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RESEARCH ARTICLE

LncRNAs are potentially involved in the immune interaction between small brown planthopper and rice stripe virus

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Abstract

Small brown planthopper (SBPH, *Laodelphax striatellus* Fallén) is an important vector of major crop pathogen rice stripe virus (RSV). Controlling SBPH population is an efficient approach to control RSV. Long non-coding RNAs (lncRNA) have been reported to block virus replication in hosts. However, the function of lncRNAs in RSV infection and replication is still unknown. Here, we aimed to study regulatory mechanisms of lncRNA in an immune system during RSV infection. First, lncRNA genes were predicted from SBPH transcriptomes using a bioinformatics pipeline based on characteristics of lncRNA. We identified 4 786 lncRNA genes corresponding to 5 790 transcripts in SBPH from an RNA-Seq dataset of 15 transcriptomes. Differential expression analysis indicated that 3, 11, and 25 lncRNA genes were highly expressed in gut, salivary gland, and ovary, respectively, of viruliferous SBPH (Student's *t*-test, $P<0.05$). We randomly selected eight lncRNAs for expression validation using quantitative real-time PCR, confirming the differential expression of these lncRNAs between viruliferous and non-viruliferous SBPH. In summary, we present evidence that the expression of lncRNA genes was induced by RSV infection, suggesting that RSV might be involved in the antivirus immune system in SBPH and participate in regulating the RSV replication mechanism. These data provide helpful information for future investigations of the interaction between lncRNA and RSV.

Keywords: lncRNA, small brown planthopper, rice stripe virus, RNA-Seq, viral infection

1. Introduction

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into proteins. ncRNAs have become a major research topic due to their unique biological function (Esteller 2011; Qureshi and Mehler 2012; Anastasiadou *et al.* 2018; Fan *et al.* 2018). A diversity of ncRNAs perform genetic functions in cells, including microRNA (miRNA), small interference RNA (siRNA), PIWI-interacting RNA (piRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), long non-coding RNA (lncRNA), circular RNA (circRNA),

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and unclassified ncRNAs (Surono *et al.* 1999; Mattick and Makunin 2006; Lauressergues *et al.* 2015). LncRNAs are defined as transcripts longer than 200 nucleotides. These short RNA molecules have been considered "transcriptional noise" due to their low abundance in gene expression and low conservation among species (Huttenhofer *et al.* 2005). However, more ncRNAs have been detected in a variety of organisms and lncRNAs are believed to have important functions involved in almost all kinds of biological processes. Increasing evidence has shown that lncRNAs are key components in eukaryotes that participate in regulating many important physiological processes, such as gene expression, chromatin modification, transcriptional interference, and post-transcriptional processing (Hung and Chang 2010; Rinn and Chang 2012; Batista and Chang 2013; Atianand and Fitzgerald 2014; Roberts *et al.* 2014). For example, lncRNA regulated mRNA precursors by isolating splicing factors when *Drosophila* cells were heat shocked (Jolly and Lakhotia 2006).

Classification of lncRNAs is mainly based on transcription location, transcript length, and biological function (Ma *et al.* 2013; Laurent *et al.* 2015). According to the transcription location in the genome, lncRNAs can be divided by Cuffcompare classification annotation information into the following types: intergenic lncRNA, intronic lncRNA, sense exonic overlap lncRNA, antisense-exonic overlap lncRNA, junction overlap lncRNA, and unclassified lncRNA (Pauli *et al.* 2012; Rinn and Chang 2012; Trapnell *et al.* 2012; Ma *et al.* 2013). With rapid development of high-throughput sequencing techniques, many lncRNA genes have been identified in many organisms, including fruit fly (*Drosophila melanogaster*), silkworm (*Bombyx mori*), diamondback moth (*Plutella xylostella*), malarial mosquito (*Anopheles gambiae*), Chinese honey bee (*Apis cerana*), and rice brown planthopper (*Nilaparvata lugens*) (Young *et al.* 2012; Padrón *et al.* 2014; Jayakodi *et al.* 2015; Legeai and Derrien 2015; Xiao *et al.* 2015; Chen *et al.* 2016; Etebari *et al.* 2016; Wu *et al.* 2016; Zhu *et al.* 2016). It is increasingly evident that lncRNAs have a very wide repertoire of involvement in insect growth and development (Li *et al.* 2019).

