Bulletin of Entomological Research

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Research Paper

Cite this article: Yang Y, Wang A, Wang M, Zhang Y, Zhang J, Zhao M (2022). ATP-binding cassette transporters *ABCF2* and *ABCG9* regulate rice black-streaked dwarf virus infection in its insect vector, *Laodelphax striatellus* (Fallén). *Bulletin of Entomological Research* **112**, 327–334. https://doi.org/10.1017/S0007485321000869

Received: 11 June 2021
Revised: 7 August 2021
Accepted: 18 August 2021
First published online: 22 September 2021

Keywords:
ABC transporters; *ABCF2*; *ABCG9*; *Laodelphax striatellus*; rice black-streaked dwarf virus

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ATP-binding cassette transporters *ABCF2* and *ABCG9* regulate rice black-streaked dwarf virus infection in its insect vector, *Laodelphax striatellus* (Fallén)

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Abstract

The majority of plant viral disease is transmitted and spread by insect vectors in the field. The small brown planthopper, *Laodelphax striatellus* (Fallén), is the only efficient vector for rice black-streaked dwarf virus (RBSDV), a devastating plant virus that infects multiple grain crops, including rice, maize, and wheat. Adenosine triphosphate (ATP)-binding cassette (ABC) transporters participate in various biological processes. However, little is known about whether ABC transporters affect virus infection in insects. In this study, RBSDV accumulation was significantly reduced in *L. striatellus* after treatment with verapamil, an effective inhibitor of ABC transporters. Thirty-four ABC transporter genes were identified in *L. striatellus* and expression analysis showed that *LsABCF2* and *LsABCG9* were significantly upregulated and downregulated, respectively, after RBSDV infection. *LsABCF2* and *LsABCG9* were expressed during all developmental stages, and *LsABCG9* was highly expressed in the midgut of *L. striatellus*. Knockdown of *LsABCF2* promoted RBSDV accumulation, while knockdown of *LsABCG9* suppressed RBSDV accumulation in *L. striatellus*. Our data showed that *L. striatellus* might upregulate the expression of *LsABCF2* and downregulate *LsABCG9* expression to suppress RBSDV infection. These results will contribute to understanding the effects of ABC transporters on virus transmission and provide theoretical basis for virus management in the field.

Introduction

In all kingdoms of life, the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family is one of the largest transporter protein superfamily; these proteins can bind and hydrolyze ATP while transporting multiple substrates on lipid membranes, including amino acids, peptides, xenobiotics, and drugs (Derumauw and Leeuwen, 2014). The functional ABC transporter consists of two cytosolic nucleotide binding domains (NBDs), which contain three conserved motifs (Walker A, Walker B, and ABC signature motifs), and two integral transmembrane domains (TMDs) (Davidson *et al.*, 2008). The NBDs can provide energy to transport substrates through binding and hydrolyzing ATP, and the TMDs participate in translocating the substrate (Rees *et al.*, 2009). The ABC protein family can be classified into eight subfamilies (ABCA to ABCH), according to the sequence similarity and the domain structure of the NBDs (Dean and Annilo, 2005; Hollenstein *et al.*, 2007). However, members of the ABCH family have been identified only in *Danio rerio*, *Dictyostelium discoideum*, and arthropods (Derumauw and Leeuwen, 2014).

ABC transporters have been widely studied in humans. Multidrug-resistance proteins (MDRs), P-glycoproteins (P-gps), multidrug-resistance associated proteins (MRPs), and the breast cancer resistance protein are related to the multidrug resistance of cancer cells to chemotherapeutics (Kartner *et al.*, 1985; Riordan *et al.*, 1985; Hopper-Borge *et al.*, 2009; Juan-Carlos *et al.*, 2021). In addition, ABC transporters participate in lipid transport (Wenzel, 2007), the import of long-branched chain acyl-CoA into peroxisomes (Morita and Imanaka, 2012), the biogenesis of cytosolic iron–sulfur clusters, heme biosynthesis, iron homeostasis, and protection against oxidative stress in humans (Zutz *et al.*, 2009). Among arthropods, the ABC transporters in *Drosophila melanogaster* are the most widely studied. In *D. melanogaster*, ABC transporters are involved in the transport of eye pigment precursors (Mackenzie *et al.*, 1999), cyclic guanosine monophosphate (Evans *et al.*, 2008), biogenic amines (Borycz *et al.*, 2008), development (Hock *et al.*, 2000) and control of the transcription of circadian clock genes (Itoh *et al.*, 2011). In *Tribolium castaneum*, it has been proven that ABCG subfamily members are associated with insect development. Injection of *TcABCG* dsRNA into *T. castaneum* larvae resulted in growth arrest and localized melanization, eye pigmentation defects, abnormal cuticle formation, egg-laying and egg-hatching defects, and mortality due to abortive molting and desiccation (Broehan *et al.*, 2013). Similar phenomena have also been found in *Tetranychus urticae* and *Bombyx mori* (Grbic *et al.*, 2011; Liu *et al.*, 2011). In addition, ABC transporters play important roles in insecticide resistance, in insect–*Bacillus thuringiensis* interaction and in the transportation of plant secondary compounds (Aurade *et al.*, 2010, 2011; Guo *et al.*, 2015; He *et al.*, 2018; Liu *et al.*, 2019; Guo *et al.*, 2020; Meng *et al.*, 2020; Rosner *et al.*, 2021; Shan *et al.*, 2021). However, little is known about the functions of ABC transporters in virus transmission in insect vectors.

