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RESEARCH ARTICLE

Rice stripe virus protein NS3 exploits synergistically insect vector importin and ubiquitin systems to promote viral replication

Lu Zhang[1], Ze Qu[1], Yihui Tan[1], Yao Li[1], Xinyi Li[1], Zhipeng Huang[1], Siyuan Ruan[1], Shimin Zuo[2], Fang Liu[1, 2, 3#], Wenxing Hu[1#]

[1] *College of Plant Protection, Yangzhou University, Yangzhou 225009, China*
[2] *Jiangsu Co-Innovation Center for Modern Production Technology of Grain Crops, Yangzhou University, Yangzhou 225009, China*
[3] *Key Laboratory of Plant Functional Genomics of the Ministry of Education, Yangzhou University, Yangzhou 225009, China*

Highlights

- RSV infection activates genes involved in both the non-classical and classical import pathways in the insect vector SBPH.
- A member of the importin β family of SBPH, importin 5, mediates nuclear entry of RSV protein NS3.
- The ubiquitin system interacts synergistically with the nuclear import pathway to facilitate RSV NS3 nuclear entry and viral replication in SBPH.

Abstract

Plant viruses pose significant threats to agriculture, with many vectored by insect pests. The entry of viruses and their encoded proteins into the host nucleus is a critical step for promoting some viral replication and enabling systemic infection. *Laodelphax striatellus*, also known as the small brown planthopper (SBPH), is an efficient vector for rice stripe virus (RSV), one of the most damaging viruses of rice. In this study, we demonstrate that RSV infection induces the expression of genes in both the classical and non-classical nuclear import pathways of SBPH. A gene belonging to the importin β family, *importin 5* (*LsIPO5*), was upregulated by 84% in SBPH midguts infected with RSV. The nuclear localization signal (NLS, ^{168}YRSPSKKRHKYV^{179}) is located within the nonstructural protein NS3 directly bound to LsIPO5, thereby facilitating NS3 nuclear entry. Moreover, a RING-type E3 ligase (LsRING) in SBPH, which mediated the ubiquitination of NS3 in the insect vector, enhanced NS3 binding to LsIPO5 and facilitated NS3 perinuclear localization. Combined treatment of SBPH with both ds*IPO5* and ds*RING* significantly reduced RSV loads, highlighting the importance of LsIPO5 and NS3 ubiquitination cooperation in facilitating viral replication. Our findings provide new insights into synergistic molecular mechanisms that govern RSV infection and suggest potential therapeutic targets to control viral transmission through their insect vectors.

Keywords: *Laodelphax striatellus*, rice stripe virus, cytoplasmic-nuclear trafficking, importin, ubiquitin

1. Introduction

Plant viruses are major threats to global agriculture and ecosystems (Tatineni and Hein 2023; Wu *et al.* 2024) and are often transmitted by insect vectors (Whitfield *et al.* 2015). Viruses are obligate intracellular parasites that can only replicate in host cells (Koonin *et al.* 2021), and the entry of arboviruses into the nucleus of insect cells is a crucial step for viral replication. Examples of arboviruses that enter the insect nucleus to replicate include maize mosaic virus (MMV), tomato yellow leaf curl virus (TYLCV), and sowthistle yellow vein virus (SYVV) (Richardson and Sylvester 1968; He *et al.* 2020; Xavier *et al.* 2024). Arboviruses that replicate in the insect vector cytoplasm include rice stripe virus (RSV) and rice black-streaked dwarf virus (RBSDV); these viruses are known to enter the insect cell nucleus for viral replication (Liu *et al.* 2007; Zhao *et al.* 2022). The mechanisms that enable plant viruses to enter the nuclei of the insect vector remain a topic of considerable importance.

The nuclear transport system is responsible for translocating macromolecules between the cytoplasm and the nucleus, thus ensuring the proper functioning of essential physiological activities (Wente and Rout 2010). Nuclear

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Lu Zhang, E-mail: Zhanglu93@yzu.edu.cn; [#]Correspondence Fang Liu, E-mail: liufang@yzu.edu.cn; Wenxing Hu, E-mail: wxh2100@163.com

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transport includes both classical and non-classical pathways; in the former, importin α (IMPα) interacts with cargo macromolecules that exhibit nuclear localization signals (NLS) and forms a heterodimer (IMPα/β1) with importin β1 (IMPα/β1) to modulate import into the nucleus. In contrast, the non-classical pathway directly binds importin β to cargo proteins, resulting in protein complexes that facilitate nuclear entry. Both pathways are crucial for viral replication and infection (Dohner *et al.* 2018; Martin and Jans 2021). Many viruses, including adenoviruses and herpesviruses, utilize the classical nuclear transport pathway to move their genomes or proteins from the host cytoplasm to the nucleus for replication and transcription (Cai *et al.* 2016; Dohner *et al.* 2018; Athukorala *et al.* 2024). Additionally, some viruses, such as porcine circovirus (PCV) and influenza A virus (IAV), hijack non-classical nuclear transport routes for viral replication (Meng *et al.* 2020; Lin *et al.* 2022). It is still not fully understood whether a virus relies on only one nuclear transport pathway (classical or non-classical), or if it may simultaneously utilize both pathways to mediate the virus' nuclear transport.

The ubiquitin-proteasome system (UPS) is widely present in eukaryotic cells and includes the ubiquitin-activating enzyme (E1), conjugating enzyme (E2), and ligase (E3) (Luo 2016). The UPS is the major system for degrading proteins (Nandi *et al.* 2006) and also has a role in the nuclear transport of viral particles. For example, small ubiquitin-like modifiers (SUMO) from the host modify the hepatitis B virus (HBV) core protein to achieve nuclear import of the viral genome (Hofmann *et al.* 2023). In Kaposi's sarcoma-associated herpesvirus (KSHV), the replication and transcription activator protein was polyubiquitinated by host cells, and this process controlled the nuclear localization of the virus (Luan *et al.* 2024). Furthermore, both bovine (BPV) and canine parvoviruses (CPV) utilize the ubiquitin–proteasome machinery for nuclear translocation (Ros and Kempf 2004). To date, a few studies have demonstrated an association between the nuclear import system and the UPS in viral nuclear entry. The monoubiquitination of the NLS motif present in the matrix protein of paramyxoviruses regulates its nucleocytoplasmic transport (Wang *et al.* 2010; Pentecost *et al.* 2015). However, it remains important to note that many aspects of the connection between the nuclear and the ubiquitin systems in viral transport to host nuclei have yet to be fully explored.

Most plant viruses depend on biotic organisms for transmission and survival, and insects often serve as vectors (Bragard *et al.* 2013). *Laodelphax striatellus*, commonly known as the small brown planthopper (SBPH), efficiently transmits RSV in rice using the persistent-propagative strategy (Falk and Tsai 1998). RSV encodes the following proteins: NSvc2 (putative membrane glycoprotein), RdRp (RNA-dependent RNA polymerase), NS3 (gene-silencing suppressor), NS2 (RNA silencing suppressor), NP (nucleocapsid protein), NSvc4 (movement protein) and SP (nonstructural disease-specific protein) (Zhao *et al.* 2016). RSV conducts replication and assembly in the cytoplasm of both rice and planthopper cells. However, recent studies have demonstrated that RSV NP and RSV genomic RNAs utilize the classical nuclear pathway to enter insect vector nuclei, activatnig an antiviral apoptosis pathway to inhibit RSV replication (Zhao *et al.* 2022). We previously demonstrated that infection of SBPH with RSV results in polyubiquitylation of NS3 by the RING ubiquitin ligase. Ubiquitylation promotes NS3 translocation from the cytoplasm to the nucleus, leading to RSV replication (Zhang *et al.* 2024). We speculate that RSV may also hijack other pathways for viral nuclear entry. It remains unclear whether the nuclear entry of NS3 is associated with import pathways in SBPH and whether this process operates collaboratively or independently of ubiquitin-mediated NS3 nuclear entry.