Small brown planthopper (SBPH, *Laodelphax striatellus* Fallén, Delphacidae) transmits the serious crop pathogen rice stripe virus (RSV) in China. Rice stripe disease caused by RSV causes extremely high morbidity and mortality in rice plants and has been called "rice cancer". This disease is widespread in temperate and subtropical regions in east Asia. Unfortunately, there is still no highly efficient management method to control RSV infection (Zhou *et al.* 2012). RSV is spread by SBPH in two major ways: first, the virus is transmitted to offspring laid by infected females; second, the virus is transmitted to plants through the proboscis of infected SBPH. These two transmission methods usually exist simultaneously, which aggravates outbreaks of rice stripe disease (Zhou *et al.* 2012). RSV is mainly distributed in the gut, salivary gland, and ovary cells. It has been reported that female SBPHs have a higher capacity of carrying RSV than the males (Suzuki *et al.* 1992; Wu *et al.* 2001, 2012; Deng *et al.* 2013) and RSV infection can lead to a decrease in fecundity of up to 15.05% of the eggs laid in a population (Wan *et al.* 2015). Recently, organ-specific transcriptomes revealed that the overall immune response was present in both gut and salivary gland after RSV infection (Zhao *et al.* 2016). RSV can manipulate the c-Jun N-terminal kinase (JNK) signaling pathway for replication in the insect vector (Wang *et al.* 2017). The host can also suppress viral replication through its antivirus immune system such as the small RNA-mediated virus defense mechanism (Xu *et al.* 2012).

LncRNAs have been reported to be induced by viral infection, suggesting that lncRNAs participate in controlling the host innate immune system (Atianand and Fitzgerald 2014; Ding *et al.* 2016). Etebari *et al.* (2016) reported that increased abundance of host lncRNAs can suppress viral replication in *Aedes aegypti* A20 cells infected with DENV-2. In contrast, DENV-2 viral replication was enhanced after silencing the *linc-1317* gene, indicating that *linc-1317* has an essential role in the host anti-viral defense. To study the functions of lncRNAs in anti-RSV immunity in SBPH, we used a computational pipeline to predict lncRNA genes from RNA-Seq data and then the differential expression of lncRNAs was estimated between viruliferous and non-viruliferous SBPH. Some lncRNAs were identified to be highly expressed in the viruliferous SBPHs, suggesting that lncRNAs might be involved in RSV transmission or replication in SBPH. These results provide novel insights for RSV management in agriculture.

2. Materials and methods

2.1. Data sources

We downloaded 34 SBPH transcriptome datasets from the NCBI Sequence Read Archive (SRA) database. In addition, we previously sequenced four SBPH transcriptomes (He *et al.* 2019). The NCBI SRA accession numbers are given below for each sample type (underlined numbers indicate the RNA-Seq data sequenced in our lab): 1) non-viruliferous samples: SRR1617622, SRR1617623, SRR1614218, SRR1617628, SRR7091177, and SRR7091179; 2) viruliferous samples: SRR1617617, SRR1617620, SRR1619428, SRR1619422, SRR7091178, and SRR7091180; 3) different temperature treatments: SRR4019001, SRR4019085, SRR4019086,

and SRR4020768; 4) *Wolbachia*-infected samples: SRR4075582, SRR4075591, SRR4075600, SRR4075601, SRR4075914, SRR4087171, and SRR4088002; 5) samples not infected with *Wolbachia*: SRR4075602, SRR4075603, SRR4075604, SRR4075605, and SRR4088021; and 6) samples of normal tissues: SRR5816374, SRR5816375, SRR5816380, SRR5816381, SRR5816382, SRR5816383, SRR5816394, SRR871533 and SRR941775.

The SBPH genome and gene annotation files were downloaded from InsectBase (Yin *et al.* 2016).