Rice black-streaked dwarf virus (RBSDV) is transmitted by *Laodelphax striatellus* (Fallén) in a persistent propagative manner (Zhang *et al.*, 2001; Wu *et al.*, 2020). RBSDV belongs to the genus *Fijivirus* of the family *Reoviridae* and is the causal agent of rice black-streaked dwarf and maize rough dwarf disease (Wu *et al.*, 2020). The epidemic of these viral diseases caused considerable yield losses in East Asia. The RBSDV genome includes ten double-stranded RNA (dsRNA) segments (*S1* to *S10*), and encodes thirteen proteins. Each segment of *S5*, *S7*, and *S9* encodes two proteins. P5-1, P6, and P9-1 proteins are components of the viroplasm (Li *et al.*, 2013; Sun *et al.*, 2013; He *et al.*, 2020). P10 protein is the outer capsid protein of RBSDV and plays key roles in viral infection (Lu *et al.*, 2019*a*; Zhang *et al.*, 2019).

In the present research, the functions of ABC transporters in RBSDV infection in the insect vector *L. striatellus* were analyzed through treatment with the ABC transporter inhibitor verapamil. A total of 34 genes encoding members of the ABC superfamily were identified based on the genome of *L. striatellus*. The relative expression levels of 34 ABC transporters were compared in RBSDV-infected and virus-free populations of *L. striatellus*. Furthermore, the roles of *LsABCF2* and *LsABCG9* were analyzed during RBSDV infection in *L. striatellus* by RNA interference (RNAi). These results will provide insight into ABC transporter functions in response to virus infection in insects.

Materials and methods

Insect and virus

The population of *L. striatellus* was originally collected from Haian (32.57°N, 120.45°E; Jiangsu, China). The population was fed on rice seedlings at 25 ± 1°C with 70–80% humidity and 16 h light:8 h dark photoperiod. The RBSDV-infected rice plants, containing typical dwarf symptoms, were collected from the field.

Verapamil treatment

The verapamil was dissolved in the dimethyl sulfoxide, and diluted to 0.5, 0.25, and 0.1 mM in acetone. Third-instar nymphs of *L. striatellus*, reared on the rice seedlings infected with RBSDV for 2 days, were used in the study. After anesthetization on the ice, a droplet (100 nl) of verapamil solution was applied topically to the prothorax notum of the nymphs by a FemtoJet microinjector (Eppendorf, Germany). Acetone treatment was applied as a control. Each experiment was carried out with 30 nymphs, and each treatment included three independent biological replicates. The treated nymphs were transferred to healthy rice seedlings for 3 days. The RBSDV accumulation was determined by detecting the expression of *S10* and the replication-related genes, *S5-1*, *S6*, and *S9-1* by quantitative real-time polymerase chain reaction (RT-qPCR).

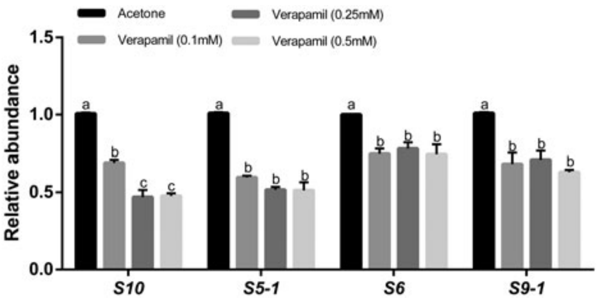

Figure 1. Relative expression of RBSDV coat protein gene *S10* and replication related genes, *S5-1*, *S6*, and *S9-1* after acetone treatment and verapamil treatment with three doses. The letters a, b, and c present significant differences ($P < 0.05$) of the expression level.

Identification of ABC transporter genes and phylogenetic analysis

Potential ABC transporter genes annotated in the *L. striatellus* genome were searched for, including ABC transporters, ABC transporters/proteins, MDRs, P-gps, and MRPs (Zhu *et al.*, 2017). The short (<500 bp) and repetitive sequences were removed, and the amino acid sequences of the remaining ABC transporter gene were blasted against the NCBI nonredundant (Nr) protein database to remove the redundant sequences and faulty annotated sequences. A phylogenetic tree was generated by the ClustalW alignment of the amino acid sequences with ABC transporter genes from other insect species, including *Nilaparvata lugens*, *B. mori*, *Diaphorina citri*, and *Helicoverpa armigera*, using the neighbor-joining method in MEGA 5.05 with 1000 bootstrap replicates.