This study demonstrates that RSV infection activates both non-classical and classical pathways for nuclear import in SBPH. NLS in RSV NS3 was shown to bind importin 5 (LsIPO5) in SBPH, which mediated the cytoplasmic-nuclear trafficking of NS3. The ubiquitination of NS3 helped position the protein near the SBPH nucleus and facilitated the entry of NS3 into the nucleus, thus promoting RSV replication. Our data reveal that RSV NS3 hijacks the nuclear import and ubiquitin systems of SBPH for nuclear entry, thereby promoting viral replication. This study reveals a unique strategy for managing plant viruses by regulating insect vectors' nuclear import and/or the ubiquitin system.

2. Materials and methods

2.1. Plants, insects, and RSV

Laodelphax striatellus was initially collected from Jiangsu Province, China. Both RSV-free and RSV-infected insect populations were reared separately on rice cv. Wuyujing 3 as detailed in a previous study (Zhang *et al.* 2024).

To verify that planthoppers were infected with RSV, a single female and male were fed independently in a test tube, and the female insect and progeny were evaluated every week for RSV using an RSV-NP monoclonal antibody and Dot-ELISA as described previously (Li *et al.* 2020). Highly viruliferous insects were selected and used as described below.

2.2. RNA interference

The TranscriptAid™ T7 High Yield Transcription Kit (K0441, Thermo Fisher Scientific, USA) was used to synthesize double-stranded RNAs (dsRNAs) from the coding sequences of *LsIPO5* and *LsRIN*G. The dsRNAs (36.8 ng) were microinjected into adult viruliferous SBPH adults as described (Li *et al.* 2020). Insects were collected two days post-injection and analyzed for expression of *LsIPO5* and *LsRING* by RT-qPCR. Planthoppers injected with the double-stranded green fluorescent protein (ds*GFP*) were used as controls.

2.3. RNA extraction, PCR, and real-time RT-qPCR

SBPH adults were collected as a single sample, and total RNA was extracted from adults using the RNA Isolater Total RNA Extraction Reagent (R401-01, Vazyme, China). RNA was reverse-transcribed into cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (R211-01, Vazyme, China) as recommended. The primers used in this study are shown in Appendix A, and PCR experiments were executed with the Phanta Super-Fidelity DNA Polymerase (P501-d1, Vazyme, China).

Real-time RT-qPCR was performed using the CFX 96

Real-Time PCR System (Bio-Rad, USA) with gene-specific primers (Appendix A) and the Hieff® qPCR SYBR Green Master Mix (11204ES03, Yeasen Biotechnology, China). PCR conditions included 95°C (5 min) followed by 40 cycles at 95°C (10 s), 55°C (20 s), and 72°C (20 s). Dissociation curves were generated with a temperature gradient from 57–95°C to assess amplification accuracy and ensure that primer dimers were absent. Expression was normalized with *LsActin* (GenBank accession no. KC683802) and analyzed by the $2^{-\Delta\Delta CT}$ protocol and CFX Maestro software (Bio-Rad, USA). Datapoints were generated as means with standard errors, which were calculated from three or more biologically independent replicates.

2.4. Plasmid construction

For yeast two-hybrid (Y2H) assays, *LsIPO5* and *LsIPOα2* were amplified by PCR from *L. striatellus* cDNA using gene-specific primers that overlapped sequences present in pGBKT7, which is the bait vector for expressing proteins fused to the GAL4 DNA-binding domain (BD). PCR products were then ligated into *Nde*I/*Bam*HI-digested pGBKT7 using the ClonExpress II One Step Cloning Kit (C112-01, Vazyme, China), resulting in constructs BD-LsIPO5 and BD-LsIPOα2. Similarly, *NS3* from RSV was amplified using cDNA of RSV-infected SBPH and ligated into *Nde*I/*Bam*HI-digested pGADT7 to generate AD-NS3. The Mut Express II Fast Mutagenesis Kit V2 (C214-01, Vazyme, China) was used to create point mutations in AD-NS3.

For prokaryotic expression, the coding sequence of NS3 was subcloned from plasmid AD-NS3 using gene-specific primers containing sequences that overlapped with pET-28a(+) and subcloned into *Nde*I/*Bam*HI-digested pET-28a(+) to generate His-NS3.

For the glutathione-*S*-transferase (GST) pull-down assay, *LsIPO5* was subcloned from BD-LsIPO5 using gene-specific primers with sequences that overlapped with pGEX-4T-1; these were recombined into *Bam*HI/*Not*I-digested pGEX-4T-1 to generate GST-LsIPO5.

2.5. Western blots and protein detection

The tissue protein extraction kit (CW0891M, CWBIO, China) was used to extract total protein from SBPH. Proteins were isolated, electrophoretically separated in 12% SDS polyacrylamide gels, and blotted to polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, USA). The membranes were blocked by incubating them at room temperature in 5% BSA for 1 h, followed by incubation with anti-GAPDH (1:2,000 dilution) or anti-RSV NP (1:1,000 dilution) at 4°C overnight. Membranes were then incubated with the second antibody at room temperature for 1 h. Proteins transferred to the membranes were treated with Clarity Western ECL Substrate (1705061, Bio-Rad, USA) and visualized using the Tanon 5200 Multi Chemiluminescent Imaging System.

2.6. Immunofluorescence microscopy

For tissue staining and imaging, the dissected midguts, salivary glands, and ovaries were fixed in 4% paraformaldehyde for 1 h, followed by three washes in PBS (pH 7.4, 10 mmol L^{-1}). A 1% solution of Triton X-100 in PBS (v/v) was used to permeabilize tissues for 1 h and blocked as previously described (Li *et al.* 2020). Tissues were then incubated overnight at 4°C with RSV NP antibodies, rinsed in PBS, and then treated with Alexa Fluor 488 secondary antibodies (A0428, Beyotime, China) for 1 h in PBS (1:1,000 dilution). Samples were briefly washed in PBS and then mounted on slides containing 4′,6 diamidino-2-phenylindole (DAPI) mounting medium (S2100, Solarbio, China). Imaging was performed with the Leica TCS SP8 STED 3X microscope (Leica, Germany) and imaged as described previously (Zhang *et al.* 2024).

2.7. Yeast two-hybrid assays

The Yeastmaker™ Yeast Transformation System 2 (630440, TaKaRa, Japan) was used to conduct Y2H assays. Yeast cells of Y2HGold were co-transformed with AD-NS3 and BD-LsIPO5 or BD-LsIPOα2 plasmid constructs. A synthetic quadruple-dropout medium was used to identify positive clones, followed by isolation and sequencing of interacting prey constructs.

2.8. GST pull-down assays

Total protein from viruliferous SBPH, GST-LsIPO5 protein, and immobilized glutathione-sepharose beads (C650031, Sangon, China) were dispensed to bind/wash buffer and incubated as described (Zhang *et al.* 2024). Negative controls consisted of insect extracts incubated with GST. Beads were rinsed, and bound proteins were released as described (Li *et al.* 2020). After separation by SDS-PAGE, proteins were detected with anti-NS3 and anti-GST (MA000031, Cusabio, China) antisera.