2.2. LncRNA identification

The prediction pipeline was modified from lncScan (Xiao *et al.* 2015). First, all RNA-Seq reads of 38 SBPH were mapped to the SBPH genome using TopHat (ver. 2.1.1) (Trapnell *et al.* 2009). Second, Cufflinks (ver. 2.2.1) (default parameters) was used to combine all assembled transcripts with SBPH genome-annotation information (Trapnell *et al.* 2012). All assembled transcripts had to satisfy two criteria: length≥200 nt and number of exons≥2. Third, the transcripts including protein coding genes and known ncRNAs were filtered using SwissProt (e-value<0.001), EMBOSS (ver. 6.6.0), CPAT (ver. 1.2.4) (score>0.39), HMMER (ver. 3.1b2), and Infernal (ver. 1.1.2) (*t*=40, e-value<0.001) (Trapnell *et al.* 2009; Nawrocki 2014; Finn *et al.* 2015). Transcripts with open reading frames (ORF)≥300 nt were treated as putative protein-coding transcripts. DEseq2 (ver. 1.14.1) was used to estimate the differentially expressed lncRNAs (Love *et al.* 2014). All pipelines were carried out on a Sugon A840 server.

2.3. Insects

SBPH of viruliferous and non-viruliferous strains were provided by Prof. Zhou Tong from the Jiangsu Academy of Agricultural Sciences, China and maintained in a climate chamber as described previously (He *et al.* 2018).

2.4. Total RNA isolation and cDNA synthesis

The fourth instar of SBPH nymphs were collected for gene expression analysis. Total RNA was extracted from five individuals of each strain with 500 μL TRIzol® reagent according to the manufacturer's instructions (Life Technologies, CA, USA). After RNA isolation, the HiScript® Q RT SuperMix Kit with gDNA wiper (Vazyme Biotech, Nanjing, China) was used to synthesize the first-strand cDNA following the manufacturer's instructions. Random primers/oligo$(dT)_{23}$ primer mix with 1 μg total RNA was used for amplifying the cDNA template of lncRNAs.

2.5. Quantitative real-time RT-PCR (qRT-PCR)

To validate the relative expression levels of the identified lncRNAs, qRT-PCR was performed with ChamQ™ SYBR® qRT-PCR Master Mix (Vazyme Biotech, Nanjing, China). Each reaction mixture included 2 μL cDNA template, 0.4 μL of forward/reverse primer (10 μmol L^{-1}), 0.4 μL ROX reference dye II, and 10 μL master mix. The qRT-PCR protocol was 1 cycle of pre-denaturing at 95°C for 3 min, then 40 cycles of denaturing at 95°C for 5 s, annealing and extension at 60°C for 31 s, followed by the melt curve dissociation stage. Each sample had three technical replicates with three biological replicates. Gene expression levels were calculated against the internal reference SBPH *β-actin* gene (GenBank accession no. AY192151) with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). All primers used in this study are listed in Table 1.

3. Results

3.1. Identification of SBPH lncRNAs

All raw reads from 38 SBPH transcriptomes, including various tissues from viruliferous and non-viruliferous SBPH; *Wolbachia*-infected and *Wolbachia*-uninfected SBPH samples; and samples treated at different temperatures, were combined and filtered by TopHat, Cufflinks, and other software programs. These steps produced 4 786 lncRNA genes. According to genome location, we divided these lncRNAs into 10 types. As a result, we found 859 lncRNAs belonging to intergenic lncRNA, which were 17.9% of the total; and 1 272 lncRNAs belonging to unclassified lncRNAs, which were 26.6% of the total. To study the effects of RSV

Table 1 Primers used for quantitative real-time RT-PCR (qRT-PCR)

Primer name	Primer sequence (5′→3′)
XLOC_041323-F	ATATGAACTACGCCCGTTTCC
XLOC_041323-R	GGCCGTTACTCTTAGCCAATTA
XLOC_007257.2-F	GACTGGCTGGCAACTAACA
XLOC_007257.2-R	GATTAGCCCAACGACGTCTAC
XLOC_007257.1-F	GTCGTAGAGTAACCAGAGTAGGA
XLOC_007257.1-R	GAGTAGGATGGGTGGATTAACTG
XLOC_027678-F	CCATCTGCTTTCTCTCTCTGAA
XLOC_027678-R	GAACCCGTCCAGGATGTTATT
XLOC_047897-F	TTGGTGTGTGTTGCTTCATTAG
XLOC_047897-R	GCTAGGCGTACACTGGTTAG
XLOC_030561-F	CGGGACTCGTGGTTTGATTAG
XLOC_030561-R	GCCCAACTACACACACGTATAA
XLOC_005909-F	TCGAGTACACCGACGACTAA
XLOC_005909-R	CGACTACACACACGTCTAATCAA
LsACT-F	GCGAGAAATCGTCCGAGACAT
LsACT-R	AACTGGAGGAGGCGGCTGT

on lncRNAs, we focused on three groups (15 RNA-Seq datasets): tissues of viruliferous SBPH, non-viruliferous SBPH, and normal SBPH body tissues (Table 2).

