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# Maintenance of persistent transmission of a plant arbovirus in its insect vector mediated by the Toll-Dorsal immune pathway

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**Throughout evolution, arboviruses have developed various strategies to counteract the host's innate immune defenses to maintain persistent transmission. Recent studies have shown that, in addition to bacteria and fungi, the innate Toll-Dorsal immune system also plays an essential role in preventing viral infections in invertebrates. However, whether the classical Toll immune pathway is involved in maintaining the homeostatic process to ensure the persistent and propagative transmission of arboviruses in insect vectors remain unclear. In this study, we revealed that the transcription factor Dorsal is actively involved in the antiviral defense of an insect vector (*Laodelphax striatellus*) by regulating the target gene, *zinc finger protein 708* (*LsZN708*), which mediates downstream immune-related effectors against infection with the plant virus (Rice stripe virus, RSV). In contrast, an antidefense strategy involving the use of the nonstructural-protein (NS4) to antagonize host antiviral defense through competitive binding to Dorsal from the MSK2 kinase was employed by RSV; this competitive binding inhibited Dorsal phosphorylation and reduced the antiviral response of the host insect. Our study revealed the molecular mechanism through which Toll-Dorsal-ZN708 mediates the maintenance of an arbovirus homeostasis in insect vectors. Specifically, ZN708 is a newly documented zinc finger protein targeted by Dorsal that mediates the downstream antiviral response. This study will contribute to our understanding of the successful transmission and spread of arboviruses in plant or invertebrate hosts.**

Rice stripe virus | *Laodelphax striatellus* | toll immune pathway | dorsal | zinc finger protein

## Significance

The innate immune system, especially the Toll pathway, plays a vital role in defending against pathogenic microorganisms, including viruses. Nevertheless, whether the classical Toll immune pathway is involved in maintaining the homeostatic process to ensure the persistent and propagative transmission of arboviruses in insect vectors remains unclear. In our study, we unveiled the molecular mechanism through which Toll-Dorsal-ZN708 (zinc finger protein 708) mediates the maintenance of homeostasis of a plant arbovirus in the insect vector. Specifically, ZN708 is a newly documented zinc finger protein targeted by Dorsal that mediates the downstream antiviral response. In this study, we also present evidence of the antiviral role of the Toll immune system in an insect vector active against plant arboviruses.

Innate immunity plays a crucial role in protecting vertebrates and invertebrates against various pathogens, such as bacteria, fungi, and viruses (1). Several evolutionarily conserved immune signaling pathways, including the RNA interference (RNAi), Toll, immune deficiency (IMD), and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways, have been identified in invertebrates (2–5). The Toll and IMD pathways, which are both NF-κB immune effector pathways, are activated because pattern recognition receptors recognize signature molecules of pathogens known as pathogen-associated molecular patterns (6–10). Key components of the canonical Toll immune pathway include the extracellular factor Spatzle (Spz), Toll-like receptors (TLRs), the ligands Tube and MyD88, the kinases Pelle and Cactus, and the transcription factors Dorsal and Dorsal-related immunity factor (Dif). In invertebrates, when pathogens infect hosts, activated Spz binds to the receptor Toll, which is followed by the recruitment of MyD88, Tube, and Pelle to form the receptor proximal oligomeric complex (11–13). In *Drosophila*, the complex further causes the phosphorylation and degradation of Cactus, which leads to the nuclear translocation of Dorsal and the subsequent regulation of the expression of different antimicrobial peptide genes (14). Previous studies have indicated that the Toll pathway plays a pivotal role in various biological processes, including the development of the embryonic dorsoventral axis in *Drosophila*, as well as in the response to antibacterial and fungal infections (15–17). Interestingly, recent studies have provided compelling evidence showing that the Toll immune pathway also has antiviral functions in both mammals and model insects (2, 18–23). In vertebrates, TLR7 and TLR13 trigger antiviral responses during viral infection (18–20). In whiteleg shrimp (*Litopenaeus vannamei*), the Toll-4 receptor recognizes white spot syndrome virus (WSSV) and thus participates in antiviral activities (21). In *Drosophila*, the Toll-7 receptor interacts with vesicular stomatitis virus (VSV) on the cytoplasmic membrane to induce autophagy (2), while the Toll pathway is activated against dengue virus in various *Aedes* mosquitoes (22, 23).

During evolution, RNA viruses have also developed various counterdefense strategies to combat the antiviral responses of hosts. RNA silencing suppressors (VSRs) are typical examples of proteins that inhibit host RNAi, thus facilitating the replication of RNA viruses within host cells (24, 25). Moreover, several counterdefense strategies of WSSV fight against

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host Toll-mediated antiviral defense. In shrimp, multiple NF-κB binding motifs were discovered in the promoters of WSSV genes, and the NF-κB system has been hypothesized to be annexed by WSSV to facilitate its replication in shrimp (26–30). In addition, during WSSV infection, the virus can induce the expression of microRNA in the host (miR-1959). This miRNA targets and inhibits the transcription of the NF-κB inhibitor Cactus, thus leading to the activation of Dorsal to promote viral replication (31).

One of the distinctive characteristics of arbovirus vectors is their ability to tolerate high levels of virus proliferation, and this adaptation plays a critical role in vector competence and disease transmission (32). While the antiviral functions of various immune signaling pathways, such as the RNAi, Toll, IMD, and JAK-STAT pathways, have been demonstrated in arboviral infections, our understanding of the mechanisms by which arboviruses maintain immune homeostasis in insect vectors is still in its early stages (33). For mosquitoes, the most important arbovirus vector, virus-derived nucleic acids effectively enhance the RNAi response mounted by mosquitoes of the genus *Aedes* against dengue or chikungunya infections, thus promoting the maintenance of immune homeostasis during persistent infection (34). For the plant virus vector whitefly (*Bemisia tabaci*), two STAT-activated effector genes (*BtCD109-2* and *BtCD109-3*) mediate antiviral activity against tomato yellow leaf curl virus (TYLCV), whereas the coat protein (CP) of TYLCV inhibits the JAK/STAT pathway to maintain persistent viral infection (35). Another example of immune homeostasis in whiteflies is mediated by phosphatidylethanolamine-binding protein, which balances apoptosis and autophagy in *B. tabaci* to facilitate the transmission of TYLCV (36). Although previous studies have demonstrated that Toll plays essential roles in the antiviral response (3, 22, 23), whether this immune pathway is involved in the homeostatic process required for the persistent transmission of arboviruses in insect vectors has not been determined.

Rice stripe virus (RSV) is a negative-sense RNA virus of the genus *Tenuivirus* and family *Phenuiviridae* that causes severe disease in rice crops (37, 38). RSV is transmitted by the insect vector *Laodelphax striatellus* in a persistent propagative manner (39). When the virus particles are ingested by *L. striatellus* through feeding on RSV-infected rice plants, RSV disseminates and replicates in the midgut epithelium to establish infection. Subsequently, RSV spreads into the hemolymph and ultimately disseminates to healthy plants after dissemination into the salivary glands (SG). Moreover, RSV virions can also invade the reproductive system of *L. striatellus* (the ovaries and testes), facilitating vertical transmission to the offspring (39, 40). The developmental time of *L. striatellus* instars spans approximately 2 wk under typical rearing conditions at 25 °C and exhibiting noteworthy variability with different temperature (41, 42). It has been reported that planthopper instars are more efficient in transmitting tenuiviruses than the adults and the second instar nymph of *L. striatellus* is commonly used for plant virus acquisition (37, 43, 44). Moreover, persistent propagative transmitted plant virus must undergo a latent period in its insect vector before successful transmission to a healthy plant (39). A previous study indicated that the latent period of RSV ranges from 3 to 10 d (45) while a recent work precisely suggested a latent period of 4 d (96 h) for RSV in *L. striatellus* (46).

The genome of RSV consists of four RNA segments encoding seven proteins (47, 48). As a persistent infected plant arbovirus in planthopper, RSV and *L. striatellus* were used as an excellent model for studying the intricate interaction between the plant virus and insect vector. Previous studies have revealed that several antiviral immune pathways are involved in *L. striatellus* against plant virus infection. Abundant virus-derived small interfering RNAs were detected in *L. striatellus* during infection with RSV and Rice black-streaked dwarf virus (RBSDV), suggesting activation of the sRNA-mediated RNAi response in host insects (49). Moreover, activation of the c-Jun N-terminal kinase (JNK) promotes the replication and proliferation of RSV (50), whereas silencing of *Atg8* inhibits the phosphorylation of the JNK protein in *L. striatellus* (51). In addition, RSV inhibits the activity of the PPO pathway to maintain persistent proliferation in insect hemolymph (52), and proteins in the importin α family of *L. striatellus* interact with the nucleocapsid protein (NP) of RSV on the cell membrane, thus promoting RSV transmission (5). A recent report indicated that the JAK-STAT pathway contributes to persistent RSV infection by activating apoptosis in *L. striatellus* (53). Although our previous study indicated that the Toll pathway can be activated by RSV infection through the interaction between the Toll receptor and viral NP (54), the specific details of the downstream antiviral response responsible for maintaining the persistent viral transmission remain to be explored.