2.9. *In vitro* ubiquitination of NS3

Ubiquitination was conducted as described previously (Fan *et al.* 2013) at 30°C for 3 h in a 40 μL volume containing 40 mmol L^{-1} Tris-HCl (pH 7.5), 5 mmol L^{-1} $MgCl_2$, 40 μmol L^{-1} ubiquitin (BML-UW9920-0001, Enzo, USA), 2 mmol L^{-1} DTT, 50 nmol L^{-1} hE1 (BML-UW9410, Enzo Life Sciences USA), 5 mmol L^{-1} ATP (BML-EW9805, Enzo, USA), 200–600 nmol L^{-1} E2s (BML-UW9920-0001, Enzo, USA), 20 μL of NS3 and purified LsRING. The reaction was terminated with 4× SDS-PAGE as described (Fan *et al.* 2013). Samples were separated by SDS-PAGE, and immunoblotting was used to detect ubiquitinated NS3 as described (Zhang *et al.* 2024).

2.10. Statistical analysis

Results from three independent experiments are presented as mean±SEM. The two-tailed Student's *t*-test was used for statistical analyses. $P<0.05$ was considered statistically significant.

3. Results

3.1. RSV infection induces expression of both classical and non-classical nuclear import pathways in SBPH

To investigate the potential association of the SBPH nuclear

transport system in the entry of RSV NS3 into the insect nucleus, we identified the importin genes in the SBPH genome using InsectBase 2.0 (http://v2.insect-genome.com/). There are three importin α genes (*importin α1*, *α2* and *α3*) and six importin β genes (*importin β1*, *4*, *5*, *7*, *11* and *13*) in the SBPH genome (Fig. 1). Among these, *Lsimportin α1* (*LsIPOα1*), *α2* (*LsIPOα2*), *α3* (*LsIPOα3*), and *β1* (*LsIPOβ1*) were classified as part of the classical nuclear transport pathway, whereas *Lsimportin 4* (*LsIPO4*), *5* (*LsIPO5*), *7* (*LsIPO7*), *11* (*LsIPO11*), and *13* (*LsIPO13*) belong to the non-classical nuclear transport pathway (Fig. 1).

The expression of *LsIPOs* in RSV-infected and RSV-free SBPHs was conducted to investigate their potential roles in RSV infection. Real-time quantitative PCR (RT-qPCR) analysis showed that *LsIPO*α2 and *LsIPOβ1* of the classical nuclear transport pathway were significantly upregulated by 225 and 89%, respectively, in RSV-infected SBPH as compared to RSV-free SBPH (Fig. 2-A). In the non-classical pathway, *LsIPO4* was significantly downregulated by 45% and *LsIPO5* was significantly upregulated by 57% in response to RSV infection (Fig. 2-B). Next, we measured *LsIPOα2*, *LsIPOβ1*, *LsIPO4*, and *LsIPO5* transcript levels in ovaries, midguts, and salivary glands. Expression levels of *LsIPOα2*, *LsIPOβ1*, and *LsIPO5* were significantly higher in the midguts (834, 185 and 84%, respectively) of RSV-infected SBPH when compared to RSV-free SBPH. No significant differences were observed for expression levels in the ovaries and salivary glands of RSV-infected vs. RSV-free SBPH (Fig. 2-C–E). *LsIPO4* transcription was significantly downregulated in the midguts (43%) and salivary glands (80%) of viruliferous as compared to nonviruliferous SBPH, but no differences were observed in ovaries (Fig. 2-F). These results indicate that RSV infection upregulates *LsIPOα2* and *LsIPOβ1* in the classical import pathway, while it upregulates *LsIPO5* but downregulates *LsIPO4* in the non-classical import pathway of SBPH.

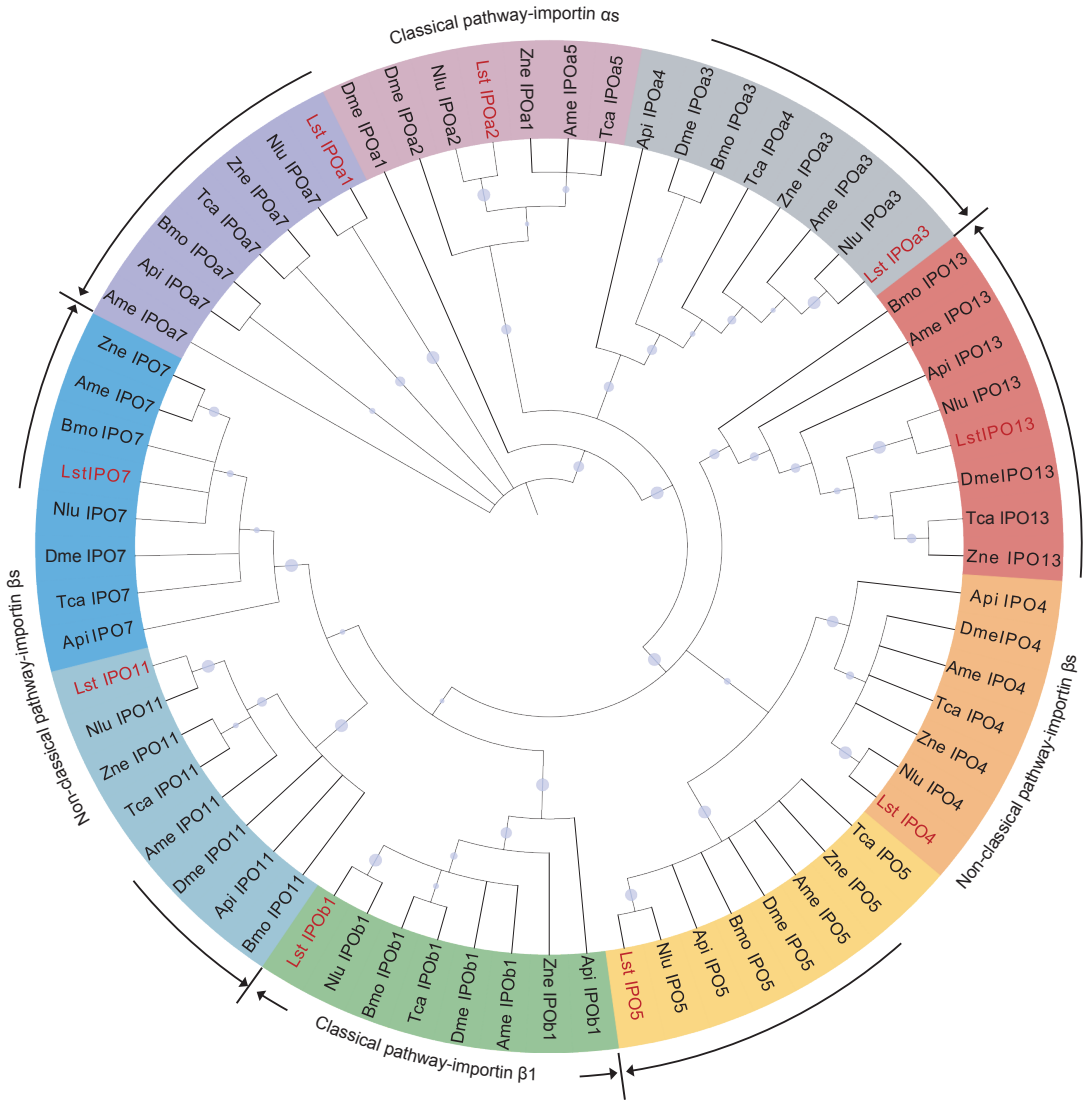


Fig. 1 Phylogenetic analysis of importins in different insect orders. A maximum likelihood tree was created from aligned amino acid sequences using MEGA 7.0. Nodes with distance bootstrap values (100 replicates) are shown. Importins noted in red font are present in *Laodelphax striatellus*. Lst, *Laodelphax striatellus*; Zne, *Zootermopsis nevadensis*; Nlu, *Nilaparvata lugens*; Dme, *Drosophila melanogaster*; Tca, *Tribolium castaneum*; Api, *Acyrthosiphon pisum*; Bmo, *Bombyx mori*; and Ame, *Apis melifera*.

