid enzyme genes of insects or mammals. Then these known sphingolipid genes in GenBank were used to search for their homologous genes in the *L. striatellus* transcriptome database that was built in our previous work (Zhou et al. 2015). To confirm the identity of the putative sphingolipid genes in *L. striatellus*, BLASTX was performed to compare them against the non-redundant database at NCBI (Table 1).

**Sphingolipid Enzymes Cloning**

First, second-, third-, fourth-, fifth-instar nymphs and their male or female adults of non-viruliferous *L. striatellus* and viruliferous *L. striatellus* were collected and total RNA were extracted from female adults of non-viruliferous *L. striatellus* and viruliferous *L. striatellus* colonies were maintained at around 90% and the high-viruliferous rate was screened. The viruliferous rate of viruliferous *L. striatellus* was reared in glass beakers (size: 500 mL) as stock population.

| Name                                      | Abr.  | Length (bp) | Identity (%) | Species                        |
|-------------------------------------------|-------|-------------|--------------|---------------------------------|
| Alpha-N-acetylgalactosaminidase1          | LsNAGA1 | 1258        | 67           | Megachile rotundata             |
| Alpha-N-acetylgalactosaminidase2          | LsNAGA2 | 782         | 75           | Cimex lectularis                |
| Ceramide glucosyltransferase1             | LsCGT1 | 1085        | 70           | Monomorium pharaonis            |
| Ceramide glucosyltransferase2             | LsCGT2 | 1275        | 69           | Bactrocera dorsalis              |
| Ceramide kinase                           | LsCK  | 1774        | 67           | Diachasma alloeum                |
| Ceramide synthase6                       | LsCS6  | 1241        | 70           | Cimex lectularis                |
| Glucosylceramide                         | LsGSC  | 1631        | 73           | Python bivittatus                |
| Serine palmitoyltransferase               | LsSPT  | 2537        | 75           | Zootermopsis nevadensis         |
| Sphingomyline synthase                    | LsSMS  | 953         | 79           | Fopius arisanus                  |
| Sphingomyelin phosphodiesterase 2         | LsSMPD2 | 1582        | 69           | Atta cephalotes                 |
| Sphingomyelin phosphodiesterase 4         | LsSMPD4 | 624         | 72           | Microplitis demolitor           |
| Sphingosine 1 phosphate phosphatase        | LsSGPP | 523         | 69           | Bombex mori                     |

**Quantitative Real-Time PCR**

To investigate RSV proliferation in different life stages of *L. striatellus*, the primer pair specific for the RSV CP gene was designed based on its sequence (GenBank accession no. X53563). The primers for sphingolipid enzymes used in quantitative RT-PCR (qPCR) were also designed based on the sequences of PCR products (Table 2). The qPCR reaction was performed in Bio-Rad CFX96 real time system (Bio-Rad, Hercules, USA) using TransStart Top Green qPCR SuperMix (Transgen, China) according to the instructions from the manufacturer. Briefly, 10 ng of cDNA, 10 μL 2 × TransStart Tip Green qPCR SuperMix, 0.4 μL of each primer and ddH2O were added to make a 20 μL reaction, and PCR was performed under the following conditions: denaturation at 94 °C for 30 s, 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The *actin* gene of *L. striatellus* was used as an internal gene to normalize the target gene expression and to correct the variation of sample-to-sample. The relative expression levels of sphingolipid enzymes and RSV CP were calculated based on the relative quantitative method (2-ΔΔCt) (Livak and Schmittgen 2001). Statistical analyses were performed using the Data Processing System (DPS) software (Tang and Zhang 2013). A two-way analysis of variance with least significant difference test was applied to compare differences in gene expression among multisamples. The level of significant difference was set at < 0.05.