In this study, we revealed that the transcription factor Dorsal was actively involved in the Toll pathway of *L. striatellus* by regulating the target *zinc finger protein 708* (*LsZN708*) gene, which mediates the downstream antiviral response against RSV infection. In contrast, a counterdefense strategy was employed by RSV, in which the nonstructural protein (NS4) of the virus antagonizes host antiviral defense through competitively binding to Dorsal from the MSK2 kinase and inhibiting Dorsal phosphorylation, thus hindering the antiviral response mediated by the Toll pathway. Our results reveal the molecular mechanism by which the Toll pathway helps to maintain arbovirus homeostasis in the insect vector, which facilitates arbovirus transmission in the field.

## Results

***Dorsal* Knockdown Promotes RSV Replication and Transmission in *L. striatellus*.** The *Dorsal* gene in the Toll pathway of insects plays a crucial role in immune response. Three functional domains of Dorsal in *L. striatellus* were predicted (RHD-n, RHD_dimer, and AidA) with NCBI conserved domain database. To elucidate the function of LsDorsal in RSV infection, RSV-free *L. striatellus* was injected with RSV crude extracts (derived from infected planthoppers), and the injected *L. striatellus* were then transferred to healthy rice seedlings to detect *LsDorsal* transcripts as illustrated in Fig. 1*A*. The results showed that the transcript level of *LsDorsal* significantly increased at 2, 4, and 8 days post injection (dpi), whereas a decrease in *LsDorsal* transcripts was observed at 6 dpi compared to that in the controls (Fig. 1*B*). Next, a mixture of ds*LsDorsal* and RSV crude extracts was injected into RSV-free *L. striatellus* (ds*GFP* and RSV crude extracts used as control). The relative transcript levels of *RSV-NP* were significantly increased at 3 and 6 dpi compared to control (Fig. 1*C*), and the increased viral abundance was further confirmed by measuring the protein levels of RSV-NP at 6 dpi (Fig. 1*D*). Moreover, RSV acquisition ratio was determined at 3 d after RSV-free *L. striatellus* was injected with the mixture of ds*LsDorsal* and RSV crude extracts. The results indicated that RSV acquisition ratio was increased in ds*LsDorsal* treated planthoppers (52.5%) compared to ds*GFP* control (35.0%). Subsequently, RSV transmission experiment was performed using the injected (ds*LsDorsal* and RSV crude extracts) instar nymph planthoppers and viral disease symptom was recorded for each of the single rice seedling 15 d after innoculation. Our results showed that the RSV transmission ratio also increased from 7.5% (ds*GFP*) to 20.0% (ds*LsDorsal*) for *L. striatellus* (Fig. 1*E*). Consistently, immunofluorescence analysis conducted using dissected SG and ovaries of *L. striatellus* (6 dpi) exhibited notably increased fluorescence signals of RSV-NP in

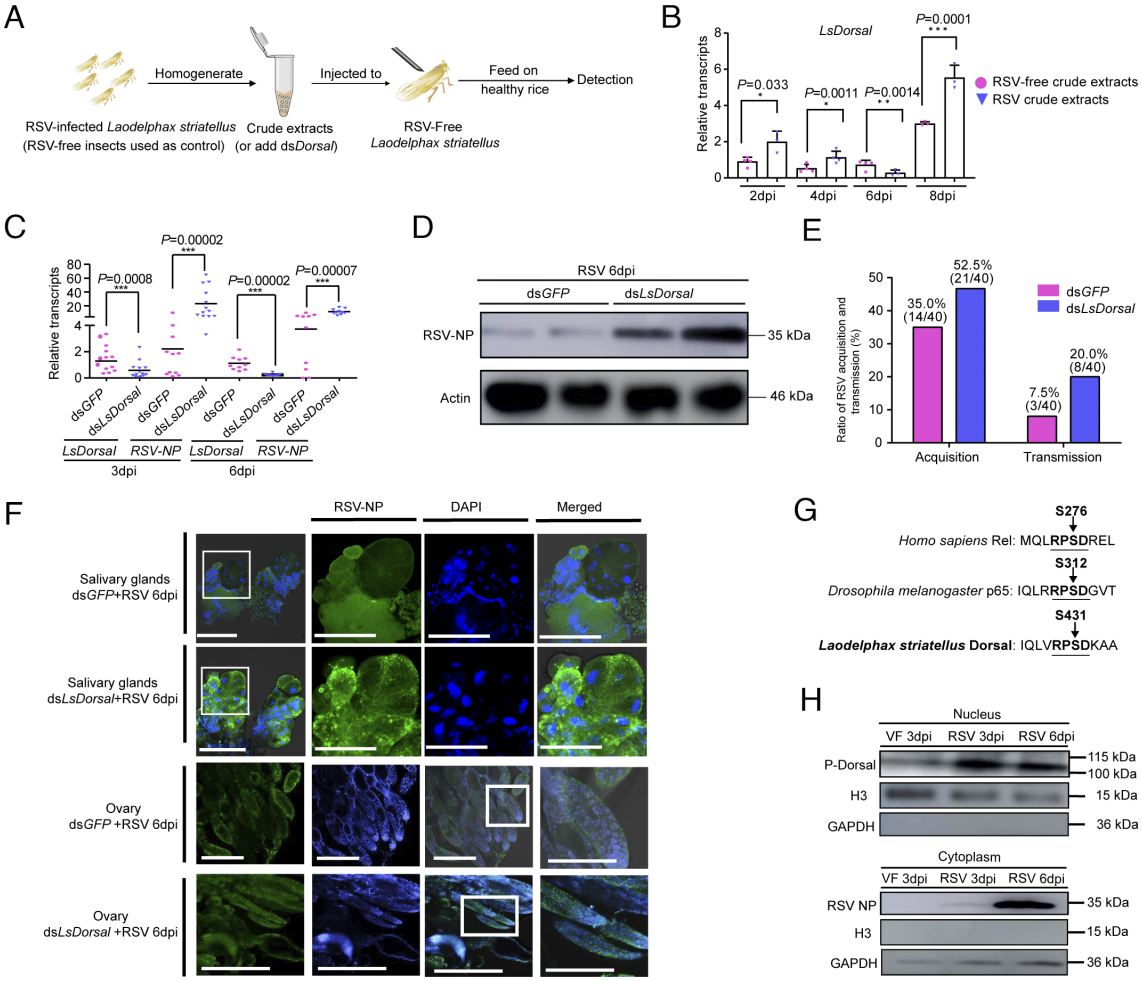


**Fig. 1.** *LsDorsal* knockdown promotes RSV replication and transmission in *L. striatellus*. (*A* and *B*) Detection for the transcript levels of *LsDorsal* injected with crude extracts of RSV-free and RSV-infected *L. striatellus* at various time points. (*C*–*E*) Effects of *LsDorsal* knockdown on the expression levels of RSV-NP in *L. striatellus* treated with ds*LsDorsal* and RSV crude extracts for transcripts at 3 and 6 dpi (*C*), protein at 6 dpi (*D*), and ratio of virus acquisition (3 dpi) and transmission (*E*). (*F*) Immunofluorescence staining of RSV NP in the SG and ovary of *L. striatellus* at 6 dpi after treatment with ds*GFP* or ds*LsDorsal* and RSV crude extracts. (Scale bar, 50 μm.) (*G*) Identification of Dorsal phosphorylation site in *L. striatellus* based on its homologous to Rel (*Homo sapiens*) and p65 (*Drosophila melanogaster*). (*H*) Phosphorylation level of Dorsal protein in the nucleus of *L. striatellus* at different time points after RSV infection. "VF 3 dpi" indicated that nonviruliferous *L. striatellus* were injected with RSV-free insect crude extracts for 3 d. "RSV 3 dpi" and "RSV 6 dpi" indicated that nonviruliferous *L. striatellus* were injected with RSV-infected insect crude extracts for 3 d and 6 d, respectively. H3 and GAPDH antibodies represent specific marker of the cell nucleus and cytoplasm, respectively. Three biological replicates were performed for each of the experiment (10 to 15 *L. striatellus* for each of the replicate). The *t* test method was used for significance analysis. * represents significant difference ($P < 0.05$), ** and *** represent extremely significant difference ($P < 0.01$ and $P < 0.001$). The error bars represent the SE of the mean.

both tissues of planthoppers treated with ds*LsDorsal* compared to that in the control (Fig. 1*F*). These results suggest that LsDorsal might play an essential role in inhibiting RSV replication and transmission in *L. striatellus*.

The phosphorylation and nuclear translocation of Dorsal are key steps in the activation of the Toll signaling pathway (55). To ascertain the conserved phosphorylated sites of Dorsal in *L. striatellus*, the predicated amino acid sequence of Dorsal in *L. striatellus* (LsDorsal), p65 in *Drosophila melanogaster* (DmDorsal), and Rel in *Homo sapiens* (HsDorsal) were aligned. The results indicated that the three proteins, LsDorsal, DmDorsal, and HsDorsal, possess "RPSD" phosphorylation sites, located at S431, S312, and S276, respectively (Fig. 1*G*). Subsequently, the phosphorylation level of LsDorsal protein (p-Dorsal) was evaluated at 3 and 6 dpi following RSV infection in the nucleus of the planthopper cells. The results demonstrated that p-Dorsal levels increased significantly in the nucleus at 3 and 6 dpi, indicating that LsDorsal was phosphorylated and subsequently transported to the nucleus during RSV infection (Fig. 1*H*).