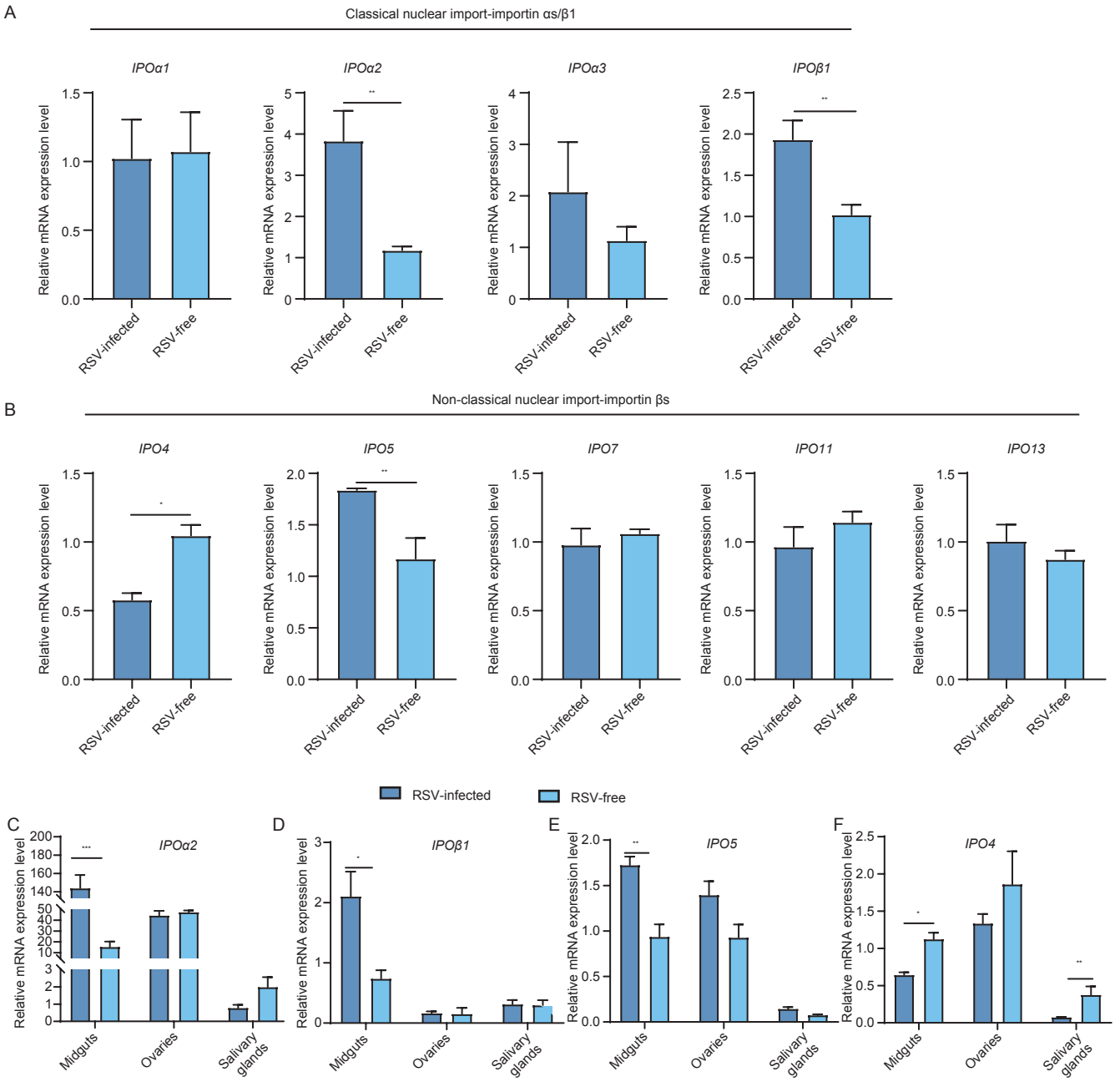


Fig. 2 Expression of classical and non-classical nuclear import pathways during rice stripe virus (RSV) infection in small brown planthopper (SBPH). A, RT-qPCR analysis of small brown planthopper (SBPH) importin genes in the classical nuclear import pathway. Expression levels are shown for *importin α1* (*IPOα1*), *importin α2* (*IPOα2*), *importin α3* (*IPOα3*), and *importin β1* (*IPOβ1*) in RSV-infected (*n*=20) and RSV-free (*n*=20) SBPHs. B, RT-qPCR analysis of SBPH importin genes in the non-classical nuclear import pathway. Expression levels are shown for *importin 4* (*IPO4*), *importin 5* (*IPO5*), *importin 7* (*IPO7*), *importin 11* (*IPO11*), and *importin 13* (*IPO13*) in RSV-infected (*n*=20) and RSV-free SBPHs (n=20). C–F, RT-qPCR analysis of expression levels for *IPOα2* (C), *IPOβ1* (D), *IPO4* (E), and *IPO5* (F) in the ovaries (*n*=150), midguts (*n*=150), and salivary glands (*n*=150) of RSV-infected and RSV-free SBPH, respectively. Data are mean±SEM. *, $P<0.05$; **, $P<0.01$; *t*-test analysis.

3.2. RSV NS3 interacts directly with LsIPO5 of SBPH

In the classical import pathway, substrates are recognized by the importin α subunit, and in the non-classical pathway, substrates bind directly to one of the importin βs (Lu *et al.* 2021). To further investigate these associations, we cloned *LsIPOα2* (GenBank accession no. MT036050) and *LsIPO5* (GenBank accession no. PQ898999). Alignment of the LsIPO5 deduced amino acid sequence with other IPO5 proteins indicated that the LsIPO5 sequence is highly conserved when compared to IPO5s in other insect species (Appendix B).

Our previous study demonstrated that NS3 enters the cell nuclei of *L. striatellus* (Zhang *et al.* 2024). In the present study, we investigate whether the nuclear transport system of SBPH mediates RSV NS3 entry and if LsIPOα2 and LsIPO5 directly interact with RSV NS3. The Y2H assays did not indicate interactions between NS3 and LsIPOα2 but demonstrated that NS3 interacts with LsIPO5 (Fig. 3-A). GST