**HPLC-MS/MS Analysis of Sphingolipids**

Total sphingolipids was extracted as described (Bielański et al. 2006), and 0.3 g of third and fourth instar (mixed 1:1) nymphs were prepared for sphingolipids extraction. Total sphingolipids of two *L. striatellus* colonies were extracted in 50 ml iso-propanol: water: ethyl acetate (30:10:60) for 10 min. The organic phase containing total sphingolipids was dried under nitrogen and dissolved in 500 μl MeOH with 25 mmol/l ammonium formate. HPLC-MS/MS analysis was performed using agilent 6460 triple quadruple mass spectrometer coupled with agilent 1200 infinity LC modules. ZORBAX Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 μm particle size, water) was chosen for the separation of sphingolipid and the injection volume was 10 μl. The mobile phases were as follows: phase A (25 mmol/l ammonium formate) and phase B (acetonitrile). The flow rate was 0.3 ml/min at 30 °C with the following gradient elution conditions of phase B: 20–95%, 0–15 min; 95–100%, 15–30 min; 100%, 30–40 min. The ESI was carried out with gas temperature of 350 °C and the drying gas flow rate was 10 l/min, leading to nebulization pressure of 50 psi. The capillary voltage and fragmentor voltage was 3500 V and 100 V, respectively, and the collision energy was 20 V. Sphingolipid Mix I (Avanti Polar Lipids, Alabama, USA) was used as a standard.

**Results**

**Identification and Analysis of Sphingolipid Genes from *L. striatellus***

Twelve different transcripts of sphingolipid enzymes were identified from the transcriptome datasets (Zhang et al. 2010) of *L. striatellus* according to a similarity in protein sequence to known sphingolipid-metabolizing enzymes from mammals. They are alpha-N-acetylgalactosaminidase 1 (*LsNAGA1*), alpha-N-acetylgalactosaminidase 2 (*LsNAGA2*), ceramide glucosyltransferase 1 (*LsCGT1*), ceramide glucosyltransferase 2 (*LsCGT2*), ceramide kinase (*LsCK*), ceramide synthase (*LsCS6*), glucosylceramide (*LsGSC*), serine palmitoyltransferase (*LsSPT*), sphingomyelins synthase (*LsCS6*), sphingomyelins phosphodiesterase 2 (*LsSMPD2*), sphingomyelins phosphodiesterase 4 (*LsSMPD4*), sphingosine 1 phosphate phosphatase (*LsSGPP*), glucosylceramide (*LsGSC*), and ceramide glucosyltransferase (*LsCGT1*).
glucosyltransferase 2 (LsCGT2), ceramide kinase (LsCK), ceramide synthase 6 (LsCS6), glucosylceramidase (LsGSC), serine palmitoyltransferase (LsSPT), sphingomyline synthase (LsSMS), sphingomyelin phosphodiesterase 2 (LsSMPD2), sphingomyelin phosphodiesterase 4 (LsSMPD4), and sphingosine-1-phosphate phosphatase (LsSGPP) (Table 2). Using the BLASTN program of NCBI, we searched for homologous sequences of these transcripts in other insects and found that LsSPT contains the full-length open reading frame whereas the other transcripts contain part of the open reading frame. Sequence comparisons revealed that LsNAGA1 shared a 67% similarity with its ortholog in Megachile rotundata, LsNAGA2 a 75% in Cimex lectularius, LsCGT1 a 70% in Monomorium pharaonis, and LsCGT2 a 69% in Bactrocera dorsalis. Similarities of the other genes are listed in Table 1 and the percentage of identity varied from 66% to 79%.