**LsDorsal Binds to the Promoter of the Zinc Finger Protein ZN708 and Regulates Its Expression.** Chromatin immunoprecipitation sequencing was performed to identify the candidate target genes that interacted with LsDorsal in the nucleus. The top eight genes (Zinc Finger Protein Z*N708*, autophagy-related gene *Atg13*, RAT Ras associated guanosine triphosphate binding protein *RRAGA*, Bispecic mitogen-activated protein kinase *DSOR1*, RNA binding protein 45 *RBM45*, synaptophysin *SNG*, Bystin protein *BYST*, and G-protein-coupled receptors *MTH*) were selected (based on the q-value cutoff) and their binding ability to LsDorsal was subsequently evaluated by ChIP–qPCR and yeast one-hybrid assays. The results demonstrated that LsDorsal bound specifically to the promoter region of *ZN708* in both RSV-free and RSV-infected *L. striatellus* compared to the control (IgG) (Fig. 2 *A* and *B*), which was widely present in diverse arthropods and mammals (*SI Appendix*, Fig. S1). Moreover, the binding strength of LsDorsal to *LsZN708* was significantly higher in viruliferous planthoppers compared to that in the nonviruliferous planthoppers (Fig. 2*C*). In addition, results of yeast one-hybrid assays showed that LsDorsal and its RHD-n

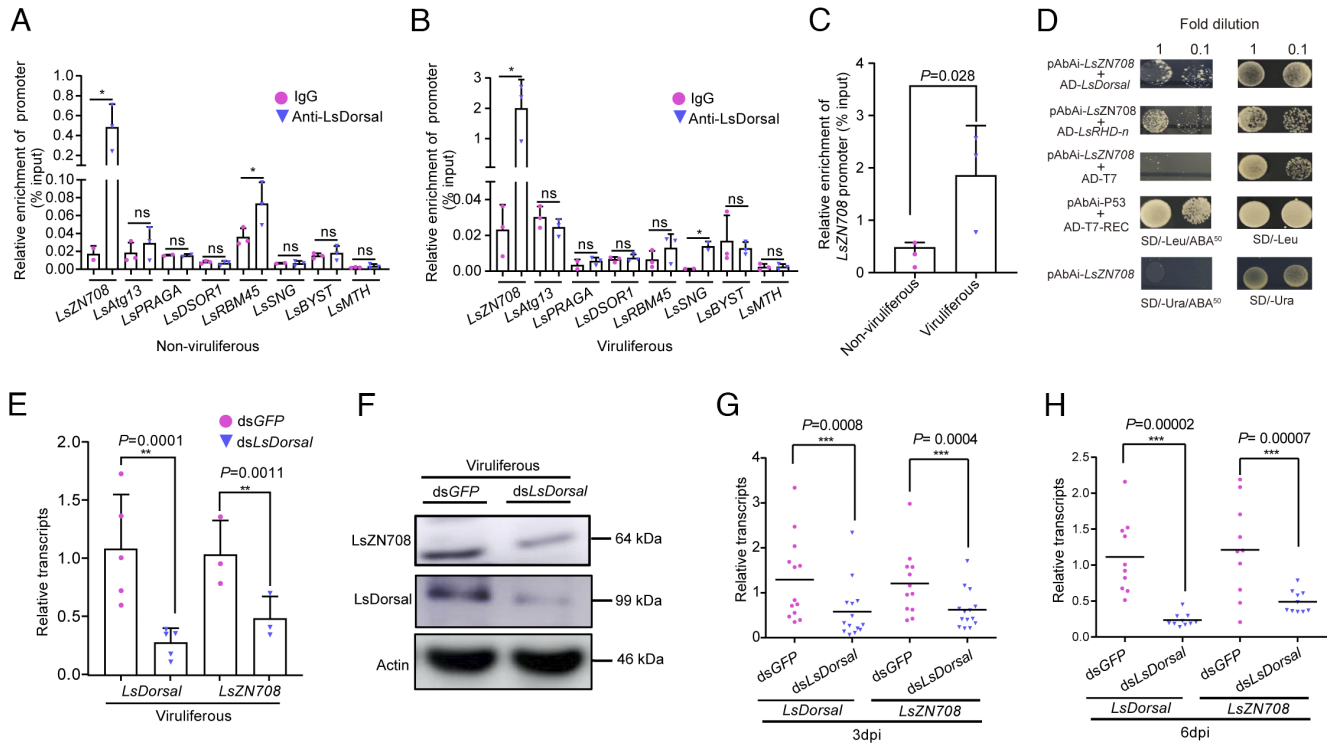


**Fig. 2.** LsDorsal binds to the promoter of the zinc finger protein LsZN708 and regulates its expression. (*A* and *B*) Evaluation for the binding ability of LsDorsal to the promoters of eight candidate genes in RSV-free (*A*) and RSV-infected *L. striatellus* (*B*) by Chip-qPCR. (*C*) Comparative analysis for the LsDorsal binding ability to the *LsZN708* promoter in nonviruliferous and viruliferous *L. striatellus*. (*D*) Verification for the binding of LsDorsal to the promoter of the Zinc Finger Protein LsZN708 using yeast one-hybrid assay. pAbAi-*LsZN708* indicated that the promoter of *LsZN708* was constructed to pAbAi vector. AD-*LsDorsal* and AD-*RHD-n* suggested that the full length of genes was constructed to pGAD-T7 vector. pAbAi-*P53* and AD-T7-*REC* represented a group of positive control. pAbAi-*LsZN708* and AD-T7 represented a group of negative control. ABA screening concentration was set at 50 ng/μL (inhibiting self-activation). The self-activation assay was performed on selective medium SD/-Ura. The different combinations of constructs transformed into yeast cells were grown on selective medium SD/-Leu, and interactions were detected on SD/-Leu/-ABA (50). Pictures were taken after 3 d of incubation at 30 °C. IgG antibody was used as a negative control, and three biological replicates were performed. (*E* and *F*) Effects of *LsDorsal* knockdown on the expression levels of transcript (*E*) and protein (*F*) of LsZN708 in the viruliferous *L. striatellus* injected with ds*LsDorsal*. (*G* and *H*) Effects of *LsDorsal* knockdown on the transcripts of *LsZN708* in nonviruliferous *L. striatellus* treated with ds*GFP* or ds*LsDorsal* and RSV crude extracts at 3 and 6 dpi, respectively. Three biological replicates were performed for each experiment. The *t* test method was used for significance analysis. * represents significant difference ($P < 0.05$), ** and *** represent extremely significant difference ($P < 0.01$ and $P < 0.001$), n.s. means no significance. The error bars represent the SE of the mean.

domain (conserved functional domain of LsDorsal) only interacted with the promoter region of *LsZN708* (Fig. 2*D*) and did not interact with the other seven candidate genes (*SI Appendix*, Fig. S2). Furthermore, knockdown of *LsDorsal* in viruliferous *L. striatellus* resulted in the significantly reduced LsZN708 expression at both the transcription (Fig. 2*E*) and protein (Fig. 2*F*) level, suggesting that the expression of LsZN708 might be regulated directly by LsDorsal. Consistently, when nonviruliferous planthoppers were injected with a mixture of ds*LsDorsal* and RSV crude extracts, *LsZN708* expression was also significantly downregulated compared to the control (ds*GFP*+RSV) at 3 (Fig. 2*G*) and 6 dpi (Fig. 2*H*), confirming that LsDorsal promotes the expression of LsZN708.

***ZN708* Knockdown Promotes RSV Replication and Transmission in *L. striatellus*.** Previous studies have demonstrated that zinc finger protein are actively involved in the host antiviral process in response to various viruses (56–60). In our study, we showed that the relative transcript level of *LsZN708* was significantly higher in viruliferous *L. striatellus* compared to that in nonviruliferous *L. striatellus* (Fig. 3*A*). Further analysis indicated that knocking down *LsZN708* effectively increased the transcription of *RSV-NP* in viruliferous planthoppers at 3 and 6 dpi (Fig. 3 *B* and *C*). Moreover, when nonviruliferous *L. striatellus* were treated with a mixture of ds*LsZN708* and RSV crude extracts, the relative transcription of *RSV-NP* was significantly higher at 3 and 6 dpi compared to the control (treated with a mixture of ds*GFP* and RSV crude extracts) (Fig. 3*D*). This result was further confirmed by measuring the protein level of RSV-NP, which was obviously increased compared to that in the control at 6 dpi (Fig. 3*E*).

Similarly, the effects of *LsZN708* knockdown on RSV acquisition and transmission in *L. striatellus* were also evaluated. The results showed that both the RSV acquisition ratio (43.3%) and transmission ratio (30.0%) were increased in ds*LsZN708* treated planthoppers compared to corresponding controls (ds*GFP*) (Fig. 3*F*). Additionally, immunofluorescence analysis of the dissected SG of *L. striatellus* (6 dpi) demonstrated increased RSV-NP fluorescence in the SG of *LsZN708* knockdown planthoppers (Fig. 3*G*). Similar to the function of LsDorsal, these results suggest that LsZN708 also inhibits RSV replication and transmission in *L. striatellus*.