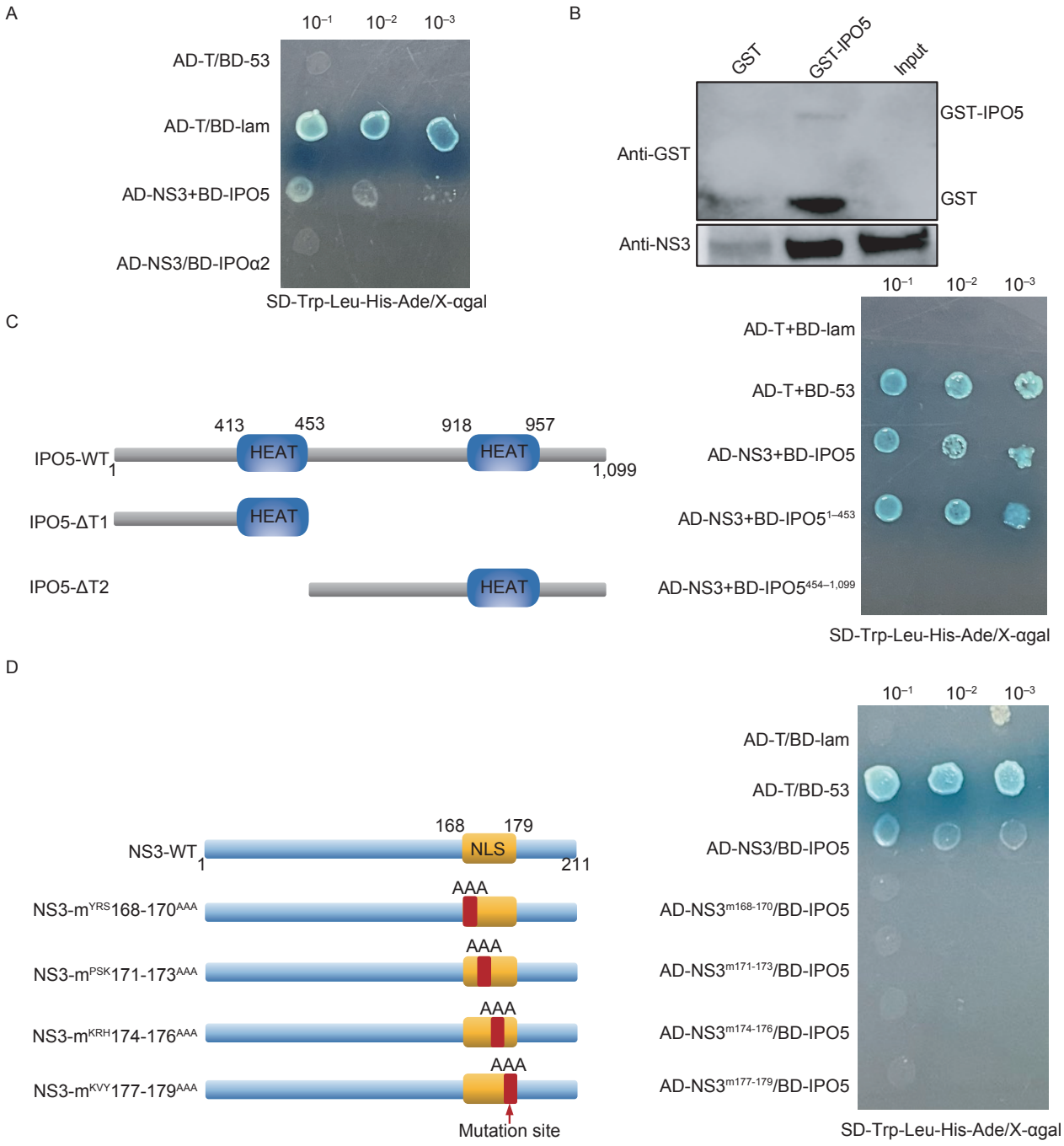


Fig. 3 Rice stripe virus (RSV) nonstructural protein 3 (NS3) directly interacts with importin 5 (IPO5) in small brown planthopper (SBPH). A, the interaction of IPO5 from SBPH with the RSV NS3 using Y2H assays. IPO5 or IPOα2 was co-transformed with NS3 in yeast cells, dilutions were plated onto quadruple-dropout SD-Trp-Leu-His-Ade-20 mmol L^{-1} 3-AT medium, and colonies were analyzed for β-galactosidase activity as described previously (Zhang *et al.* 2024). B, pull-down assays with glutathione *S*-transferase (GST). Purified GST-IPO5 was used as bait, and GST was the control. Total proteins from RSV-infected SBPH were combined with GST-IPO5 or GST and incubated with glutathione-S beads. Proteins were released from beads, separated by SDS-PAGE, and immunoblotted with anti-GST and anti-NS3 antibodies. C, Y2H with two truncated variants of IPO5 missing one of the two HEAT domains. D, the schematic of wild-type NS3 and four NS3 variants containing alanine substitutions in the nuclear localization sequence (NLS) of IPO5. The four variants containing alanine substitutions were used in Y2H assays with LsIPO5. AD-T+BD-Lam (T/Lam) and AD-T+BD-53 (T/53) served as negative and positive controls, respectively.

pull-down results further verified the interaction between NS3 and LsIPO5 (Fig. 3-B).

LsIPO5 encodes 1,099 amino acids and contains two HEAT domains (Fig. 3-C), which provide binding sites for substrates by altering their conformation (Lu *et al.* 2021). To determine the region of LsIPO5 that interacts with NS3, we conducted Y2H assays with two IPO5 deletion variants: IPO5-ΔT1 (containing residues 1–453) and IPO5-ΔT2 (residues 454–1,099) (Fig. 3-C). The Y2H assays indicated that only yeast strains harboring IPO5-ΔT1 and NS3 grew (Fig. 3-C),

which demonstrated that amino acid residues 1–453 of LsIPO5 interact with NS3. When the NS3 protein sequence was analyzed by NLS Mapper, a nuclear localization signal was identified at amino acid residues 168–179 in NS3 (Fig. 3-D). Four NS3 variants were generated containing alanine substitutions in the NS3 NLS and used in Y2H assays with LsIPO5. The results demonstrated that mutations at these four sites within NLS abolished the interaction between NS3 and IPO5 (Fig. 3-D). Collectively, these results suggest that the first HEAT domain of IPO5 directly binds to the NLS of NS3. Since NLS sequences are conserved peptides that interact with importin to facilitate protein entry into the nucleus, our findings suggest that LsIPO5 may mediate RSV NS3 cytoplasm-nuclear trafficking.

3.3. RSV NS3 nuclear entry is mediated by LsIPO5 in SBPH

To investigate whether LsIPO5 mediates RSV NS3 nuclear entry, we evaluated the localization of NS3 in the midguts of RSV-infected SBPHs treated with ds*IPO5* or ds*GFP* by immunofluorescence microscopy. In ds*GFP*-treated SBPH, we observed NS3 signals in cell nuclei; however, in the ds*IPO5*-treated group, NS3 signals were primarily present in the cytoplasm or near the nucleus (Fig. 4-A). NS3 protein levels in the nuclei and cytoplasm of ds*GFP*- and ds*IPO5*-treated SBPH were evaluated by immunoblot assays. In nuclei, NS3 levels in ds*IPO5*-treated SBPHs were lower than those in ds*GFP*-treated SBPHs; however, in the cytoplasm, NS3 levels were much higher than in ds*GFP*-treated SBPHs (Fig. 4-B). Taken together, these results demonstrate that LsIPO5 mediates the nuclear transport of NS3 in SBPH.