Analyzing the expression of ABC transporters during RBSDV infection

The virus-free nymphs of *L. striatellus* were reared on rice seedlings infected with RBSDV for 2 days, and then transferred to healthy rice seedlings until molting into fifth-instar nymphs. The surviving nymphs were collected as RBSDV-infected samples. In addition, the virus-free nymphs of *L. striatellus* were reared on healthy rice seedlings until molting into fifth-instar nymphs, and collected as a control. Each treatment contained thirty nymphs, and each treatment included three independent biological replicates. The expression of ABC transporters was detected in both RBSDV-infected and virus-free samples.

L. striatellus *tissue and instar samples*

For tissue dissection, the *L. striatellus* were rinsed three times with 75% ethanol and washed three times with sterilized deionized water. With a stereomicroscope, the tissues of the midgut, fat body, ovary, and salivary gland of *L. striatellus* were dissected in chilled 1× phosphate-buffered saline (pH 7.4) with sterile forceps. Tissues dissected from 50 virus-free *L. striatellus* and each tissue sample included three independent biological replicates.

The first-instar to fifth-instar nymphs, and male and female adults of *L. striatellus* were collected. Each treatment included 50 insects, and was processed in three independent biological replicates.

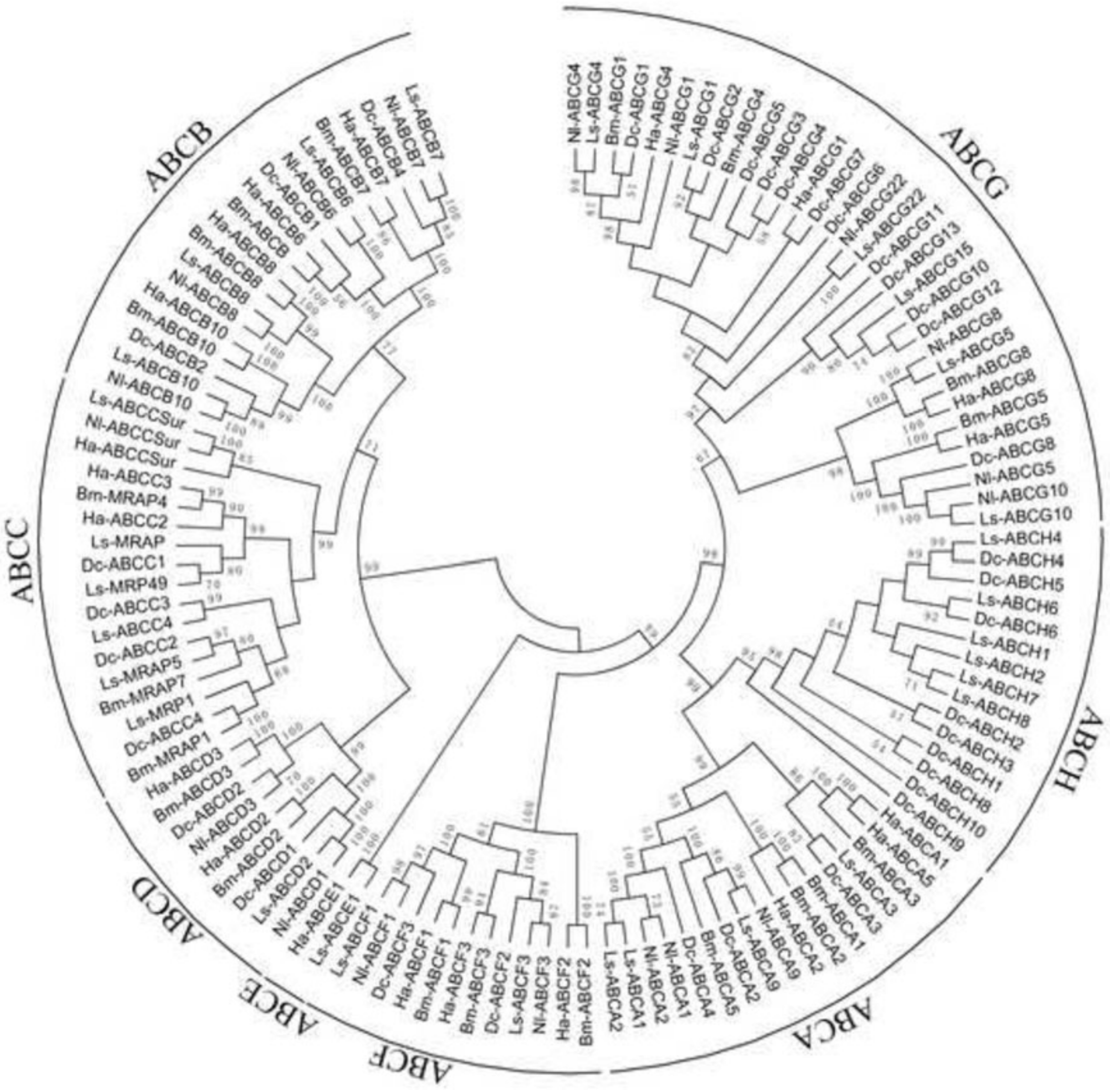

Figure 2. Phylogenetic analysis of ABC transporter genes. The numbers above the branches indicated the support for the phylogenies, and only values above 50% are shown. Ls, *L. striatellus*; Nl, *Nilaparvata lugens*; Bm, *Bombyx mori*; Dc, *Diaphorina citri*; Ha. *Helicoverpa armigera*.

Quantitative real-time PCR

L. striatellus total RNA was extracted by TRIzol reagent (Invitrogen, United States). The RNA quantity and quality were detected by spectrophotometry (NanoDrop 2000, Thermo Scientific) and agarose gel electrophoresis, respectively.

The PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan) was used to synthesize cDNA. RT-qPCR experiments were performed using a SYBR PrimeScript™ RT-PCR Kit (Takara, Japan) and an IQ™5 multicolor real-time PCR detection system (Bio-Rad, USA). Each RT-qPCR reaction included three independent technical and biological replications. *RPL5* (encoding ribosome protein L5) was used as an internal reference gene. The $2^{-\Delta\Delta Ct}$ method was used for data analysis. The primers were designed by using Beacon Designer 7.7 and are listed in table S1.

RNA interference

The primers, amplifying the target fragment for RNAi, are listed in table S1. According to the manufacturer's instructions, the T7 high-yield transcription kit (Invitrogen, United States) was used to synthesize the dsRNA. After anaesthetization on ice, virus-free third-instar nymphs were injected with 100 nl dsRNA ($2\,\mu g\,\mu l^{-1}$) by using a FemtoJet microinjector (Eppendorf, Germany) in conjunction with the prothorax and mesothorax of *L. striatellus*. Enhanced green fluorescent protein dsRNA was used as a control. The RNAi efficiency of target genes was determined by calculating their relative expression levels at 1 and 3 days after dsRNA injection. The mortality rate of *L. striatellus* was checked at 1 and 3 days after dsRNA injection. Furthermore, virus-free third-instar nymphs reared on rice seedlings infected with RBSDV for 2 days were injected with 100 nl dsRNA ($2\,\mu g\,\mu l^{-1}$). After injection, the nymphs were transferred to healthy rice seedlings for 3 days. RBSDV accumulation was determined by detecting the relative expression levels of *S5-1*, *S6*, *S9-1*, and *S10* by RT-qPCR. Each experiment was carried out with 30 insects and three independent biological replicates.

Statistical analysis

SPSS 20.0 (IBM Corporation, United States) was used for the statistical analysis. One-way analysis of variance with the least significant difference test was used in data analysis. The *P* values <0.05

Table 1. Summary of the 34 ABC transporters identified from *L. striatellus* genome