Table 2. Primers used in reverse transcription PCR and quantitative RT-PCR

| Gene      | Primers | Sequence (5′−3′) | Accession no. |
|-----------|---------|------------------|---------------|
| Reverse transcription PCR |          |                  |               |
| LsNAGA1   | F       | CCTCAAAATCAAGACAGACG | KU757057      |
|           | R       | CTCATTCCTATATGTTTATTTTCTT |           |
| LsNAGA2   | F       | CTTTGAAAGATGCAGGCTGAAT | KU757058      |
|           | R       | CCTGCAATAGTTGCACTGTC |               |
| LsCGT1    | F       | TCCAGAATCTCCAGACTACAG | KU757055      |
|           | R       | CTGTCAGACCCCTCCAACTA |               |
| LsCGT2    | F       | TGCCAAAAACTCTACATATGCAA | KU757056     |
|           | R       | CAAATGTTCGACAGCTTTT |               |
| LsCK      | F       | AGCACCACTTTGAAAGTTTCTCTTG | KU757064    |
|           | R       | ATTTTCCAATTCTACGCAGAAAGC |           |
| LsCS6     | F       | CGTATCCACTAAGGTTGCAGAT | KU757063      |
|           | R       | GGGCGAAGATGCAGATTACATCA |           |
| LsGSC     | F       | CGATCCAGGATTCAGGCTGTG | KU757062      |
|           | R       | GGCCATCAAGGCTGTGCTTGTG |           |
| LsSMPD2   | F       | CGTCTGAGGAGGCTTAAAGAAA | KU757061     |
|           | R       | GAAGGATGTGCTTGCAGCA |               |
| LsSPT     | F       | TGAAGGCGGCCCCTGTCATTTA | KU757060      |
|           | R       | AAAACAGTCCTCATCTCCGTCTTTCA |           |
| LsSMS     | F       | ATTTTCTCAAGGATACGGAACAGC | KU757059    |
|           | R       | GTGTTATTTATTCCATCCACACCTC |           |
| LsSMPD4   | F       | CTCAACTACCATTTGGGATCTC | KU757058      |
|           | R       | GCTGCTGAGGAAAACAAATAAG |           |
| LsSGPP    | F       | CCACACAGTAAGGCTGACTG | KU757057      |
| R         | CTAGTCATCTGACCTCTTG |               |
| R         | ATGTTGACCACAGAGGAGC |               |
| Quantitative RT-PCR |          |                  |               |
| LsNAGA1   | F       | GCTGAGTGGGAGGTTGATTAA |               |
|           | R       | CGCTGTAATTTTGCGATCTCTT |           |
| LsNAGA2   | F       | ACAGGAGAACCGATGGTACG |               |
|           | R       | GGAAGGATGCTGCTGACAT |               |
| LsCGT1    | F       | ACTGTTTGTTGGGCAACAGTG |               |
|           | R       | TGCCAAAACACACGGTACTG |               |
| LsCGT2    | F       | GTGGGAGGCCATACAGATT |               |
|           | R       | TCCTACCTGCCATCTTTG |               |
| LsCK      | F       | GCACATGATTGGTATGAAGG |               |
|           | R       | ATCTTCCGAGACGCAAGAAG |           |
| LsCS6     | F       | GTTGTCGCGTTGCTTCTCTT |               |
|           | R       | ATCCGAGTGGTCTCAAAAGAT |           |
| LsGSC     | F       | TGAAGGTTTGGCCCATACTACA |               |
|           | R       | TAGGATGCTGCTGAAATGTC |           |
| LsSMPD2   | F       | TTGGCCCTGTTGTGCGAAGAC |               |
|           | R       | CCTCTATCGCCACATCTTGA |           |
| LsSPT     | F       | TTCATAGGGCCACCTTACC |               |
|           | R       | CCATTTGCGCCCTAAATGAC |           |
| LsSMS     | F       | CGGACACAGAATCCTGCAA |               |
|           | R       | TTTCGTGACTCGTTGGAAAC |           |
| LsSMPD4   | F       | CCCACCTCCAAAGACCTCAT |               |
|           | R       | CACACAAGGCTGCTACATAT |           |
| LsSGPP    | F       | GTCCTCGGTTACACAGGCTA |               |
| R         | ATCCACAGGCGCCACATGAG |           |
| R         | ATGACATTGCCGCACTTTG |               |
| LsActin   | F       | CCCGACCTCCACATCTCAC |               |
| R         | TGGTCGACGGGACCTC |               |
RSV Proliferation in the Different Life Stages of *L. striatellus*

To understand the characteristics of RSV replication, investigation of RSV proliferation in different life stages of viruliferous *L. striatellus* was performed using the RSV CP gene. The relative transcript level of the CP gene was undetectable in non-viruliferous *L. striatellus*. In contrast, the significant accumulation of the virions in viruliferous *L. striatellus* were found at the second, third, and fourth instar nymphs, and reached the maximum at the third instar nymph, followed by the fourth instar nymph (Fig. 2). The transcript levels of CP were lower in male adults than in female adults and other nymphs (Fig. 2).