**Regulation of Downstream Immune-Related Effectors by the Toll-Dorsal Pathway in the Antiviral Response of *L. striatellus* against RSV Infection.** To further elucidate the specific antiviral defense mechanism mediated by Dorsal-ZN708, differentially expressed genes (DEG) analysis was performed for the nonviruliferous planthoppers at 3 and 6 dpi following injection with ds*LsDorsal* or ds*LsZN708* and RSV crude extracts compared to the corresponding controls (a mixture of ds*GFP* and RSV crude extracts). The results revealed that 1,122 and 900 genes (225 common genes) were differentially expressed for ds*LsDorsal* and ds*LsZN708* treated planthoppers compared to that in the controls at 3 dpi, respectively (Fig. 4*A*). When these two genes were knockdown at 6 dpi, 3,504

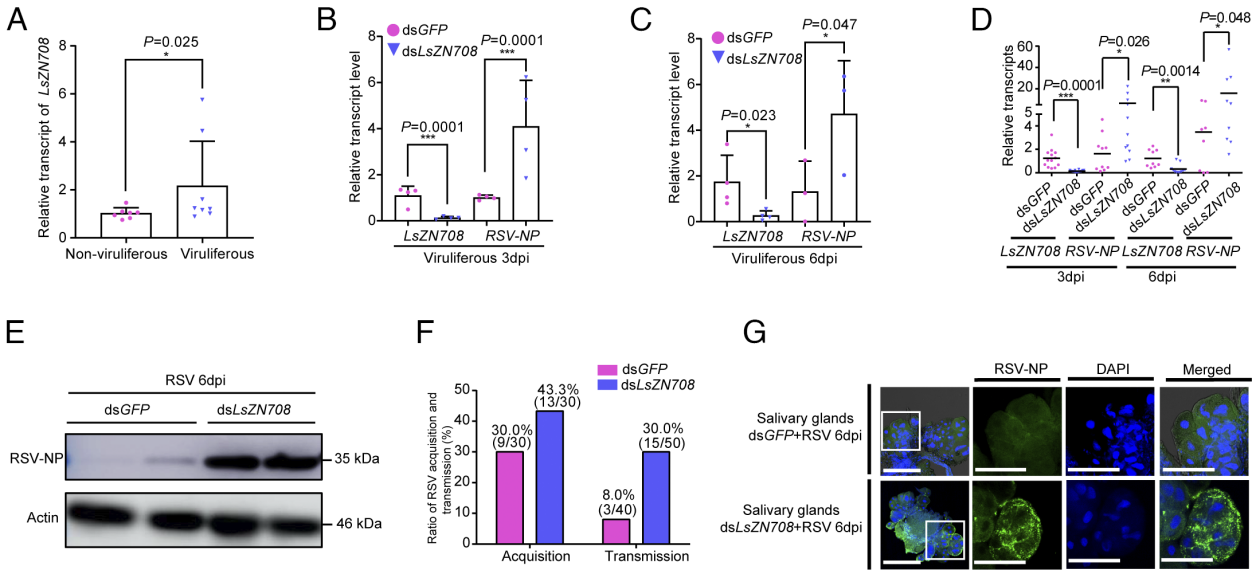


**Fig. 3.** *LsZN708* knockdown promotes RSV replication and transmission in *L. striatellus*. (*A*) Transcription level of *LsZN708* in nonviruliferous and viruliferous *L. striatellus*. (*B* and *C*) Relative transcription levels of *RSV-NP* in viruliferous *L. striatellus* treated with ds*GFP* or ds*LsZN708* at 3 dpi and 6 dpi, respectively. (*D–F*) Effects of ds*LsZN708* knockdown on the expression levels of RSV-NP in *L. striatellus* treated with ds*LsZN708* and RSV crude extracts for transcripts at 3 and 6 dpi (*D*), protein at 6 dpi (*E*), and ratio of virus acquisition (3 dpi) and transmission (*F*). (*G*) Immunofluorescence staining of RSV NP in the SG of *L. striatellus* treated with ds*LsZN708* or ds*LsDorsal* and RSV crude extracts at 6 dpi. The scale bar represents 50 μm. Three biological replicates were conducted for each experiment. Significance was determined using the *t* test method. * represents significant difference ($P < 0.05$), ** and *** represent extremely significant difference ($P < 0.01$ and $P < 0.001$). The error bars represent the SE of the mean.

and 1,792 differential expressed genes (675 common genes) (Fig. 4*B*) were observed for ds*LsDorsal* and ds*LsZN708* knockdown insects, respectively. KEGG pathway enrichment analysis of the common DEGs indicated that two immune-related pathways, autophagy, and exosome, were stably enriched in ds*LsDorsal* or ds*LsZN708* treated planthoppers at both 3 and 6 dpi (Fig. 4 *A* and *B*), implying that these two pathways might play essential roles in the antiviral response to RSV infection.

Previous studies have demonstrated that the zinc finger protein-mediated autophagy pathway is essential in resistance to different viruses; the key genes of autophagy, including *Atg3*, *Atg5*, *Atg8*, *Atg9*, *Atg12*, *ULK1*, and *Sqstm1*, are especially important (61–63). In our study, subsequent yeast two-hybrid (Y2H) indicated that planthopper LsZN708 interacts specifically with LsAtg8 and not with the other autophagy-related proteins (Fig. 4*C*). Furthermore, this interaction was verified by an in vitro pull-down assay. Primarily, recombinant His-Atg8 fusion protein and MBP beads with MBP-ZN708 protein coincubated overnight at 4 °C. Then the MBP beads were washed 5 to 6 times with corresponding buffer, and detected using His-tag antibody by western blot. MBP fusion protein and His-Atg8 fusion protein were used as a negative control (Fig. 4*D*). Moreover, knockdown of either *LsZN708* (Fig. 4 *E* and *F*) or *LsDorsal* (Fig. 4 *G* and *H*) resulted in significantly reduced transcription of *LsAtg8*, *LsAtg5*, and *LsAtg3* in *L. striatellus* at both 3 and 6 dpi, suggesting regulation of the autophagy pathway by Dorsal-ZN708. Importantly, inhibition of LsDorsal following treatment with a QNZ inhibitor resulted in a remarkable reduction in LsAtg8-II protein levels in RSV-infected *L. striatellus* (Fig. 4*I*).

To further investigate the function of autophagy in RSV infection, the level of *LsAtg8* transcripts was determined in *L. striatellus* challenged by RSV infection (injected with RSV crude extract). The results demonstrated that *LsAtg8* transcripts of the insects were significantly increased at 2, 4, and 8 dpi compared to the control samples (*SI Appendix*, Fig. S3*A*). Subsequently, RSV-infected *L. striatellus* were treated with 3-MA (an autophagy pathway inhibitor) or Rapamycin (an autophagy pathway activator) through membrane feeding for 24 h and viral accumulation levels were determined by RT-qPCR. The results indicated that the relative transcript levels of *RSV-NP* was significantly increased when planthoppers were treated with 3-MA (*SI Appendix*, Fig. S3*B*) whereas decreased transcripts of *RSV-NP* were observed with Rapamycin treatment (*SI Appendix*, Fig. S3*C*), implying the antiviral role of autophagy pathway in *L. striatellus*. Furthermore, the relative transcript levels of *RSV-NP* exhibited a significant increase when RSV-free *L. striatellus* was injected with a mixture of RSV crude extracts and ds*LsAtg8* at 3 and 6 dpi (*SI Appendix*, Fig. S3*D*) or ds*LsAtg3* at 6 dpi (*SI Appendix*, Fig. S3*E*) compared to the control (RSV crude extracts and ds*GFP*). Moreover, the elevated viral abundance was also confirmed by the protein levels of RSV-NP when treated with ds*LsAtg8* (*SI Appendix*, Fig. S3*G*) or ds*LsAtg3* (*SI Appendix*, Fig. S3*H*) at 6 dpi. Conversely, effective silencing of *LsSqstm1* (a negative regulator of the autophagy pathway) resulted in a significant decrease in the relative expression levels of RSV-NP transcripts (*SI Appendix*, Fig. S3*F*) and protein (*SI Appendix*, Fig. S3*I*). Additionally, increased or decreased protein levels of RSV-NP were observed when viruliferous *L. striatellus* was injected with ds*LsAtg3* or ds*LsSqstm1* compared to control (ds*GFP*) (*SI Appendix*, Fig. S3*J*), respectively, consolidating the above results. These results collectively suggest that autophagy functions as downstream antiviral effectors within the Toll pathway in planthoppers, which act against RSV infection.