Subfamily	Gene ID	Gene name	ORF (bp)	Blasted species	Acc. number	Score	*E*-value
A	Contig1075.1	*LsABCA2*	1653	*L. striatellus*	AIO05331.1	1301	0.00
	Contig377.47	*LsABCA3*	4935	*L. striatellus*	AIN44094.1	3323	0.00
	Contig157.68	*LsABCA9*	5148	*N. lugens*	XP_022184316.1	2284	0.00
B	Contig18.97	*LsABCB6*	2520	*N. lugens*	XP_022195861.1	1459	0.00
	Contig238.26	*LsABCB7*	2262	*L. striatellus*	AIN44095.1	1541	0.00
	Contig0.124	*LsABCB8*	1458	*L. striatellus*	AIN44100.1	1377	0.00
	Contig686.5	*LsABCB10*	1536	*L. striatellus*	AIN44099.1	1341	0.00
C	Contig17.199	*LsABCCSur*	4896	*N. lugens*	XP_022207582.2	2533	0.00
	Contig490.9.1	*LsABCC4*	4317	*L. striatellus*	AIO05327.1	2955	0.00
	Contig92.71	*LsABCC12*	897	*N. lugens*	XP_022183913.1	545	5×10^{-139}
	Contig40.16	*LsMRP1*	4914	*N. lugens*	XP_022190395.1	2379	0.00
	Contig167.10	*LsMRP49*	4032	*L. striatellus*	AIN44101.1	2734	0.00
	Contig666.8	*LsMRAP*	4191	*L. striatellus*	AIN44103.1	2867	0.00
	Contig86.86	*LsMRAP5*	4410	*L. striatellus*	AIN44105.1	2983	0.00
D	Contig58.174	*LsABCD2*	2217	*L. striatellus*	AIN44106.1	1526	0.00
E	Contig522.5	*LsABCE1*	1212	*L. striatellus*	AIN44108.1	916	0.00
F	Contig30.131	*LsABCF1*	1854	*L. striatellus*	AIO05328.1	1274	0.00
	Contig226.17	*LsABCF2*	1872	*L. striatellus*	AIN44109.1	1300	0.00
	Contig772.5	*LsABCF3*	2202	*Zootermopsis nevadensis*	XP_021913879.1	1293	0.00
G	Contig13.430	*LsABCG1*	2049	*L. striatellus*	AIN44111.1	1407	0.00
	Contig26.100.1	*LsABCG4*	2136	*L. striatellus*	AIN44115.1	1471	0.00
	Contig167.40	*LsABCG5*	2880	*L. striatellus*	AIO05329.1	1938	0.00
	Contig1023.1	*LsABCG9*	609	*N. lugens*	XP_022197235.1	303	$1 \times 10^{-}95$
	Contig167.43	*LsABCG10*	1929	*L. striatellus*	AIO05330.1	1297	0.00
	Contig248.9	*LsABCG15*	1833	*D. citri*	QAA95931.1	642	0.00
	Contig1082.12	*LsABCG22*	2094	*L. striatellus*	AIN44120.1	1264	0.00
H	Contig447.11	*LsABCH1*	1701	*L. striatellus*	AIN44128.1	1177	0.00
	Contig447.12.1	*LsABCH2*	2106	*L. striatellus*	AIN44122.1	1448	0.00
	Contig447.9	*LsABCH3*	1362	*L. striatellus*	AIN44127.1	944	0.00
	Contig84.134	*LsABCH4*	2271	*L. striatellus*	AIN44124.1	1542	0.00
	Contig1612.4	*LsABCH5*	1860	*L. striatellus*	AIN44123.1	1108	0.00
	Contig447.8	*LsABCH6*	2082	*L. striatellus*	AIN44126.1	1432	0.00
	Contig46.21	*LsABCH7*	1332	*L. striatellus*	AIN44125.1	880	0.00
	Contig20.121	*LsABCH8*	2046	*L. striatellus*	AIN44129.1	1402	0.00

ORF, open-reading frame; Ls, *Laodelphax striatellus*.

and <0.01 were regarded as the thresholds of significant and very significant differences, respectively.

Results

The role of verapamil in RBSDV accumulation

Verapamil, an inhibitor of ABC transporters, was applied to explore the potential functions in RBSDV accumulation in *L. striatellus*. Compared to the control, the relative expression of the RBSDV coat protein gene *S10* and replication-related genes *S5-1*, *S6*, and *S9-1* were significantly reduced after verapamil treatment at three doses (fig. 1). These results indicated that inhibition of ABC transporters suppressed RBSDV accumulation in its insect vector.

Transcriptional response of 34 ABC transporters to RBSDV infection

In insects, ABC proteins are classified into eight subfamilies, ABCA to ABCH. In this study, 34 ABC transporter genes were identified based on the genome of *L. striatellus*, including three