Transcription Profiling of Sphingolipid Enzymes in Different Life Stages

To investigate the relative expression levels of the 12 genes encoding sphingolipid-metabolizing enzymes in different life stages, qPCR assays were performed using specific primers. Both RSV-free and RSV-infected *L. striatellus* were used for the comparison analysis of transcription profiles, and the stage-specific expression levels were determined in a total of six stages, including five instar nymph stages, female, and male adults. According to the expression profiles of these genes in different life stages of RSV-free *L. striatellus* (Fig. 3), three genes (*LsCK*, *LsCS6*, and *LsGSC*) were more highly expressed in first instar. Moreover, other three genes (*LsCGT2*, *LsNAGA1*, and *LsSGPP*) were more highly expressed in second instar and *LsNAGA2* showed extremely high mRNA level in fifth instar. Interestingly, *LsCGT1* was hardly detected in all stages. However, the expression pattern of these genes in different life stages of RSV-infected *L. striatellus* showed a different result from RSV-free *L. striatellus*. Four genes (*LsCK*, *LsCGT2*, *LsSMPD4*, and *LsSPT*) were more highly expressed in the adults while two genes (*LsNAGA1* and *LsSMS*) were higher in the third- and fourth-instar nymphs. Moreover, *LsCGT1* and *LsSMPD2* showed extremely higher expression in third-instar, while three genes (*LsCS6*, *LsSGPP*, and *LsNAGA2*) and *LsGSC* were significantly higher in the fourth and second instar nymphs, respectively (p < 0.05).

When comparing RSV-infected *L. striatellus* with RSV-free *L. striatellus*, six genes (*LsCGT1*, *LsNAGA1*, *LsSGPP*, *LsSMPD4*, *LsSMS*, and *LsSPT*) were more highly expressed in most stages of RSV-infected *L. striatellus*. In the adult stages, seven genes (*LsCK*, *LsCGT1-2*, *LsNAGA1*, *LsSMPD4*, *LsSMS*, and *LsSPT*) were expressed at greater levels in RSV-infected *L. striatellus*. Furthermore, *LsSGPP* and two genes (*LsSMPD2* and *LsCGT1*) showed extremely higher expression in the fourth and third viruliferous nymphs, respectively. *LsNAGA1* and *LsSMS* were extremely higher in both two stages. However, *LsCK* and *LsCS6* were significantly higher in non-viruliferous nymphs than viruliferous nymphs. *LsCGT2* showed a higher expression level in early non-viruliferous nymphs (first and second instar) compared with the early nymphs of viruliferous *L. striatellus*.

Relative Content of Sphingolipids in Third and Fourth Nymph of Two *L. striatellus* Colonies

There are great differences in the mRNA levels of most sphingolipid enzyme genes between third or fourth instar nymphs of viruliferous and non-viruliferous *L. striatellus*. Moreover, the relative transcript levels of the RSV CP gene were higher in the third and fourth nymphs than in the other stages. Thus, we analyzed the relative contents of sphingolipids in the third and fourth nymphs (mixed with 1:1) of both viruliferous and non-viruliferous colonies by HPLC-MS/MS. As shown in Fig. 4D, C18:0-sphinganine (dSph18:0) is more abundant than dSph 17:0 in both two colonies. The relative content of dSph18:0 is significantly higher (2.4 fold) in non-viruliferous *L. striatellus* than in viruliferous *L. striatellus* whereas there is no difference in the relative content of dSph17:0 between the two colonies (Fig. 4D), so the total content of sphinganines (dSph) was significantly higher in non-viruliferous nymphs than viruliferous nymphs (Fig. 4A). The ceramide species Cer18:0 and Cer22:0 are the most abundant ceramides in the two colonies, followed by Cer20:0 and Cer24:1 species (Fig. 4H), and all these ceramide species and total ceramides were higher in viruliferous nymphs than in non-viruliferous nymphs (Fig. 4B). The levels of C16-dihydroceramide (dCer16:0) were significantly higher in non-viruliferous
L. striatellus than in RSV-infected L. striatellus, whereas the opposite was true with dCer18:0 and dCer18:1 (Fig. 4G), so there was no difference in the total levels of dihydroceramides ($P = 0.97$) (Fig. 4B). Sphingomyelins are the main sphingolipid class in both colonies with the highest relative content (>1700 ng/g) and their total levels were similar between the two colonies (Fig. 4C). Although the levels of SM18:0 species were slightly lower in viruliferous L. striatellus compared to non-viruliferous L. striatellus (Fig. 4F), there was no difference in the total levels of sphingomyelins between the two colonies (Fig. 4C). The amount of Sph16:1 is much higher than Sph18:1 in two colonies, the relative levels of Sph16:1 were 20% higher in viruliferous L. striatellus than in non-viruliferous L. striatellus, whereas the opposite was true with Sph18:1 species (Fig. 4E), so there was no significant difference in the total levels of sphingosines (Fig. 4A).