3.2. Characteristics of lncRNA in SBPH

We analyzed the characteristics of SBPH lncRNAs and found that 73% of lncRNA transcripts had only two exons. In contrast, 15.5% of protein coding genes had two exons (Fig. 1-A). The exon lengths of lncRNAs (284 bp) are significantly shorter than protein-coding genes (320 bp) (P<0.005, t-test; Fig. 1-B). Because lncRNA transcripts had fewer exons than protein-coding genes, the average lengths of lncRNA transcripts (320 bp) are much shorter than the transcripts of protein-coding genes (1 289 bp) (P<1.1e–166, t-test; Fig. 1-C). Alternative splicing occurred in about 26.4% of the lncRNA of *L. striatellus* (Fig. 1-D).

3.3. lncRNAs highly expressed in viruliferous SBPH

RSV needs to cross three important barriers: salivary gland, gut, and ovary of the host SBPH (Wu *et al.* 2001, 2012; Deng *et al.* 2013). According to differential expression analysis, 39 lncRNA genes were highly expressed in tissues of viruliferous SBPH ($|\log_2 FPKM[HV/NV]|>1$, P<0.05). The genes are shown in Table 3. Fig. 2 shows that 26 lncRNA genes were highly expressed in various viruliferous tissues (Fig. 2; Boxes 2, 3, 5, and 7) while 13 lncRNA genes were highly expressed in various non-viruliferous tissues (Fig. 2; Boxes 1, 4, and 6). Specifically, seven, three, and seven lncRNA genes were highly expressed in viruliferous gut, salivary gland, and ovary at 24 h after emergence (ovary 24 HAE), respectively. In addition, nine lncRNAs were highly expressed in the ovary at 48 h after emergence (ovary 48 HAE). Four, three, and six lncRNA genes were highly expressed in non-viruliferous gut, ovary 24 HAE, and ovary 48 HAE, respectively. Eleven lncRNA genes showed opposite expression patterns between viruliferous and non-viruliferous SBPH (Fig. 2). lncRNAs also showed different expression patterns at different development stages in the same viruliferous tissue (Fig. 2-C–D). Protein-coding genes adjacent to some differentially expressed lncRNA genes were related to antiviral immunity.

3.4. Validation of highly expressed lncRNAs in viruliferous SBPH

Eight lncRNA genes were randomly selected and their expression levels in viruliferous and non-viruliferous SBPH were validated by qRT-PCR (Fig. 3). According to the differential expression analysis, XLOC_041323 and XLOC_007257 showed high expression in gut of viruliferous SBPH; XLOC_007257.1 and XLOC_027678 were highly expressed in salivary gland of viruliferous SBPH; XLOC_047897 and XLOC_030561 were highly expressed in ovary 24 HAE of viruliferous SBPH; and XLOC_005909 and XLOC_030561 were highly expressed in ovary 48 HAE of viruliferous SBPH. Consistent with RNA-Seq analysis, qRT-PCR confirmed the high expression of XLOC_041323, XLOC_007257.1, XLOC_027678, and XLOC_030561 in viruliferous SBPH and XLOC_007257.2 and XLOC_005909 in non-viruliferous SBPH. However, expression of

Table 2 Types of predicted long non-coding RNA (lncRNA) among RNA-Seq samples[1)]