It should be noted that significant enrichment of exosome pathway–related genes were observed in ds*LsDorsal* and ds*LsZN708* knockdown planthoppers (Fig. 4 *A* and *B*), and the antiviral roles of these genes against RSV were also evaluated. The results showed that the expression of these genes was significantly downregulated when nonviruliferous *L. striatellus* was treated with ds*LsDorsal* or ds*LsZN708* and RSV crude extracts (*SI Appendix*, Fig. S4 *A* and *B*). Interestingly, the protein level of RSV-NP was markedly decreased after the knockdown of exosome pathway–related genes (*SI Appendix*, Fig. S4*C*); this result was also verified at the transcriptional level

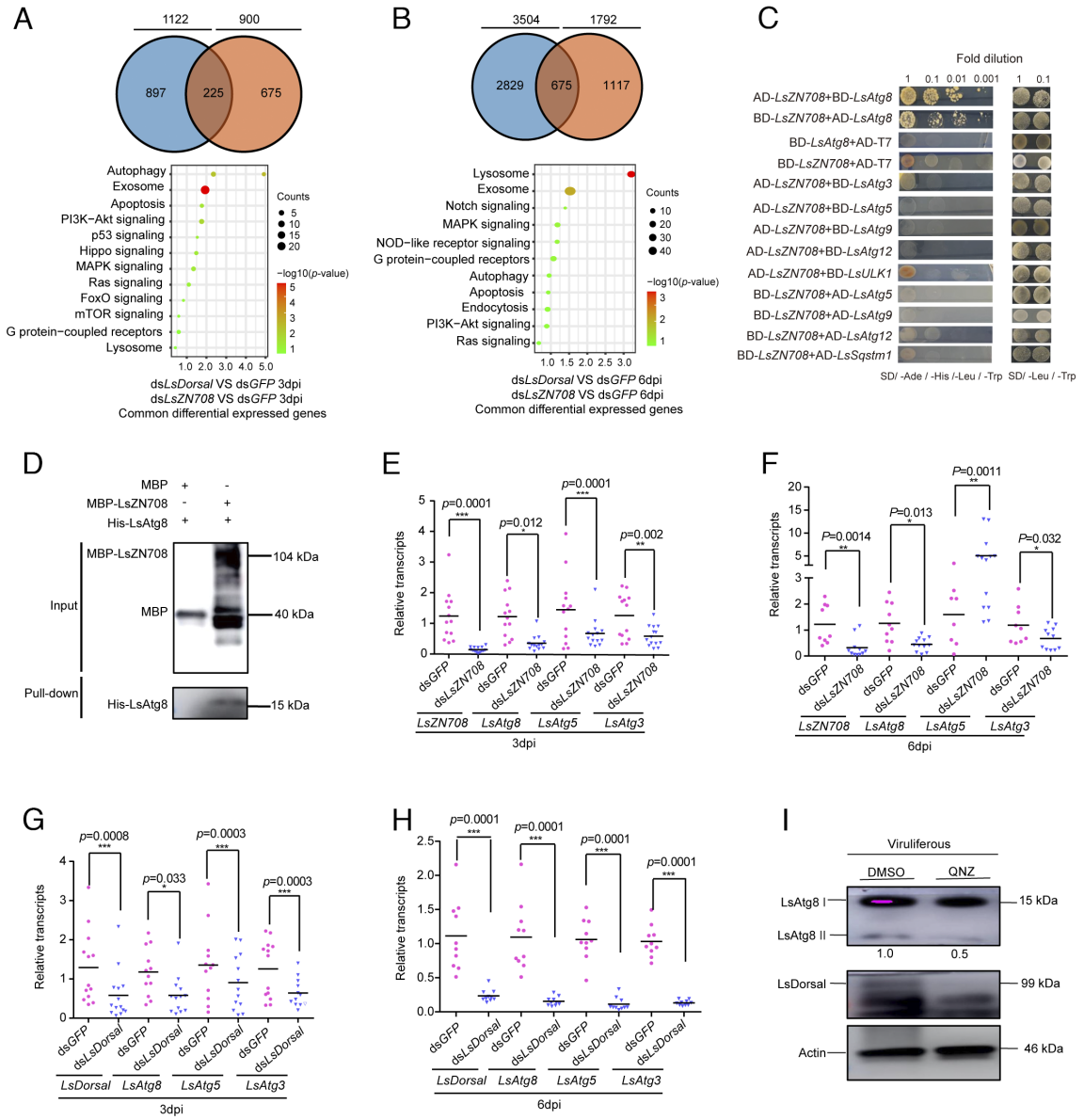


**Fig. 4.** Downstream immune-related effectors of toll pathway that potentially involved in the antiviral response of *L. striatellus* against RSV infection. (*A* and *B*) KEGG pathway enrichment analysis of the common DEG in nonviruliferous *L. striatellus* treated with a ds*LsDorsal* or ds*LsZN708* and RSV crude extracts at 3 dpi (*A*) and 6 dpi (*B*). Significant differences were indicated when $\log_2$ (fold change) ratio was ≥1 and $P \leq 0.05$. (*C*) The interaction between LsZN708 and autophagy protein LsAtg8 through Y2H assay. The different combinations of constructs transformed into yeast cells were grown on selective medium SD/-Leu/-Trp, and interactions were detected on SD/-Ade/-His/-Leu/-Trp. The images were taken after 3 d of incubation at 30 °C. (*D*) The interaction between LsZN708 and LsAtg8 protein through an in vitro pull-down assay. MBP-ZN708 fusion protein was used to pull-down with His-Atg8. MBP was used as negative control and His-Atg8 was further detected with anti-His antibody. (*E–H*) Effects of *LsZN708* or *LsDorsal* knockdown on the transcription levels of autophagy-related genes (*LsAtg8*, *LsAtg5* and *LsAtg3*) in nonviruliferous *L. striatellus* treated with ds*LsZN708* (*E* and *F*) or ds*LsDorsal* (*G* and *H*) and RSV crude extracts at 3 or 6 dpi. (*I*) Protein level of LsAtg8II in viruliferous *L. striatellus* treated with QNZ inhibitor for 48 h (DMSO was used as control). Three biological replicates were performed for each experiment. Significance analysis was performed using the *t* test method. * represents a significant difference ($P < 0.05$), ** and *** represent extremely significant differences ($P < 0.01$ and $P < 0.001$). The error bars represent the SE of the mean.

(*SI Appendix*, Fig. S4 *D–I*). Nevertheless, determining the precise roles of the exosome pathway in the regulation of Toll-Dorsal-ZN708 antiviral immunity still requires further investigation.

Additionally, knockdown of *LsToll*, an upstream receptor in the Toll pathway, notably decreased the transcript levels of *LsDorsal*, *LsZN708*, and *LsAtg8* in *L. striatellus* at both 3 and 6 dpi (*SI Appendix*, Fig. S5 *A* and *B*), concomitant with a significant increase of RSV accumulation at 6 dpi compared to that in the control (*SI Appendix*, Fig. S5*B*). These results consolidate our finding that the Dorsal-ZN708 mediated Toll pathway modulates the downstream antiviral response such as autophagy in *L. striatellus*. Furthermore, to evaluate the effects of RSV persistent infection on the survival of *L. striatellus*, dsRNA of *LsDorsal*, *LsZN708*, or *LsAtg8* mixed with the crude extracts of viruliferous planthoppers were injected into virus-free *L. striatellus* (the mixture of ds*GFP* and the crude extracts as the control). Survival rates were recorded everyday until 13 d post injection. The results indicated that there was no significant difference in the mortality of *L. striatellus* treated with the ds*LsDorsal* mixture (*SI Appendix*, Fig. S6*A*) and limited effects on the insect mortality were observed when treated with ds*LsZN708* (*SI Appendix*, Fig. S6*B*) or ds*LsAtg8* (*SI Appendix*, Fig. S6*C*) compared to ds*GFP*. These results suggest that these key genes of Toll pathway have limited effects on the insect susceptibility to RSV infection and the downstream genes (such as *LsAtg8*) exhibit higher efficacy against RSV.

**Non-Structural Protein of RSV (RSV NS4) Participates in the Viral Counterdefense Strategy by Inhibition LsDorsal Phosphorylation in *L. striatellus*.** To investigate potential interactions between RSV and LsDorsal, we utilized LsDorsal and its functional domains (RHD-n, RHD_dimer, and Aida) as bait to conduct a Y2H interaction assay with RSV-encoded proteins. The results revealed that, in contrast to the other RSV proteins (*SI Appendix*, Fig. S7), only NS4 interacted with LsDorsal through its specific RHD-n domain (301 to 339 aa) (Fig. 5*A*). Bimolecular fluorescence complementation (BiFC) assays were conducted to verify this interaction in tobacco cells. There were strong Yellow fluorescent protein (YFP) fluorescence signals in the nucleus and cytoplasm when nYFP-*LsDorsal* and cYFP-*NS4* or cYFP-*LsDorsal* and nYFP-*NS4* were transiently coexpressed in *Nicotiana benthamiana* leaves, whereas no detectable signal was observed in the negative control (nYFP-*LsDorsal* and cYFP) (Fig. 5*B*). In addition, the interaction was further confirmed by an in vitro pull-down assay. Recombinant His-NS4 fusion protein and MBP beads with MBP-Dorsal or RHD-n protein were coincubated overnight at 4 °C. MBP fusion protein and His-NS4 fusion protein were used as negative control (Fig. 5*C*). Moreover, a coimmunoprecipitation (Co-IP) assay was performed to verify the interaction between LsDorsal and NS4 in vivo in *L. striatellus*. Crude extracts of *L. striatellus* were incubated with ProteinG-NS4 and the coimmunoprecipitated proteins were identified using Dorsal antibody (Fig. 5*D*). These results conclusively confirm the interaction between LsDorsal and RSV NS4 in *L. striatellus*.