3.4. Ubiquitinated NS3 acts synergistically with LsIPO5 to promote NS3 nuclear import in SBPH

Our previous study demonstrated that LsRING-mediated NS3 ubiquitination facilitates NS3-mediated nuclear transport in SBPH (Zhang *et al.* 2024). To investigate the relationship between nuclear transport as a function of NS3 ubiquitination and that mediated by LsIPO5, we examined NS3 levels in the cell nucleus of ds*IPO5*-treated SBPH and ds*IPO5*+ds*RING*-treated SBPH. Immunoblots revealed that RSV NS3 accumulated at lower levels in nuclei when SBPHs were treated with both ds*IPO5* and ds*RING* as compared to ds*IPO5* alone (Fig. 5-A). Therefore, we hypothesized that LsRING-mediated ubiquitination of NS3 might facilitate the nuclear transport of NS3 mediated by LsIPO5. To test this hypothesis, an IP assay was conducted to assess the impact of NS3 ubiquitination on the binding efficiency between LsIPO5 and NS3. The results showed that the ubiquitination of NS3 enhanced its binding affinity for LsIPO5 (Fig. 5-B). Furthermore, immunofluorescence assays showed that NS3 accumulated near the nucleus in

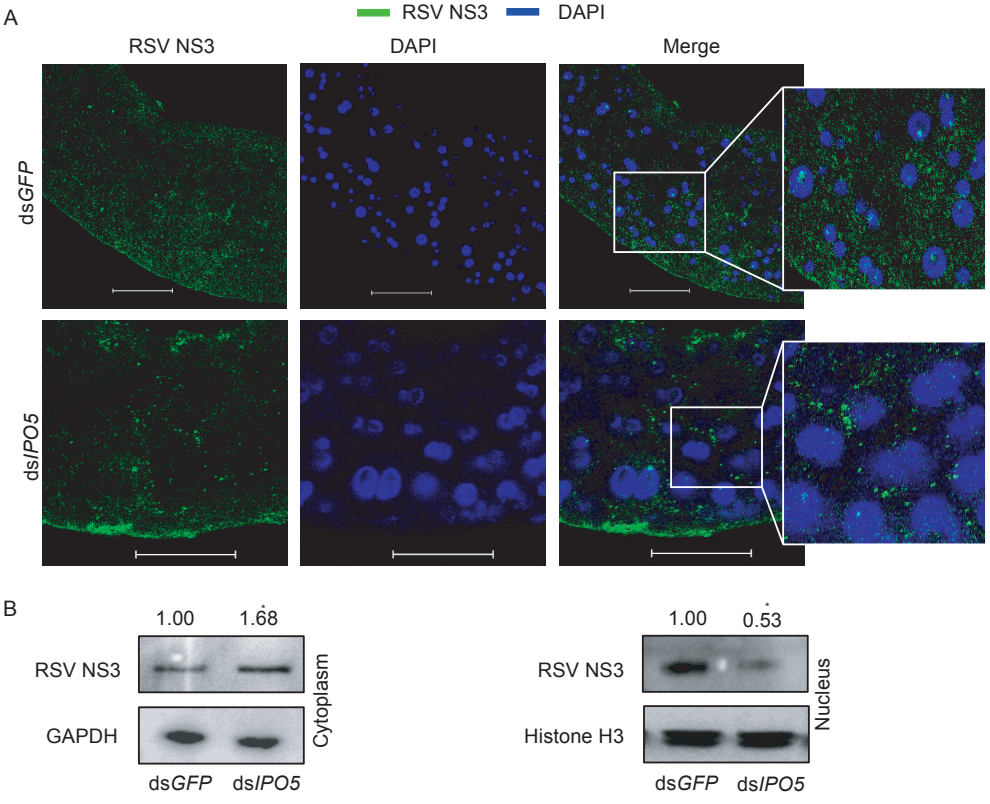


Fig. 4 LsIPO5 mediates rice stripe virus (RSV) nonstructural protein 3 (NS3) nuclear entry in small brown planthopper (SBPH). A, localization of NS3 in the midguts of RSV-infected planthoppers treated with ds*IPO5* or ds*GFP* and subjected to immunofluorescence microscopy. Antisera labeled with Alexa Fluor 488 (green) was used to label RSV NS3. DAPI (blue) shows nuclear staining. Scale bar, 50 μm. B, NS3 protein levels in the nuclei and cytoplasm of ds*GFP*- and ds*IPO5*-treated SBPH. Immunoblot analysis of NS3, GAPDH, and histone H3 levels in the nuclei and cytoplasm of ds*GFP*- and ds*IPO5*-treated SBPH infected with RSV. Data are mean±SEM. *, $P<0.05$; *t*-test analysis.

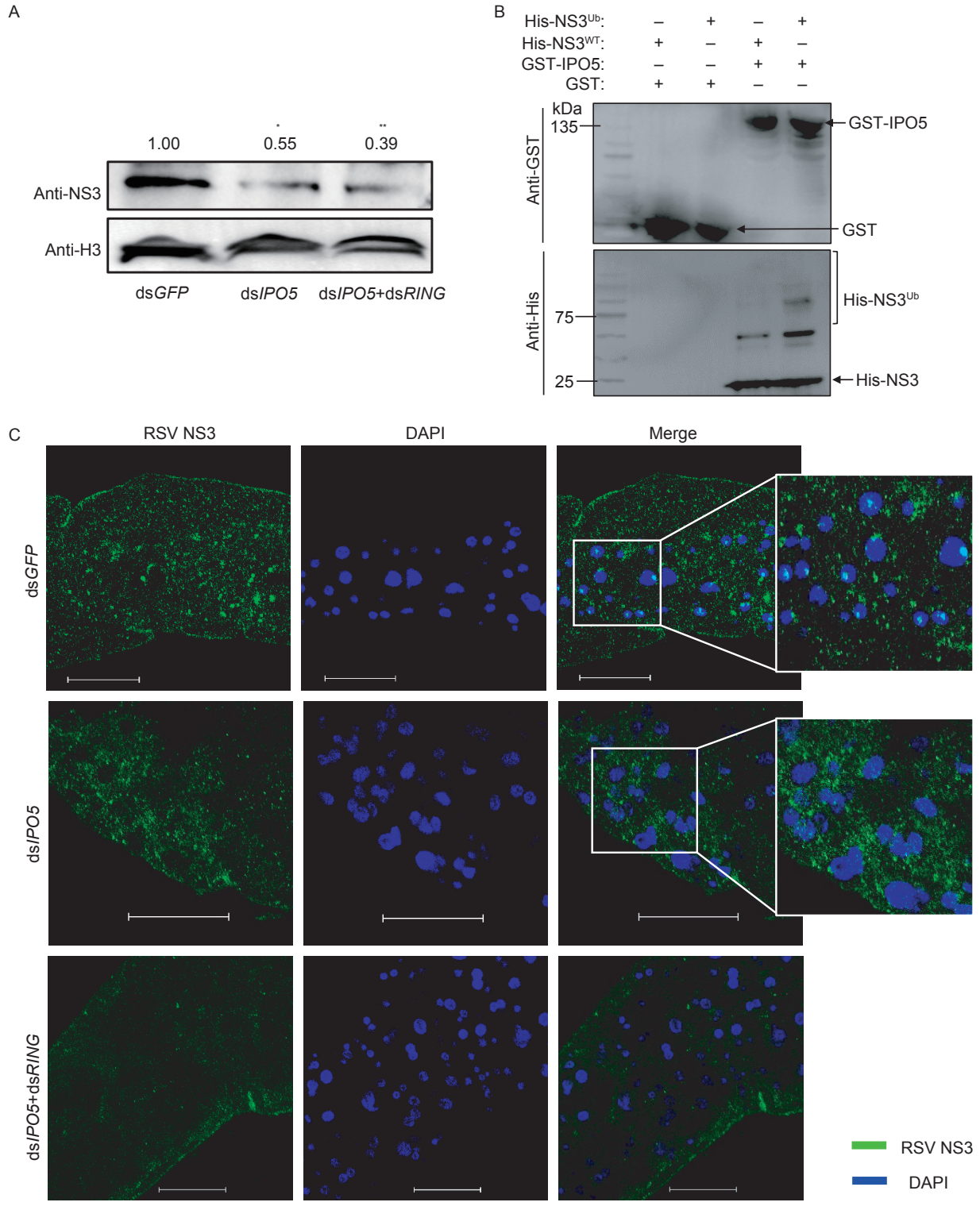


Fig. 5 Ubiquitinated nonstructural protein 3 (NS3) acts synergistically with LsIPO5 to promote NS3 nuclear import in small brown planthopper (SBPH). A, Western blot analysis of NS3 and histone H3 proteins in the nuclei of ds*GFP*-, ds*IPO5*- and ds*IPO5*+ds*RING*-treated SBPH infected with RSV. B, binding efficiency of His-tagged NS3 and His-tagged ubiquitinated NS3 for GST-labeled IPO5 in pull-down assays. Western blots were probed with anti-GST and anti-His antibodies. C, immunofluorescence assays of interactions between NS3 and ds*IPO5* and ds*IPO5*-ds*RING*. RSV NS3 was labeled with antisera conjugated to Alexa Fluor 488 (green). Nuclei were stained with DAPI (blue). Scale bar=50 μm. Data are mean±SEM. *, P<0.05; **, P<0.01; t-test analysis.

ds*IPO5*-treated SBPH, while NS3 was primarily present in the cytoplasm of ds*IPO5*+ds*RING*-treated SBPH (Fig. 5-C). These results indicated that ubiquitination of NS3 increased its binding with LsIPO5 and facilitated transport to the nuclear periphery, thereby promoting the entry of NS3 into the SBPH nucleus.