**Discussion**

Increasing studies demonstrate that sphingolipids and their metabolizing enzymes may have a role in controlling viral infection in mammalian cells (Schneider-Schaulies and Schneider-Schaulies 2015). However, it remains unclear whether this is also true with viral infection in insects. In this study, we identified 12 genes that encode putative 12 distinct sphingolipid-metabolizing enzymes in L. striatellus and found that some of these genes and the contents of several sphingolipid species were regulated by RSV infection, suggesting that sphingolipids and their metabolizing enzymes may also play a role in viral infection of insects.

All protein sequences of 12 sphingolipid metabolizing enzymes (shown in Fig. 1) display significant homology to those found in other insects. Since sphingolipid biosynthetic pathway (Kihara et al. 2007) and their metabolizing enzymes are evolutionarily conserved (Huitema et al. 2004; Levy and Futerman 2010; Pitson 2011), we suggest that these 12 genes are highly conserved among insects (Table 1).

According to the results of transcriptional profiling of six life stages, 12 genes were differentially expressed in two colonies of L. striatellus and had great variances between viruliferous and non-viruliferous L. striatellus (Fig. 3). LsCK showed lower expression in nymphs of viruliferous L. striatellus than in nymphs of non-viruliferous L. striatellus (Fig. 3), which may lead to ceramide accumulation in nymphs of viruliferous L. striatellus. We postulated that the increased ceramides may play roles in RSV infection or replication in L. striatellus since ceramide accumulation has been shown to facilitate Japanese encephalitis virus entry and infection in Huh cells (Bollinger et al. 2005; Tani et al. 2010). Moreover, the transcripts of LsCGT1, LsNAGA1, LsSGPP, LsSMPD4, LsSMS, and LsSPT were elevated in most stages of viruliferous L. striatellus compared with the non-viruliferous L. striatellus, suggesting that RSV infection may induce the expression of these six genes. It has been shown that suppressing SPT expression can inhibit HCV replication (Katsume et al. 2013), so we predicted that the increase in the expression of LsSPT may have a role in facilitating L. striatellus infection by RSV. As SMPD4 activation has been implicated in facilitating measles virus to infect cells (Mueller et al. 2014), it is possible that the increased expression of LsSMPD4 may also assist RSV to infect L. striatellus. The role for the other four genes in RSV infection remains to be determined.
Nymphs of *L. striatellus*, especially the third and fourth instar nymphs, were proposed to be a more efficient vector to RSV than adults (Li et al. 2015). In this study, the transcriptional levels of RSV CP gene reached the peak at the third instar, so did the transcripts of four genes (*LsCGT1*, *LsSMPD2*, *LsNAGA1*, and *LsSMS*). This correlation may indicate a potential role of these genes in RSV replication in the third nymph. Viral infection and replication not only influence the transcript levels of sphingolipid enzymes but also the content of sphingolipid species. For instance, in HCMV infected cells, viral infection results in alteration of the content of several sphingolipid species, including ceramide, dihydrosphingosine-1-phosphate, and dihydrosphingosine (Machesky et al. 2008). In line with these observations, we observed the higher levels of ceramides in RSV-infected nymphs of *L. striatellus*, suggesting that RSV infection or replication may induce the accumulation of ceramides. It was suggested that ceramide-enrichment domains may create an environment enhancing virus infection (Schneider-Schaulies and Schneider-Schaulies 2015), we predicted that the increased ceramides may facilitate RSV to infect *L. striatellus*. In contrast to ceramides, the content of dihydrosphingosines (dSph) was decreased in virus-infected nymphs. Consistently, HCMV infection also reduced dSph levels in MRC-5 human primary fibroblasts cells (Machesky et al. 2008). It remains to be determined whether cells attempt to combat viral infection by lowering the level of dSph and sphingosine or decreased dSph facilitates viral infection.

In conclusion, many sphingolipid-metabolizing enzymes are highly conserved between *L. striatellus* and other insects. The expression of these sphingolipid-metabolizing enzymes is regulated during infection of *L. striatellus* by RSV, which leads to changes in the levels of ceramides and dSph in *L. striatellus*. The alteration in these sphingolipids may facilitate RSV to infect *L. striatellus* or is an adaptive mechanism by which *L. striatellus* limits RSV infection.
The identification of these genes lays a molecular foundation to define the role of sphingolipids in controlling \textit{L. striatellus} infection by RSV.

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