As the phosphorylation of Dorsal is a key regulatory step in the Toll antiviral pathway, it's interesting to investigate whether RSV NS4 can affect the phosphorylation of LsDorsal and thus interfere with the host subsequent antiviral response activated by LsToll. The results showed that knocking down RSV *NS4* in viruliferous *L. striatellus* resulted in an increase in both the transcript and phosphorylation levels of LsDorsal (Fig. 5 *E* and *F*). Conversely, the LsDorsal phosphorylation was clearly decreased in nonviruliferous *L. striatellus* when injected with purified NS4 protein of RSV compared to that in the GFP control (Fig. 5*G*). Additionally, the phosphorylation level of LsDorsal was considerably increased at 5 dpi following injection with ds*NS4* and RSV crude extracts in nonviruliferous *L. striatellus* (Fig. 5*H*). Notably, when *NS4* was knocked down in viruliferous *L. striatellus*, more p-Dorsal protein translocated into the nucleus compared to the cytoplasm, suggesting that NS4 could inhibit the translocation of p-Dorsal (Fig. 5*I*). Moreover, similar to the effects of LsDorsal, knocking down RSV *NS4* in

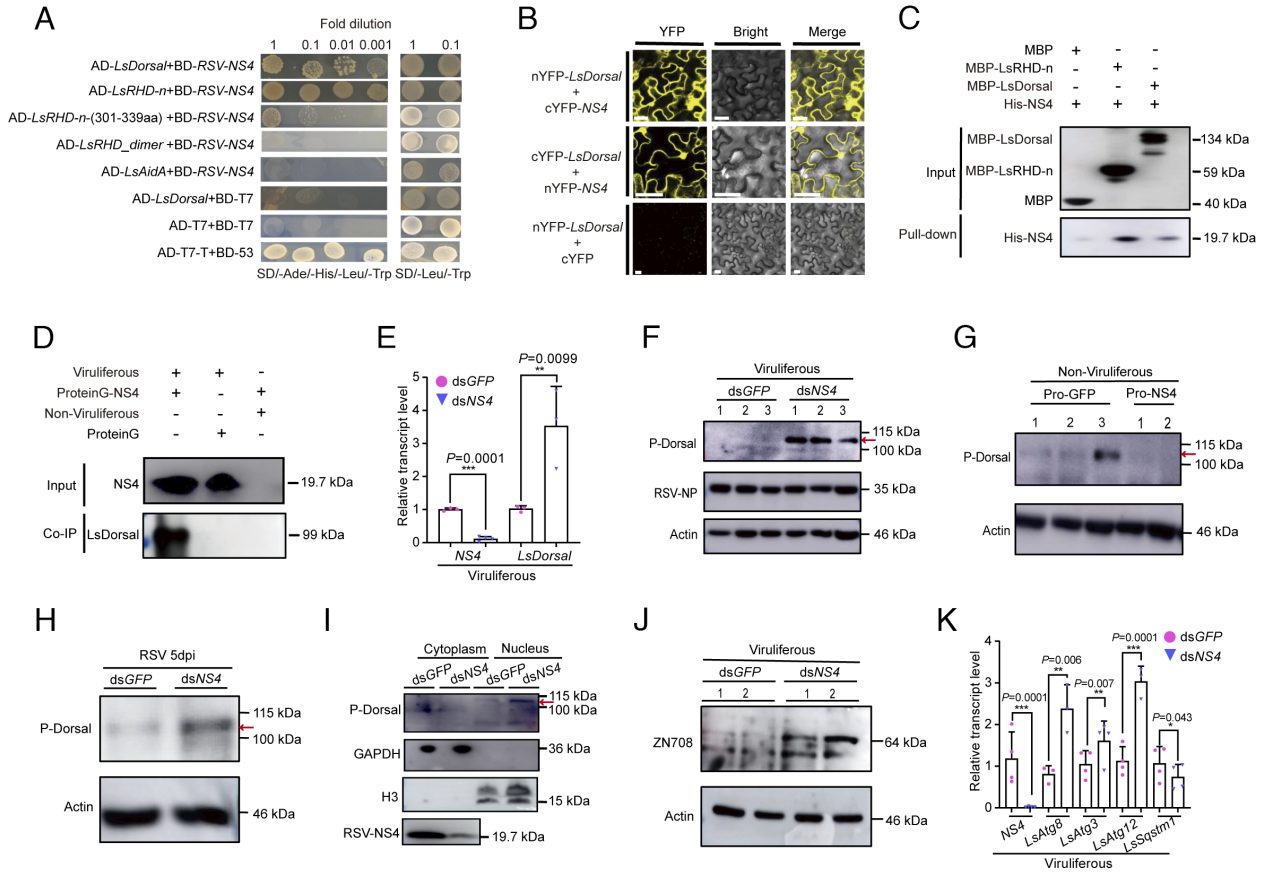


**Fig. 5.** Nonstructural protein NS4 of RSV (RSV NS4) participates in the viral counterdefense strategy through the inhibition of LsDorsal phosphorylation. (*A*) Interaction of RSV NS4 with LsDorsal or RHD-n domain (301 to 339 aa) of LsDorsal was confirmed by Y2H assay. (*B*) BIFC assays verified the interaction between LsDorsal and NS4 in the cell nucleus and cytoplasm of *Nicotiana benthamiana* leaves. (*C*) The interaction between LsDorsal and NS4 was confirmed by an in vitro pull-down assay. MBP-Dorsal and MBP-RHD-n proteins were used to pull-down with His-NS4. His-NS4 was further detected with anti-His antibody. (*D*) The Co-IP assay confirmed the interaction between LsDorsal and NS4 of *L. striatellus* in vivo. The crude extracts of *L. striatellus* were prepared and immunoprecipitated by ProteinG-NS4 combinations. The coimmunoprecipitated proteins were detected with LsDorsal antibody. (*E* and *F*) Effect of *NS4* knockdown on the transcription (*E*) and phosphorylation (*F*) levels of LsDorsal in viruliferous *L. striatellus* (ds*GFP* as control). (*G*) Phosphorylation level of LsDorsal in nonviruliferous *L. striatellus* treated with purified NS4 protein (GFP protein as control). (*H*) Phosphorylation level of LsDorsal in nonviruliferous *L. striatellus* treated with ds*NS4* and RSV crude extracts at 5 dpi. (*I*–*K*) Effect of *NS4* knockdown on the protein level of p-Dorsal in the nucleus and cytoplasm (*I*), the protein level of LsZN708 (*J*), and relative transcription levels of autophagy genes *LsAtg8*, *LsAtg3*, *LsAtg12* and *LsSqstm1* (*K*) in viruliferous *L. striatellus*. Three biological replicates were performed for each experiment. The *t* test method was used for significance analysis. * represents significant difference ($P < 0.05$), * and *** represent extremely significant difference ($P < 0.01$ and $P < 0.001$), n.s. means no significance. The error bars represent the SE of the mean.

viruliferous *L. striatellus* led to a significant increase in the level of LsZN708 protein (Fig. 5*J*) and the transcription of autophagy-related genes (*LsAtg8*, *LsAtg3* and *LsAtg12*) (Fig. 5*K*) compared to that in the ds*GFP* control; however, the expression for the negative regulator gene of autophagy (*LsSqstm1*) was significantly downregulated (Fig. 5*K*). In addition, *NS4* knockdown also resulted in a significantly decreased viral loads of RSV at both transcription and protein levels in viruliferous *L. striatellus* (*SI Appendix*, Fig. S8). These results provided evidence that NS4 is actively involved in the counterdefense mechanism of RSV by inhibiting the phosphorylation of LsDorsal, thereby regulating the expression of LsZN708 and the downstream immune-related effectors.

**RSV NS4 Competes with LsMSK2 for Dorsal Binding.** Previous studies have indicated that p65 phosphorylation can be directly targeted to the S276 site by kinases such as Mitogen- and stress-activated protein kinase-1 (MSK1), MSK2, and PKAc in mammals (64–66). In this study, MSK2 of *L. striatellus* (LsMSK2) was identified and the interaction between the C-terminus of LsMSK2 (C-terminus) and LsDorsal (RHD-n domain) was confirmed by Y2H assay (Fig. 6*A*). The interaction was further confirmed by an in vitro pull-down assay. Recombinant His-LsMSK2-C fusion protein and GST beads with GST-LsRHD-n protein were coincubated overnight at 4 °C. GST fusion protein and His-LsMSK2-C fusion protein were used as negative control (Fig. 6*B*). In addition, in vitro phosphorylation experiments showed that, with increasing levels of LsDorsal protein, the level of phosphorylation of LsDorsal protein by LsMSK2 increased steadily (Fig. 6*C*). To further investigate the binding of NS4 and LsMSK2 to LsDorsal, a competitive assay was performed. The result showed that increased concentrations of MBP-NS4 protein led to a decrease in binding to LsRHD-n and His-MSK2-C (Fig. 6*D*). However, the concentration of MBP-NS4 bound to LsRHD-n did not significantly change with increasing concentrations of His-MSK2-C protein (Fig. 6*E*). Furthermore, knockdown of *LsMSK2* in viruliferous *L. striatellus* resulted in a significantly increase in the transcription of *RSV-NP* (Fig. 6*F*). Similarly, nonviruliferous *L. striatellus* injected with ds*LsMSK2* and RSV crude extracts also had a significantly increased level of *RSV-NP* transcripts at 6 dpi (Fig. 6*G*). These findings suggest that the RSV NS4 protein competes with LsMSK2 for binding to LsDorsal, thereby inhibiting the phosphorylation of LsDorsal, and contributes to the counterdefense and persistent RSV infection in host insects.