3.5. Ubiquitinated NS3 acts synergistically with LsIPO5 to promote RSV replication in SBPH

To explore the role of NS3 ubiquitination and LsIPO5-mediated transport of NS3 into the SBPH nucleus, we measured RSV load levels in ds*IPO5*-treated and ds*IPO5*+ds*RING*-treated planthoppers. Compared to ds*GFP*-treated controls, *LsIPO5* expression levels in ds*IPO5*-treated SBPHs and ds*IPO5*+ds*RING*-treated SBPHs were significantly reduced by 97 and 98%, respectively (Fig. 6-A). *LsRING* mRNA levels in ds*IPO5*+ds*RING*-treated SBPH were significantly reduced by 93% as compared to ds*GFP* controls (Fig. 6-A). The combined treatment of SBPH with ds*IPO5*+ds*RING* reduced RSV loads by 75% as compared to the ds*IPO5*-treated group (Fig. 6-A). Furthermore, immunoblots confirmed that RSV load was reduced when SBPH was treated with both ds*IPO5* and ds*RING* as compared to ds*IPO5* alone (Fig. 6-B). RSV loads were then analyzed in different SBPH tissues treated with ds*GFP*, ds*IPO5*, or ds*IPO5*+ds*RING* in immunofluorescence assays (Fig. 6-C–E). The ds*IPO5* treatment significantly reduced RSV accumulation in the midguts and ovaries (Fig. 6-D and E) but had no effect on RSV accumulation in the salivary glands (Fig. 6-C) when compared to the ds*GFP* control. The ds*RING*+ds*IPO5* dual treatment reduced RSV accumulation in the ovaries, midguts, and salivary glands when compared to the ds*GFP* and ds*IPO5* treatments. These results indicate that LsIPO5 and ubiquitinated NS3 cooperate to promote NS3 nuclear entry during RSV replication.

4. Discussion

A few studies have demonstrated that some viruses utilize both classical and non-classical nuclear import pathways to facilitate their nuclear entry. For example, human immunodeficiency virus type 1 (HIV-1) promotes nuclear entry by hijacking genes involved in classical (*importin α* and *β1*) and non-classical nuclear import pathways (*importin 5*, *transportin 3* and *importin 7*) (Arnold *et al.* 2006; Levin *et al.* 2010). Similarly, Simian immunodeficiency virus (SIV) is also imported into the nucleus *via* both classical and non-classical nuclear import pathways (Singhal *et al.* 2006). In this study, we found that genes encoding *importin α2* (*LsIPOα2*), *importin β1* (*LsIPOβ1*), and *importin 5* (*LsIPO5*) are upregulated in response to RSV infection in SBPH (Fig. 2). Among these, LsIPOα2 and LsIPOβ1 are part of the classical nuclear transport pathway, while LsIPO5 belongs to the non-classical pathway. Our data demonstrated that LsIPO5 could directly bind to NS3, which mediated NS3 nuclear entry (Figs. 3 and 4). LsIPOα2 does not interact with NS3 (Fig. 3), suggesting that LsIPOα2 is responsible for the nuclear entry of other RSV proteins. A recent study reported that LsIPOα2 mediates RSV NP nuclear entry in SBPH (Zhao *et al.* 2022). These findings indicate that plant viruses can exploit both classical and non-classical nuclear import pathways in insect vectors for nuclear entry.

While ubiquitination is traditionally known for its role in protein degradation, its emerging function in regulating nuclear entry *via* the nuclear import system is gaining increasing attention. In the study of paramyxoviruses, the ubiquitin system has been shown to regulate nuclear transport by ubiquitinating the NLS of the M protein encoded by paramyxoviruses (Wang *et al.* 2010; Pentecost *et al.* 2015). However, the current study and our previous report (Zhang *et al.* 2024) reveal a different mechanism in RSV. Specifically, we identified that the ubiquitination site of NS3 (K127) is located outside its NLS (residues 168–179) (Fig. 3), indicating that RSV employs an ubiquitination-synergistic strategy, distinct from that of paramyxoviruses, to facilitate its nuclear entry. Our investigation further demonstrates that NS3 ubiquitination facilitates its perinuclear localization in SBPH and enhances binding affinity for LsIPO5 (Fig. 5). Interestingly, this mechanism bears similarity to the nuclear import regulation of the tumor suppressor protein PTEN, where monoubiquitination mediates importin binding (including importin α, importin β, and IPO5) to facilitate nuclear translocation (Xie *et al.* 2021). These findings collectively highlight a potential synergistic relationship between UPS and the nuclear import pathway in promoting protein nuclear entry. However, the mechanism of ubiquitination that modulates substrate-importin interactions remains elusive. The mechanistic basis of synergism between the nuclear import system and UPS warrants further investigation.

RSV replication occurs in the midgut of insect vectors (Dow 1987), which concurs with our finding that *LsIPO5* was highly expressed in the midgut of RSV-infected SBPH (Fig. 2). We speculate that importin 5 of SBPH may also be involved in RSV replication. There are many examples where IPO5 plays a role in viral replication. For example, the Rev protein of HIV-1 is critical for HIV replication and is an essential shuttle protein for the nuclear export of unspliced viral mRNAs; moreover, Rev interacted with IPO5 at nanomolar concentrations (Levin *et al.* 2014). In the case of the influenza A virus (IAV), IPO5 was proposed to function as an import factor for newly synthesized polymerase enzymes to traffic the PB1-PA dimer to the nucleus, thus promoting viral replication (Deng *et al.* 2006). In addition, IPO5 also interacts with human papillomavirus type 16 (HPV16) (Nelson *et al.* 2003; Krawczyk *et al.* 2008) and hepatitis C virus (HCV) (Levin *et al.* 2014). These findings further support our hypothesis that LsIPO5 and ubiquitination synergistically mediate NS3 nuclear entry, thereby facilitating RSV replication in SBPH.