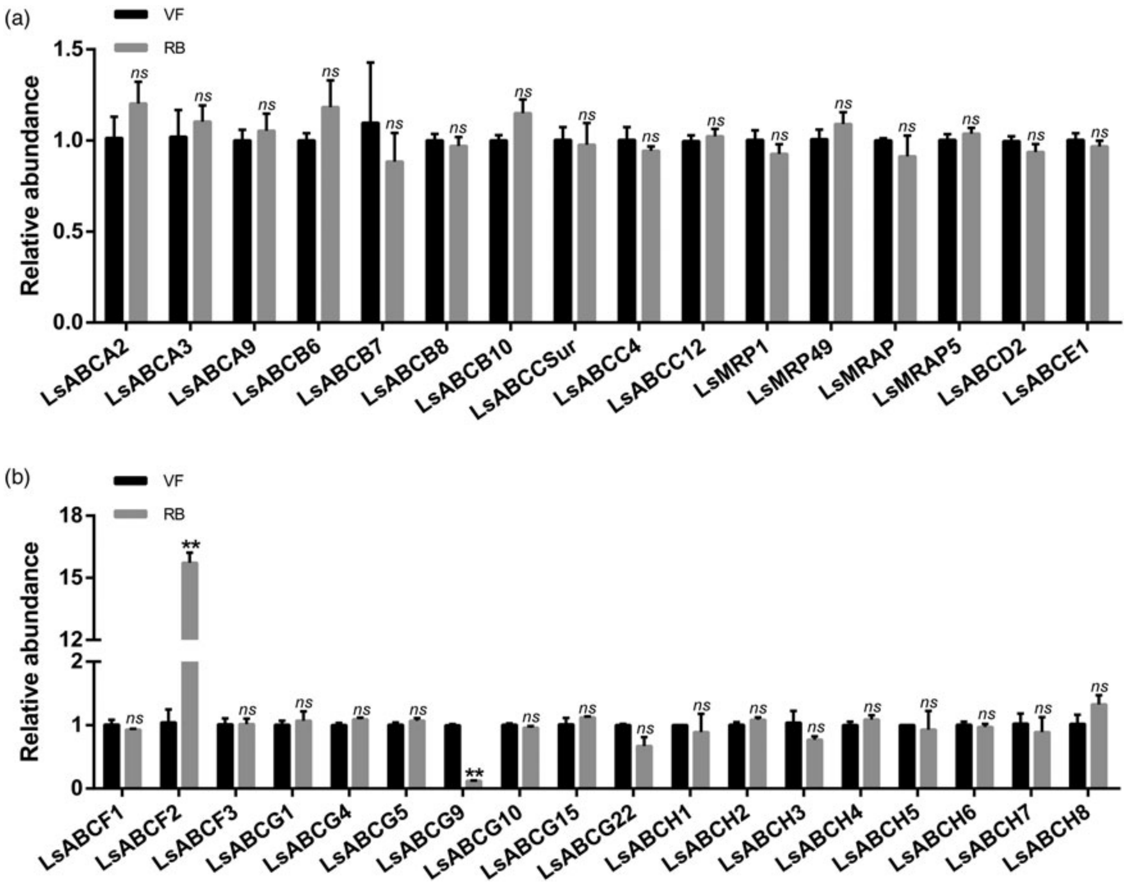

Figure 3. Expression induction of 34 ABC transporter genes between RBSDV-infected and virus-free populations. (a) The fold changes in the expression of ABC transporter genes from ABCA, ABCB, ABCC, ABCD, and ABCE subfamily. (b) The fold changes in the expression of ABC transporter genes from ABCF, ABCG, and ABCH subfamily. The significant differences were marked by asterisk. **Significantly at the 0.01 level. The *ns* represents no significant difference.

ABCAs, four ABCBs, seven ABCCs, one ABCD, one ABCE, three ABCFs, seven ABCGs, and eight ABCHs (fig. 2, table 1). To identify the ABC transporters that might be involved in RBSDV infection, the relative expression levels of 34 ABC transporters in RBSDV-infected and virus-free populations of *L. striatellus* were compared by RT-qPCR. Two ABC transporters were significantly differently expressed after RBSDV infection (fig. 3). After RBSDV infection, the relative expression level of *LsABCF2* was upregulated more than 15-fold, while *LsABCG9* was reduced by 85% (fig. 3b).

Expression profiles analysis of LsABCF2 *and* LsABCG9

The tissues of the fat body, midgut, ovary, and salivary gland and different developmental stages were examined to determine the expression profiles of *LsABCF2* and *LsABCG9*. *LsABCF2* was expressed in all developmental stages and expressed at a high level in females (fig. 4a). The tissue expression profiles revealed that *LsABCF2* had no expression differences in the tissues of the fat body, midgut, ovary, and salivary gland (fig. 4b). *LsABCG9* was expressed in all developmental stages and had high levels in fourth-instar and fifth-instar (fig. 4c). The tissue expression profiles revealed that *LsABCG9* was highly expressed in the midgut (fig. 4d).

LsABCF2 *and* LsABCG9 *regulate RBSDV infection in* L. striatellus

To confirm whether *LsABCF2* and *LsABCG9* regulated RBSDV infection in *L. striatellus*, the expression of *LsABCF2* and *LsABCG9* was knocked down via RNAi. The expression levels of *LsABCF2* and *LsABCG9* were significantly decreased at 1 and 3 days after dsRNA injection (fig. 5a, b). The mortality rate of *L. striatellus* was no significant difference at 1 and 3 days after dsRNA injection (fig. S1a, b). Knockdown of *LsABCF2* significantly increased the relative expression of RBSDV *S10* and the RBSDV replication-related genes, *S5-1*, *S6*, and *S9-1* (fig. 5c, e). The results suggested that inhibition of *LsABCF2* promoted RBSDV accumulation in *L. striatellus*. In addition, knockdown of *LsABCG9* significantly reduced the relative expression of RBSDV *S10* and the RBSDV replication related genes, *S5-1*, *S6*, and *S9-1* (fig. 5d, f). The results suggested that inhibition of *LsABCG9* suppressed RBSDV accumulation in *L. striatellus*.