LncRNA types[2)]	Non-viruliferous SBPH				Viruliferous SBPH				Tissue type								
	Gut	Ovary 24 HAE	Ovary 48 HAE	Salivary gland	Gut	Ovary 24 HAE	Ovary 48 HAE	Salivary gland	Adult	Nymph	Antenna	Brain	Egg	Fat body	Gonad	Midgut	Mixed sample
Intergenic	31	56	37	5	18	41	41	8	43	21	19	34	39	15	73	7	21
Intronic	25	28	25	14	25	22	34	12	29	28	22	28	23	27	43	10	25
Intronic overlap (–)	1	2	2	0	2	4	3	0	1	2	1	1	2	1	1	0	2
Exonic overlap (+)	40	48	38	21	39	45	42	17	48	39	44	51	48	35	45	16	54
Exonic overlap (–)	29	37	35	11	32	40	62	9	34	23	28	29	34	28	68	11	23
Junction overlap	101	100	81	51	94	88	104	40	100	78	67	101	77	82	132	42	132
Contained	35	43	25	22	30	40	33	18	37	21	30	40	25	33	39	49	92
Repeats	9	18	16	13	14	15	16	7	18	15	12	13	11	10	12	4	19
Match intron chain	25	32	25	17	29	32	34	18	37	36	28	31	32	34	34	15	32
Unclassified	454	613	490	240	441	540	604	207	578	509	438	544	460	469	658	213	549

[1)] SBPH, small brown planthopper; Ovary 24 HAE, ovary at 24 h after emergence; Ovary 48 HAE, ovary at 48 h after emergence.
[2)] +, overlaps with a reference sequence; –, overlaps with a reference sequence on the opposite strand.