**The Broad-Spectrum Antiviral Roles of the Toll Pathway against other Rice Viruses in Planthoppers.** To further investigate the potential antiviral role of the canonical Toll pathway in other arboviruses and insect vectors, the combination of *L. striatellus* and RBSDV, as well as another rice planthopper species (*Nilaparvata lugens*) and its transmitted virus (Rice ragged stunt virus, RRSV), were evaluated in this study. The results indicated that the LsRHD-n domain of LsDorsal can also interact with the P10 protein of RBSDV in *L. striatellus*, as evidenced by the Y2H assay (*SI Appendix*, Fig. S9*A*). In addition, the interaction was verified by an in vitro pull-down assay which included recombinant GST-RHD-n and His-RBSDV-P10 fusion protein. The combination of GST and His-RBSDV-P10 fusion protein was used as the negative control (*SI Appendix*, Fig. S9*B*). Additionally, the relative expression of *LsDorsal* was significantly upregulated when nonviruliferous *L. striatellus* was injected with RBSDV crude extracts at 4 dpi (*SI Appendix*, Fig. S9*C*). Similar to the results of RSV, after nonviruliferous insects were treated with ds*LsDorsal* and RBSDV crude extracts at 6 dpi, the relative transcription of *RBSDV-P10* increased significantly (*SI Appendix*, Fig. S9*D*), whereas a significant decrease in the level of *LsZN708* transcripts was observed in *L. striatellus* (*SI Appendix*, Fig. S9*E*). Notably, various expression levels of autophagy-related genes were observed after treatment, with decreased level of *LsAtg5*, increased level of *LsAtg8*, and no significant change for *LsAtg3* (*SI Appendix*,

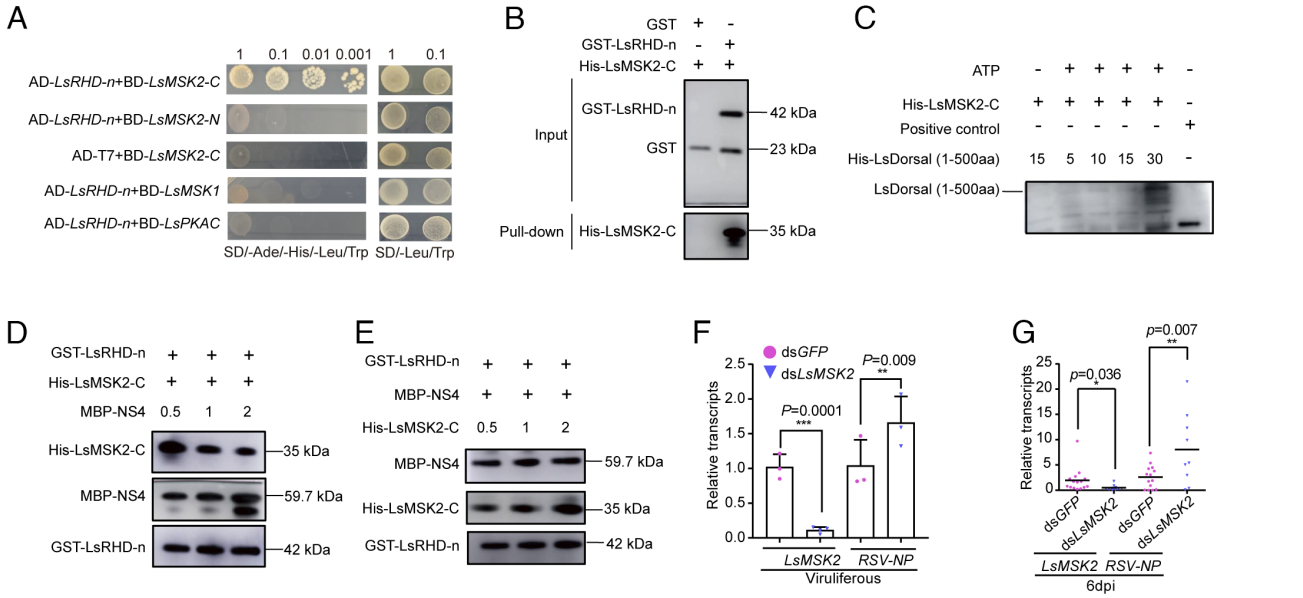


**Fig. 6.** RSV NS4 competes with LsMSK2 for LsDorsal binding. (*A*) Interaction between RHD-n domain of LsDorsal and the C-terminus of LsMSK2 kinase confirmed through Y2H assay. (*B*) An in vitro pull-down assay verified the interaction between LsRHD-n and C-terminus of LsMSK2. GST-RHD-n protein were used to pull-down with His-MSK2-C. His-MSK2-C was further detected with anti-His antibody. (*C*) The phosphorylation of LsDorsal protein by LsMSK2 kinase protein in vitro. (*D*) Binding ability of LsMSK2-C to LsRHD-n with the increasing concentration of NS4 protein. (*E*) Binding ability of NS4 to LsRHD-n with the increasing concentration of LsMSK2-C. (*F*) Effect of *LsMSK2* knockdown on the accumulation level of RSV in viruliferous *L. striatellus* treated with ds*GFP* and ds*LsMSK2*. (*G*) Effect of *LsMSK2* knockdown on the accumulation level of RSV in *L. striatellus* treated with ds*GFP* or ds*LsMSK2* and RSV crude extracts at 6 dpi. Three biological replicates were performed for each experiment. The *t* test method was used for significance analysis. * represents significant difference ($P < 0.05$), ** and *** represent extremely significant difference ($P < 0.01$ and $P < 0.001$). The error bars represent the SE of the mean.

Fig. S9*E*); this result implies that the downstream immune-related effectors of the Toll pathway might be diverse for *L. striatellus* following challenges by different RNA viruses.

Additionally, for the combination of *N. lugens* and RRSV, an interaction assay was conducted between RRSV proteins and Toll or Dorsal of *N. lugens*. Our results showed that only a specific region of NlToll, 96-891 aa, interacted with the P8 protein of RRSV in *N. lugens* (*SI Appendix*, Fig. S10*A*), but not for NlDorsal (*SI Appendix*, Fig. S10 *E–G*) or the other region of NlToll (*SI Appendix*, Fig. S10*D*). Furthermore, the relative transcript level of RRSV *P8* significantly increased in *N. lugens* injected with a mixture of ds*NlDorsal* or ds*NlToll* and RRSV crude extracts at 6 dpi compared to the ds*GFP* control (*SI Appendix*, Fig. S10 *B* and *C*), suggesting that RRSV P8 might also activate the antiviral Toll pathway in *N. lugens* by interacting with the NlToll protein.

## Discussion

Accumulating evidence has indicated that the innate Toll-Dorsal immune system plays a crucial role in resisting viral infections in invertebrates such as *Drosophila* and mosquitoes (3, 21–23, 67). Throughout evolution, arboviruses have evolved different antidefense strategies that target the innate immune system of the hosts to maintain persistent transmission (32, 33, 36). Nevertheless, the precise roles of the classical Toll immune pathway in sustaining the persistent and propagative transmission of arboviruses in insect vectors remains unclear. Our previous results showed that the Toll immune pathway was successfully activated and participated in the antiviral process by directly binding the RSV-NP to the LsToll receptor of *L. striatellus* (54). In the present study, as illustrated in the schematic diagram (Fig. 7), we revealed the molecular mechanism of the Toll-Dorsal pathway mediated homeostasis in the persistent transmission of a plant arbovirus in its insect vector. LsDorsal played a role in the antiviral defense of *L. striatellus* by regulating a new target gene, LsZN708, thus inducing the production of downstream immune response pathways against RSV infection, such as the autophagy and exosome pathways. In contrast, RSV also developed an antidefense strategy by using its NS4 protein to antagonize the host Toll antiviral defense by competitively binding LsDorsal with LsMSK2 kinase to inhibit LsDorsal phosphorylation (Fig. 7).

The transcription factor Dorsal is a key component in the Toll immune pathway, and Dorsal plays a critical role in resisting virus infections (21, 29, 67, 68). A previous study conducted in *L. Vannamei* suggested that knockdown of *Dorsal* resulted in a significant increase in the accumulation of WSSV (21). In *Drosophila*, mutation of Dorsal and Dif increased the accumulation of Drosophila C Virus in viral oral infection and led to a higher mortality rate in the host insect (67). In *A. albopictus*, knockdown of the transcription factor *Rel1* increased the accumulation of DEV-2 (69). Our results showed that the transcript level of *LsDorsal* increased significantly in nonviruliferous *L. striatellus* injected with RSV crude extracts at 2, 4, and 8 dpi (Fig. 1 *A* and *B*). In addition, knockdown of *LsDorsal* resulted in an increase in the accumulation of RSV and RSV acquisition and transmission in *L. striatellus* (Fig. 1 *C–H*). These findings confirmed that Dorsal in *L. striatellus* plays an essential role against RSV infection, which is consistent with the antiviral functions of other members in the NF-κB family (e.g., Dif, or Rel1) (67, 70). Interestingly, after 6 dpi, the transcript level of *LsDorsal* decreased significantly upon injection with RSV crude extracts (Fig. 1*B*); this response may be related to RSV counterdefense, which helps to maintain its proliferation in *L. striatellus*.