Previous studies showed that NP and several RSV nonstructural proteins (NS2, NS3, and SP) localized to nuclei in tobacco and rice (Xiong *et al.* 2008; Kong *et al.* 2014; Lian *et al.* 2014; Zheng *et al.* 2018). In SBPH, RSV has been demonstrated to utilize two distinct strategies for nuclear entry. RSV NP enters the nucleus *via* the classical nuclear transport system, where it triggers an antiviral apoptotic response to suppress excessive viral replication (Zhao *et al.* 2022). This mechanism helps maintain a balance between viral load and host immune pressure, ensuring efficient viral transmission. In contrast, RSV NS3 follows a different route, utilizing a non-classical nuclear transport pathway and the ubiquitin system to enter the nucleus, where it directly enhances viral replication (Zhang *et al.* 2024). This strategy may provide RSV with a greater capacity for replication under specific conditions. The divergence between these two

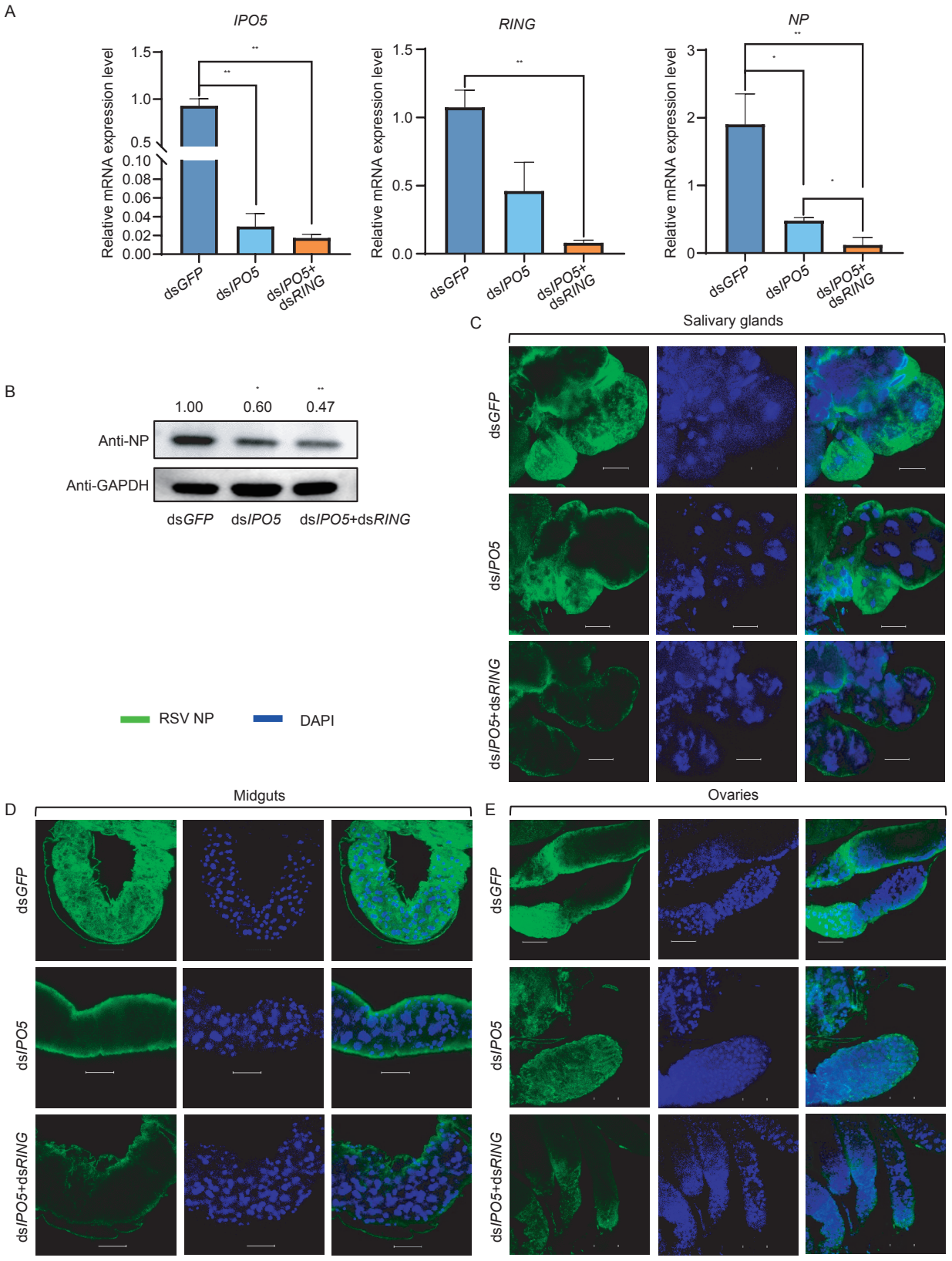


Fig. 6 Ubiquitinated nonstructural protein 3 (NS3) acts synergistically with LsIPO5 to promote RSV replication in SBPH. A, RT-qPCR analysis of *importin 5* (*LsIPO5*), RING E3 (*LsRING*), and RSV *NP* expression in ds*IPO5*-, ds*IPO5*+ds*RING*-, and ds*GFP*-treated RSV infected SBPH (*n*=20, respectively). B, immunoblot analysis of NP and GAPDH proteins in ds*GFP*-, ds*IPO5*-, and ds*IPO5*+ds*RING*-treated SBPH infected with RSV. C–E, RSV accumulation in ds*GFP*-, ds*IPO5*-, and ds*IPO5*+ds*RING*-treated SBPH in RSV-infected salivary glands (C), midguts (D) and ovaries (E). RSV NP was labeled with antisera conjugated with Alexa Fluor 488 (green). Nuclei were stained with DAPI (blue). Scale bar=50 μm. Data are mean±SEM. *, P<0.05; **, P<0.01; *t*-test analysis.

mechanisms highlights the virus's adaptive evolution in host-virus interactions, showcasing its ability to exploit diverse host cellular pathways and demonstrating the diversity and flexibility of RSV in a complex host environment. These findings suggest a potential new strategy for controlling plant viral diseases by inhibiting viral replication through the disruption of the nuclear entry system in insect vectors.

5. Conclusion

RSV infection activates both classical and non-classical nuclear import pathways in SBPHs. Within the non-classical pathway, LsIPO5 specifically binds to the NLS of RSV NS3, facilitating its nuclear import. Our previous study demonstrated that the LsRING mediates NS3 ubiquitination, further enhancing its nuclear import. Additionally, we found that NS3 ubiquitination promotes its perinuclear localization and strengthens its interaction with LsIPO5, ultimately boosting RSV replication. These findings reveal that RSV NS3 exploits the nuclear import and ubiquitin systems of SBPHs to gain nuclear entry, thereby facilitating viral replication (Fig. 7). This study highlights a novel strategy for controlling plant viruses by targeting nuclear import and ubiquitination pathways in insect vectors.

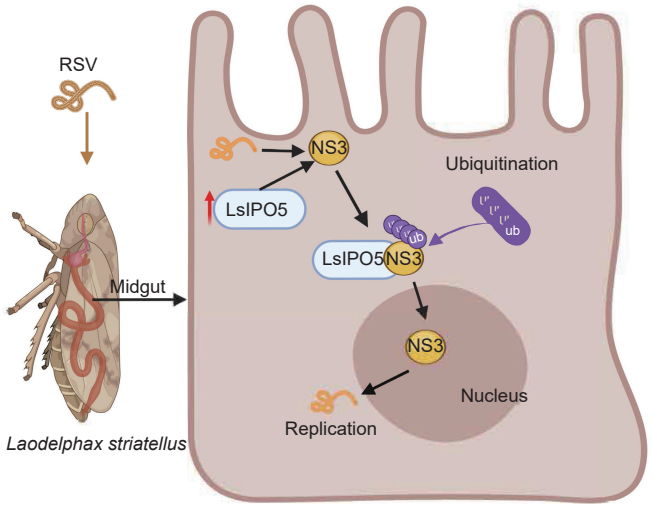


Fig. 7 Model of rice stripe virus (RSV) nonstructural protein 3 (NS3) exploits synergistically insect vector importin and ubiquitin systems to promote viral replication. Image created by BioRender.com, with permission.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in scientific writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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