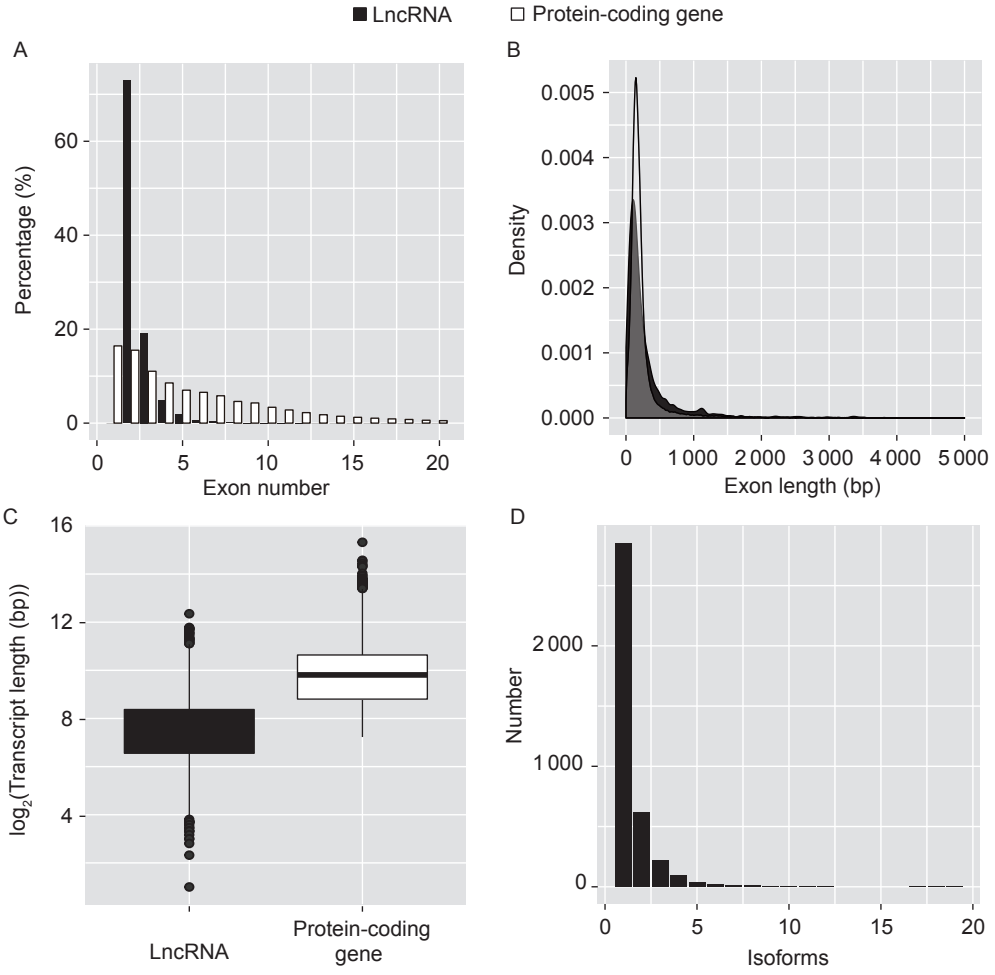

Fig. 1 Characteristics of long non-coding RNAs (lncRNA) in small brown planthopper. A, the percentage of transcript containing different exons for lncRNAs and protein-coding genes. Most lncRNAs only have two exons. B, the density distribution of exon sizes of lncRNAs and protein-coding genes. C, transcript length of lncRNAs and protein-coding genes. LncRNAs have shorter transcripts on average. D, the isoform number distribution of lncRNA genes.

Table 3 Differentially expressed long non-coding RNA genes in gut, salivary gland and ovary

Tissue[1)]	Area	Gene list
Gut	Box 1	XLOC_011155, XLOC_026412, XLOC_020124, XLOC_042264
	Box 2	XLOC_025171, XLOC_022854, XLOC_007179, XLOC_007257, XLOC_011029, XLOC_014243, XLOC_041323
Salivary gland	Box 3	XLOC_027678, XLOC_032467, XLOC_046235
Ovary 24 HAE	Box 4	XLOC_025810, XLOC_019848, XLOC_044371
	Box 5	XLOC_031376, XLOC_047897, XLOC_011016, XLOC_030561, XLOC_019417, XLOC_019142, XLOC_034802
Ovary 48 HAE	Box 6	XLOC_022425, XLOC_011220, XLOC_044371, XLOC_030561, XLOC_019848, XLOC_011789
	Box 7	XLOC_030561, XLOC_010744, XLOC_034421, XLOC_009847, XLOC_027153, XLOC_015088, XLOC_025444, XLOC_005909, XLOC_043685

[1)] Ovary 24 HAE, ovary at 24 h after emergence; Ovary 48 HAE, ovary at 48 h after emergence.

XLOC_007257.2 and XLOC_047897 was not consistent with RNA-Seq analysis. XLOC_030561 was not detected in ovary 48 HAE of viruliferous SBPH. We reasoned that these lncRNAs might be spatio-temporally expressed and the samples used for validation were not exactly the same as those used for RNA-Seq sequencing. However, the detailed mechanism underlying this difference requires further investigation.

4. Discussion

Here, a total of 4 786 lncRNA genes were identified from the transcripts of SBPH using a bioinformatics prediction pipeline. According to an analysis of lncRNA characteristics,