Discussion

Plant viral diseases cause great losses to crops worldwide every year. Most plant viruses rely on specific insect vectors for transmission and more than 76% of plant viruses are transmitted by these vectors (Hogenhout *et al.*, 2008). Virus transmission via insects is the main reason for the occurrence and prevalence of plant virus diseases in the field (Wu *et al.*, 2020). *L. striatellus* is an important insect vector that has caused epidemics of several plant viral diseases, including rice stripe disease and rice black streaked dwarf disease (Lu *et al.*, 2019*b*; Wu *et al.*, 2020). Furthermore, persistent plant viruses move from the food canal of the insect vector to the midgut, then to the hemolymph and

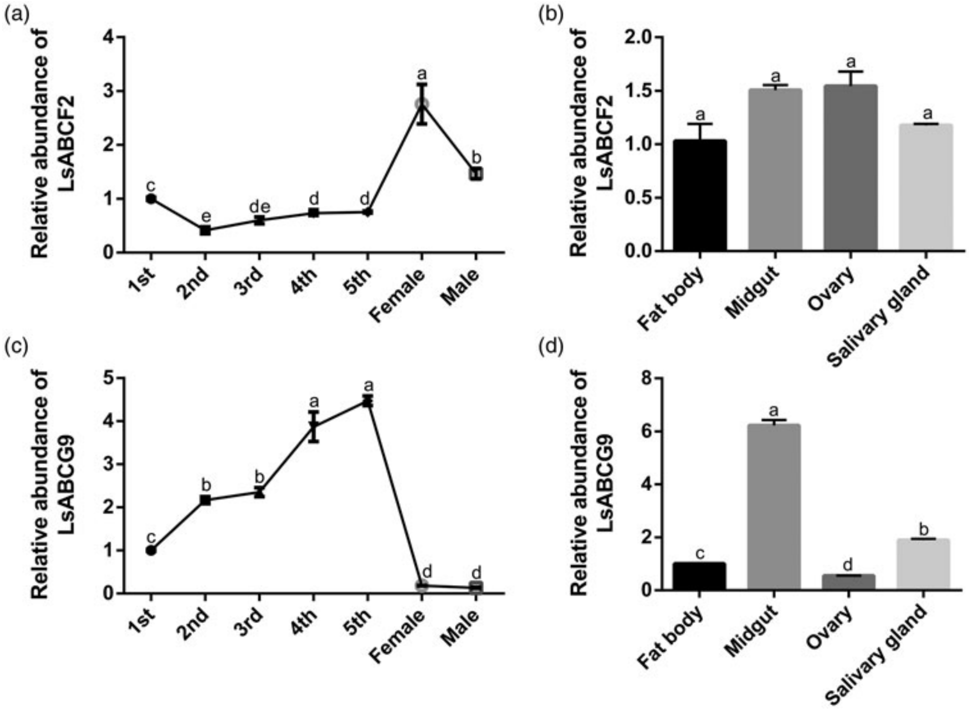

Figure 4. Expression of *ABCF2* and *ABCG9* in different developmental stages and different tissues of *L. striatellus*. (a) The relative expression of *ABCF2* in different developmental stages. (b) The relative expression of *ABCF2* in different tissues. (c) The relative expression of *ABCG9* in different developmental stages. (d) The relative expression of *ABCG9* in different tissues. The letters a, b, c, and d present significant differences ($P < 0.05$) of the expression level.