The Dorsal/REL regulate the transcription of AMPs by binding to the κB sites of their promoter regions in the nucleus, thereby inducing the production of various immune effectors. In *Drosophila*, the promoter activity of AMP genes can be activated by the Rel homology domain (RHD) of Dorsal, Dif, and Relish (71), while for *L. Vannamei*, Dorsal binds to the promoters of the AMP genes (*ALF1* and *LYZ1*) to regulate their transcription (21). Additionally, the relish of mosquito has been demonstrated to bind to the κB sites of promoters of AMP genes (72, 73). Whole genome analysis has been utilized to identify over 40 target genes of Dorsal that encode a wide range of cell signaling proteins and transcription factors (68). Interestingly, rather than the commonly reported AMP genes, our results revealed that Dorsal of *L. striatellus* bound to the promoter of a zinc finger protein, *LsZN708*, and increased its transcription and protein levels (Fig. 2). Moreover, the binding

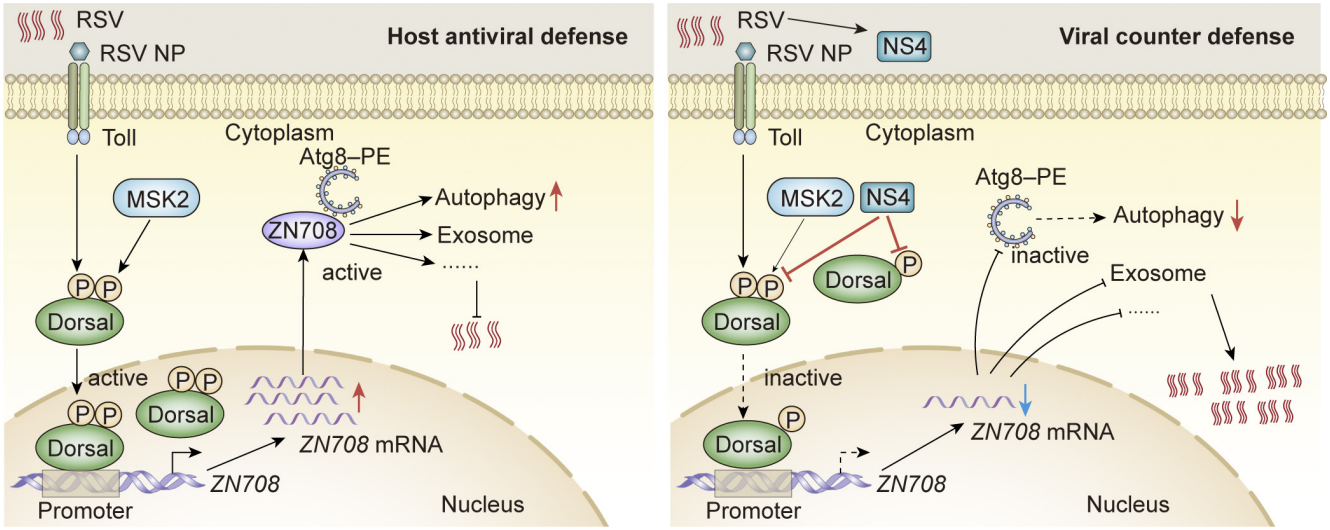


**Fig. 7.** The schematic diagram indicating toll-dorsal pathway mediated homeostasis for the persistent transmission of RSV in *L. striatellus*. The Toll pathway is activated by the interaction between the Toll receptor of *L. striatellus* and RSV NP. Then, Dorsal participated in the antiviral defense of *L. striatellus* by regulating the target gene *LsZN708* and induced the downstream immune response pathways (such as autophagy) against RSV infection (*Left*). Conversely, RSV also developed antidefense strategy by using its NS4 protein to antagonize the host Toll antiviral defense through competitively binding to LsDorsal with LsMSK2 kinase, resulting in the inhibition of LsDorsal phosphorylation and translocation to the nucleus (*Right*).

of LsDorsal to the promoter of *LsZN708* in viruliferous *L. striatellus* was significantly higher than that in nonviruliferous *L. striatellus* (Fig. 2*C*), and subsequent experiments indicated that the expression of LsZN708 might be regulated directly by Dorsal in *L. striatellus* (Fig. 2 *D–H*). These findings suggest that infection by different viruses might result in evolutionary variations in target effector genes of the Toll immune pathway, consequently leading to the binding of distinct promoter regions by Dorsal in the nucleus of host insects. The zinc finger antiviral proteins (ZAPs) are mammalian host restriction factors that inhibit the replication of various viruses through recruiting the exosome to degrade the substrate of viruses including alphaviruses (57, 59), filoviruses (58), retroviruses (56), orthohepadnaviruses (60). Consistently, knockdown of *LsZN708* led to a significant increase in the accumulation of RSV, as well as an increase in RSV acquisition and transmission in *L. striatellus* (Fig. 3), suggesting that *LsZN708* plays an important role against RSV infection.

In addition to the reported exosome that was recruited by ZAPs to degrade viral RNA (74), autophagy has also been demonstrated to be a downstream signaling pathway of the Toll immune system against viral infection. A previous study showed that MyD88-mediated autophagy can be induced by TLR7 to eliminate intracellular microbes (75). Furthermore, the accumulation of VSV was effectively inhibited through the autophagy pathway mediated by Toll7 in *Drosophila* (76). Interestingly, our KEGG analysis also showed that autophagy and exosome are stably enriched in *LsDorsal* and *LsZN708* knockdown *L. striatellus* (Fig. 4 *A* and *B*), and subsequent experiments suggested that autophagy act as an important downstream antiviral effector to limit the accumulation of RSV (Fig. 4 *C–F* and *SI Appendix*, Fig. S3); however, determining the roles of exosome in combating RSV infection will require further investigation (*SI Appendix*, Fig. S4). Moreover, a recent study has demonstrated that ZAPs regulate autophagy in cells and induce the degradation of viral RNA (61). Therefore, our results provided reliable evidence that Dorsal-ZN708 regulates immune effectors (i.e., autophagy) against RSV infection. The Dorsal-ZN708-autophagy mediated Toll antiviral pathway was also identified in *L. striatellus* infected with another plant virus, RBSDV (*SI Appendix*, Fig. S9), suggesting that this pathway might be a general antiviral mechanism in *L. striatellus*.

The coevolution of arboviruses and their hosts has led to the development of various viral counterdefense strategies and some viral proteins, such as VSRs, are used to inhibit the antiviral mechanisms of the hosts, thereby allowing the maintenance of persistent infection. For example, a viral protein of Barley stripe mosaic virus disrupts vacuolar acidification and inhibits host autophagy degradation to facilitate infection in plants (77). Moreover, the JAK/STAT immune pathway regulates the balance between whiteflies and geminiviruses. The CP of TYLCV interacts with STAT to inhibit its nuclear translocation and promote accumulation and transmission of TYLCV (35). In our study, a RSV counterdefense strategy in *L. striatellus* involves competitive binding of the viral protein NS4 to host LsDorsal with LsMSK2 kinase to inhibit Dorsal phosphorylation (Figs. 5 and 6), thus the downstream antiviral response is inhibited. The phosphorylation of Dorsal plays a critical role in regulating the transcription of target genes in the nucleus (55). In mammals, the key phosphorylation sites for p65 are S276 (RPSD), S468, and S536 (66, 78, 79). In contrast, the highly conserved serine site S312 is essential for Dorsal phosphorylation in *Drosophila* (55). Furthermore, the serine sites of Dorsal also play a role in the antiviral mechanism of *L. Vannamei* (21). In this study, comparative analysis indicated that Dorsal of *L. striatellus* also has a conserved serine site of "RPSD" at S431 (Fig. 1*G*) and protein level of phosphorylated Dorsal increased following RSV challenge (Fig. 1*H*), implying the conserved antiviral function of LsDorsal at this site of "RPSD". Additionally, RSV NS4 interacted with LsDorsal and *NS4* knockdown resulted in a significant increase in the phosphorylation of LsDorsal in viruliferous *L. striatellus* (Fig. 5 *A–I*), thereby increasing the expression of LsZN708 and activating autophagy (Fig. 5 *J* and *K*); this result suggests that the Dorsal-ZN708 antiviral pathway in *L. striatellus* was hijacked by RSV through the viral protein NS4. Phosphokinases, such as MSK1 and MSK2, are involved in the phosphorylation of serine site S276 in the p65 of mammalian cells (80). In this study, LsMSK2 was successfully identified in *L. striatellus* and the interaction between LsMSK2 and LsDorsal was confirmed; however, LsDorsal was then competitively bound by RSV NS4 (Fig. 6 *A–E*), indicating that the phosphorylation of Dorsal may vary in response to different viruses within the hosts.

In summary, the mechanism underlying Toll mediated homeostasis to maintain a persistent and propagative transmitted plant arbovirus in *L. striatellus* was revealed in this study (Fig. 7). These results will contribute to a better understanding of the arms race between arboviruses and insect vectors during coevolution. However, further investigation is necessary to elucidate the specific roles of autophagy-related genes and to understand the collaboration between autophagy and other downstream immune effectors (such as exosomes) in sustaining persistent infections of arboviruses in insect vectors.

## Materials and Methods

A full description of materials and methods, including *RSV-Infected and RSV-Free Insect Materials*, *dsRNA Microinjection*, *qRT-PCR*, *ChIP-qPCR*, *Immunohistochemistry*, *Y2H, Antibody Preparation*, *Pull-Down*, *Western Blotting*, *Co-IP*, *BiFC*, and *RSV Acquisition/Transmission*, is provided in *SI Appendix, Supplemental Materials and Methods*. Primers used in this study are listed in *SI Appendix*, Table S1.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*.

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