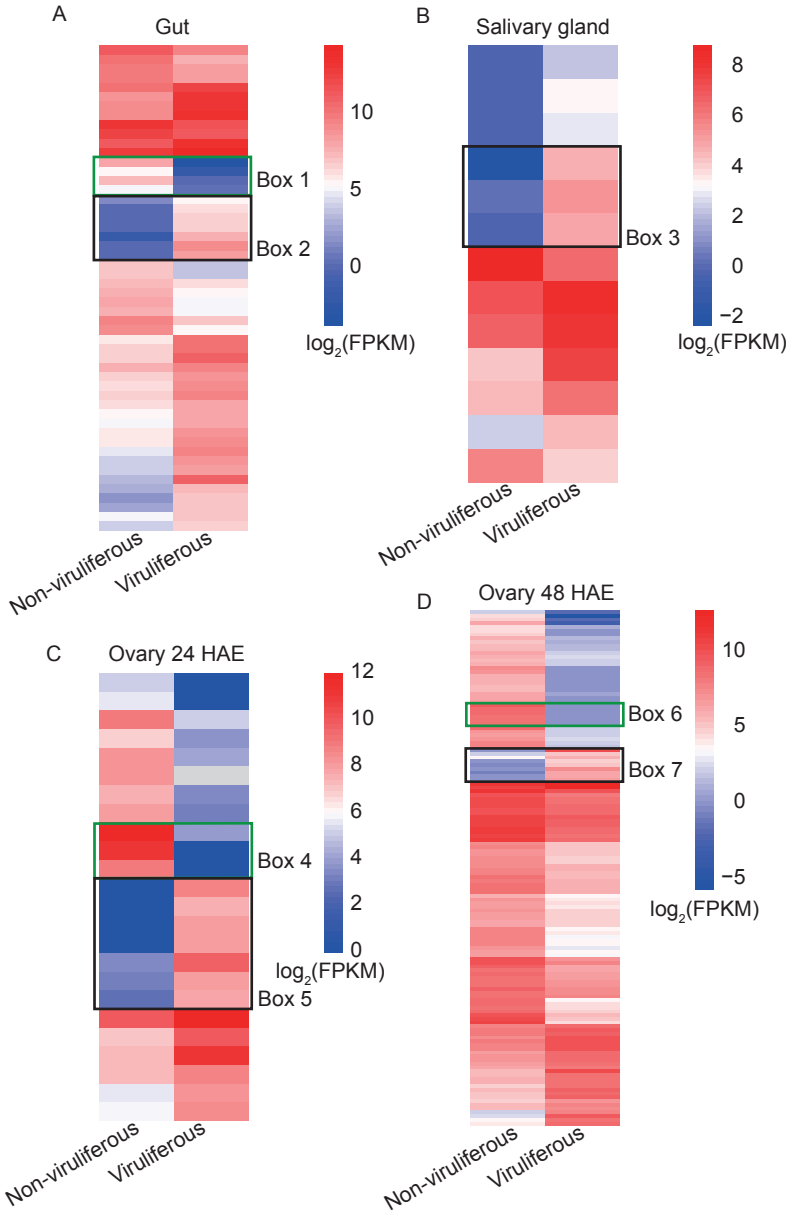

Fig. 2 Cluster analysis of long non-coding RNAs (lncRNAs) expression in viruliferous and non-viruliferous small brown planthopper. Box 2 (marked by black border) indicates that the expression of lncRNA significantly increased after virus infection, while Box 1 (marked by green border) shows that the expression of lncRNA decreased after virus infection, which proves that virus invasion can inhibit lncRNA expression. Ovary 24 HAE, ovary at 24 h after emergence; Ovary 48 HAE, ovary at 48 h after emergence.

73% of lncRNAs had two exons and shorter transcripts than protein-coding genes. These results are consistent with what has been observed in lncRNAs of rice brown planthopper (BPH), indicating that the lncRNA structural characteristics of these two species are conserved. At least three lncRNA genes that overlapped with fecundity related protein-coding genes were reported to be involved in regulating the fecundity of BPH, two genes highly expressed in a high fecundity population and one gene highly expressed in a low fecundity population (Xiao *et al.* 2015). Here, 26 lncRNAs showed significantly higher expression in viruliferous tissues while 13 lncRNAs were highly expressed in non-viruliferous tissues.

Interestingly, RSV not only induces the high expression of some lncRNAs but also suppresses the expression of other lncRNAs. For example, XLOC_007257 was highly expressed in viruliferous salivary gland, but its expression was suppressed in non-viruliferous gut. This suggests that RSV infection either induces or suppresses the expression of some lncRNAs as confirmed by qRT-PCR.

It has been reported that the expressions and functions of lncRNAs are positively associated with adjacent protein-