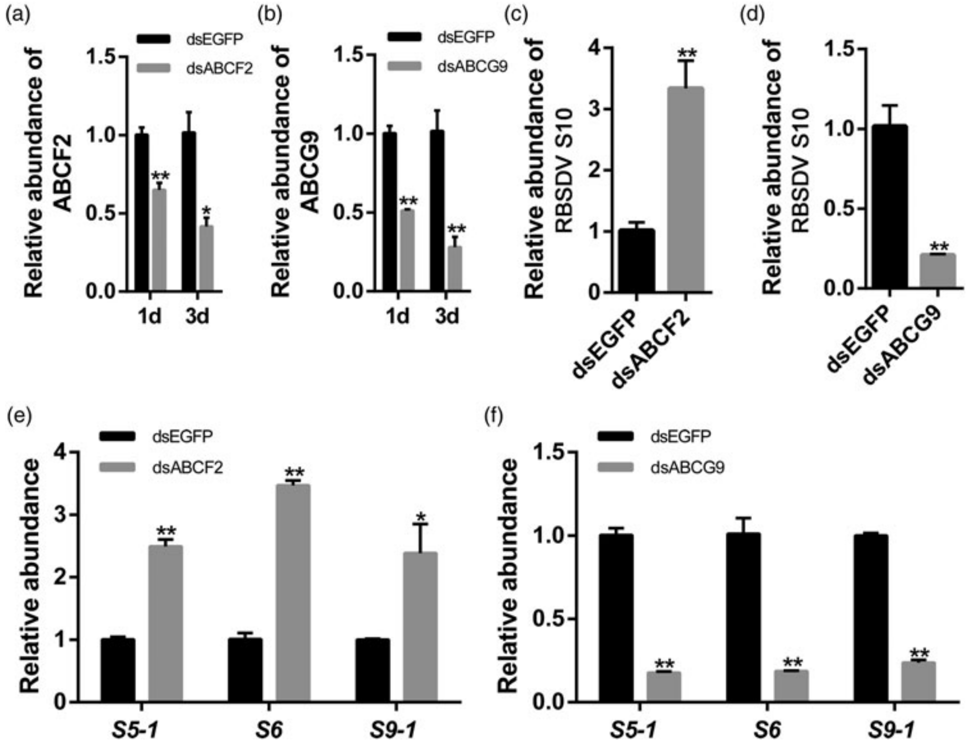

Figure 5. dsRNA-mediated suppression of ABC transporter genes and effects on RBSDV accumulation in *L. striatellus*. (a) The relative expression level of *ABCF2* at 1 and 3 days after ds*ABCF2* injection. (b) The relative expression level of *ABCG9* at 1 and 3 days after ds*ABCG9* injection. (c) The relative expression of RBSDV *S10* after knockdown of *ABCF2*. (d) The relative expression of RBSDV *S10* after knockdown of *ABCG9*. (e) The relative expression of RBSDV *S5-1*, *S6*, and *S9-1* after knockdown of *ABCF2*. (f) The relative expression of RBSDV *S5-1*, *S6*, and *S9-1* after knockdown of *ABCG9*. The significant differences were marked by asterisk. *Significantly at the 0.05 level. **Significantly at the 0.01 level.

salivary gland, and finally are introduced back into plants with saliva secretion (Hogenhout *et al*., 2008). Therefore, understanding how viruses break the infection barrier in insect vectors is the key step in controlling virus diseases.

In insects, ABC transporters are involved in trafficking a batch of substrates (Derumauw and Leeuwen, 2014). Several studies have uncovered that ABC transporters participate in the growth and development (Broehan *et al*., 2013), as well as in insecticide resistance in insects (Gahan *et al*., 2010; Yang *et al*., 2019; Shan *et al*., 2021). However, the function of ABC transporters in virus transmission is little known. Verapamil has been widely used in the functional analysis of ABC transporters in various biological processes (Meng *et al*., 2020). In this study, RBSDV accumulation was significantly reduced in *L. striatellus* after verapamil treatment, indicating that ABC transporters could regulate the infection of the virus. We identified 34 ABC transporter genes based on the genome of *L. striatellus*, which is similar to the number of ABC transporters observed in some other insect species (Xiao *et al*., 2018; Liu *et al*., 2019). In addition, the expression profiles of the 34 ABC transporters were analyzed after RBSDV infection. Only *LsABCF2* and *LsABCG9* were significantly differentially expressed after RBSDV infection. Our results indicate that these ABC transporters respond to RBSDV infection.

A previous study reported that *ABCF2* has key functions in resistance to *cis*-platin in ovarian cancer (Bao *et al*., 2017). *ABCG9* was involved in the accumulation of steryl glycosides in *Arabidopsis thaliana* (Choi *et al*., 2014). Our experiments demonstrated that *LsABCF2* and *LsABCG9* were expressed in all developmental stages, indicating that they have key biological roles in the growth and development of *L. striatellus*. In addition, *LsABCG9* was highly expressed in the midgut, and the midgut was a barrier for persistent virus infection (Hogenhout *et al*., 2008), suggesting that *LsABCG9* might be involved in RBSDV breaking the midgut barrier in *L. striatellus*. Knockdown of ABC transporter expression by RNAi has been widely used to study the contribution of these genes to various biological functions in insects. In this study, the knockdown of *LsABCF2* promoted RBSDV accumulation in *L. striatellus*, while the knockdown of *LsABCG9* suppressed RBSDV accumulation in *L. striatellus*. Therefore, it is concluded that *L. striatellus* might upregulate the expression of *LsABCF2* and downregulate *LsABCG9* expression to suppress RBSDV infection.

In conclusion, RBSDV accumulation was significantly reduced in *L. striatellus* after verapamil treatment. Thirty-four ABC transporter genes were identified in *L. striatellus* and their expression levels were analyzed after RBSDV infection. *LsABCF2* and *LsABCG9* respond to virus infection, and *L. striatellus* might upregulate the expression of *LsABCF2* and downregulate *LsABCG9* expression to suppress RBSDV infection. These results will contribute to our understanding of the effects of ABC transporters on virus transmission.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485321000869.

Acknowledgements. This study was supported by the Natural Science Foundation of Shandong Province (ZR2019BC055) and the China Postdoctoral Science Foundation (2019M661772). We thank Prof. Yijun Zhou (Jiangsu Academy of Agricultural Sciences) and Prof. Qiufang Xu (Jiangsu Academy of Agricultural Sciences) for providing RBSDV materials.

Conflict of interest. The authors declare no conflicts of interest.

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