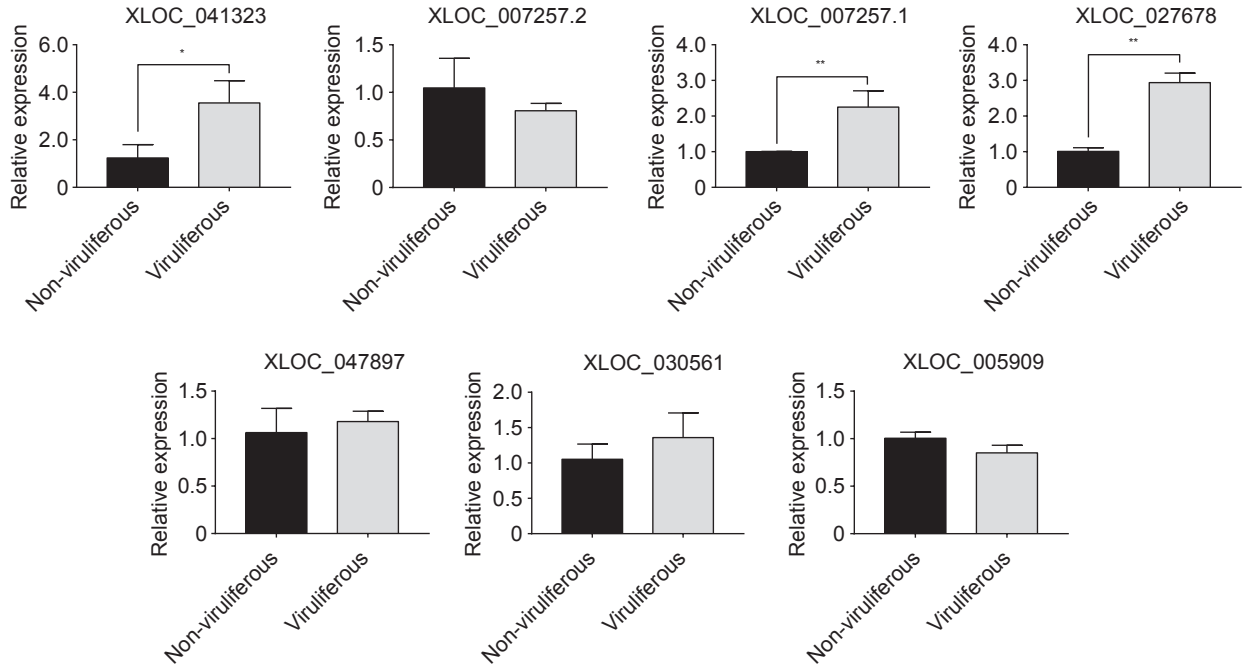

Fig. 3 Validation of differentially expressed long non-coding RNAs (lncRNAs) between non-viruliferous and viruliferous small brown planthopper by qRT-PCR. Gene expression was analyzed using Student's *t*-test. * stands for significant difference (P<0.05), and ** stands for extremely significant difference (P<0.01). Bars indicate SE with triplicates.

coding genes, with some lncRNAs that could potentially promote the transcription of a protein to affect its function (Necsulea *et al.* 2014; Spurlock III *et al.* 2015). Thus, the possible function of differentially expressed lncRNAs can be hypothesized according to the annotation of protein-coding genes adjacent to lncRNAs. XLOC_042264 is located adjacent to the down syndrome cell adhesion molecule (DSCAM), which regulates nervous system development and immune system in humans (Hattori *et al.* 2008; Smith *et al.* 2011). XLOC_041323 is located adjacent to a hemolymph glycoprotein (27 kDa hemolymph glycoprotein) produced by the braconid wasp *Cotesia congregata* polydnavirus, which helps *C. congregata* evade the host's immune response during parasitization (Harwood *et al.* 1994; Webb 1998). XLOC_031376 is adjacent to Ubiquitin-conjugating enzyme (E2 enzymes), which is used by the virus to invade the host, and affects viral survival and differentiation due to its protein degradation function (Yamamoto *et al.* 2006; Chen *et al.* 2013). The 26S protease non-ATPase modulation subunit 1 (PSMD1) is near XLOC_030561. PSMD1 is associated with the potential pathogenesis of aberrations and deregulations of the Ubiquitin-Proteasome System, and is thus relevant to neurodegenerative and myodegenerative disorders in clinical trials (Karin and Delhase 2000; Egerer *et al.* 2002). In addition, XLOC_030561 appears in Boxes 5, 6, and 7 of Fig. 2 indicating that its expression can be either induced or inhibited by RSV infection. The genome location of XLOC_015088 is near the zinc transporter gene. Zinc is the basic material for resistance to pathogen infection in the immune system, inferring that XLOC_015088 may participate in immune system activity against the virus invasion process (Shankar and Prasad 1998; Rink and Haase 2007). Collectively, these immunity-related lncRNAs genes are valid for studying gene functions possibly involved in immune interaction between RSV and SBPH during ovary development.

5. Conclusion

We identified the lncRNA genes from SBPH transcriptome data and analyzed the differential expression of lncRNAs responding to RSV infection between viruliferous and non-viruliferous SBPH. The results showed that these lncRNAs potentially interact with immune related genes, which might contribute to the success invasion of RSV in SBPH. Further investigations on regulatory mechanisms are necessary to understand the functions of lncRNAs.

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