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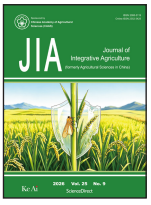

RESEARCH ARTICLE

Salivary protein NISP6935 that restricted to rice planthoppers is critical for insect survival and host defense regulation

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Highlights

- NISP6935 is a conserved salivary protein in rice planthoppers, essential for survival, feeding, and reproduction, independent of host plant resistance.
- Silencing *NISP6935* exerts effects on insect metabolism and cuticle formation, while its overexpression in plants suppresses H_2O_2 accumulation and terpenoid defenses.
- NISP6935 functions as a dual effector, critical for both insect physiology and suppression of plant immune responses, highlighting its evolutionary role in host adaptation.
- Transgenic rice expressing NISP6935 enhances planthopper attraction but fails to fully rescue RNAi-induced lethality, suggesting its indispensable endogenous function in insect biology.

Abstract

Saliva plays a crucial role in mediating plant–insect interactions, yet the functional diversity of salivary proteins remains poorly understood. Here, we identify NISP6935, a salivary gland-specific protein conserved among rice planthoppers but absent in bamboo-feeding relatives. Silencing *NISP6935* causes severe lethality, feeding impairment, and infertility in *Nilaparvata lugens*, independent of host plant resistance. Transient expression assays reveal that NISP6935 suppresses H_2O_2 accumulation in plants, while overexpression in rice downregulates terpenoid biosynthesis and enhances host attractiveness. However, transgenic NISP6935 plants only weakly rescue RNAi-induced lethality, demonstrating its dual role in insect physiology and plant defense suppression. Our findings reveal a novel effector essential for both planthopper survival and host adaptation, providing new insights into pest control strategies.

Keywords: salivary protein, pest management, defense regulation, insect–plant interaction

1. Introduction

Saliva serves as a crucial biochemical interface mediating the interaction between sap-sucking insects and their host plants (van Bel and Will 2016). During feeding, plants detect mechanical damage or insect-derived cues, which trigger a suite of physical and chemical defense responses, including the production of toxic compounds, inhibition of nutrient digestion, and activation of signals that attract natural enemies (Wu and Baldwin 2009; Guiguet *et al.* 2016; Jiang *et al.* 2019; Chen and Mao 2020). In turn, insects secrete saliva rich in bioactive molecules that play diverse roles, ranging from breaking down plant cell walls and aiding nutrient digestion to modulating or suppressing plant defenses (Ji *et al.* 2017; Bleau *et al.* 2024). Hemipteran insects produce two distinct types of saliva during feeding: gelling and watery saliva. Gelling saliva solidifies to form a protective salivary sheath that guides and stabilizes the stylet as it penetrates plant tissues (Will and Vilcinskas 2015). Disruption in the secretion of key sheath-forming proteins can lead to malformed sheaths, impairing feeding efficiency and potentially causing premature insect death (Huang *et al.* 2015; Will and Vilcinskas 2015; Shangguan *et al.* 2018). Watery saliva is primarily secreted into plant cells during stylet penetration (Tjallingii 2006). Although reduced secretion of

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various watery salivary proteins has been shown to impair insect performance, such deficiencies typically do not lead to severe lethal outcomes (Gong *et al.* 2023; Huang *et al.* 2023; Wang *et al.* 2023; Hu *et al.* 2025).

In recent decades, advances in sequencing technologies have greatly facilitated the identification of salivary components in many agriculturally important pests, including aphids, planthoppers, leafhoppers, and whiteflies (Carolan *et al.* 2011; Hattori *et al.* 2015; Huang *et al.* 2016, 2021). This progress has significantly enhanced our understanding of the functions of salivary proteins. Based on expression patterns across different tissues, salivary proteins can be classified into two categories: salivary gland (SG)-unbiased expressed genes and SG-biased expressed genes (van Bel and Will 2016). The former often serve multiple roles beyond functioning as salivary proteins, and their knockdown typically affects not only feeding but also other aspects of insect physiology (Qi *et al.* 2025a, b). Conversely, SG-biased genes have been predominantly studied for their effects on plant signaling pathways, particularly in the context of defense modulation, which indirectly determines host suitability for the insects (Bleau *et al.* 2024). As a result, the potential roles of these SG-biased salivary proteins in the internal physiology of insects remain largely unexplored. Given that saliva is a complex secretion produced by specialized glands, its components are likely to serve multifunctional roles. Expanding research to include their endogenous functions could provide deeper insights into the diversity, functionality, and evolutionary dynamics of salivary proteins.

As an important intermediary between plants and herbivores, saliva intimately interacts with plant cells after secretion and is easily shaped by natural selection (Rao *et al.* 2019; Li *et al.* 2025). A significant proportion of salivary proteins appear to be restricted to particular insect lineages, with no clear homologs identified in other taxa (Huang *et al.* 2018; Cui *et al.* 2024). Such lineage-specific salivary proteins may represent evolutionary innovations that enable insects to overcome the unique defenses or exploit the specific physiology of their preferred host plants (van Bel and Will 2016). For example, the Aleyrodidae-specific salivary protein Bt56 modulates crosstalk between salicylic acid (SA) and jasmonic acid (JA) signaling networks by directly interacting with a KNOTTED 1-like homeobox transcription factor (Xu *et al.* 2019). Similarly, the Delphacidae-specific salivary protein BISP suppresses host basal defenses by targeting receptor-like kinases (Guo *et al.* 2023). Additionally, some salivary elicitors that trigger plant defenses were expressed at lower levels in host-adapted aphid biotypes, suggesting these proteins may influence insect colonization of specific host plants (Guo *et al.* 2020). Therefore, comparative analyses of salivary proteomes across species or host-specialized populations offer a valuable approach to identify key molecular factors underlying insect–plant compatibility.

The Delphacidae, commonly known as planthoppers, comprise over 2,100 described species worldwide (https://sites.udel.edu/planthoppers, accessed on December 2023). Most species in this family feed on gramineous plants, with some posing significant threats as agricultural pests. Among the most well-known Delphacidae species are the three rice planthoppers: the brown planthopper (*Nilaparvata lugens*, BPH), the small brown planthopper (*Laodelphax striatellus*, SBPH), and the white-backed planthopper (*Sogatella furcifera*, WBPH). These insects primarily feed on rice plants and cause substantial economic losses in crop production. Comparative analyses of three rice planthopper species have revealed a high degree of overlap in their salivary composition, suggesting a conserved adaptive strategy for feeding on rice hosts (Huang *et al.* 2018). Despite this, the key salivary components, especially these specifically evolved for planthoppers adapting on rice hosts, remain poorly characterized. Given that salivary protein gene family expansion and novel gene acquisition are key mechanisms for host adaptation in insects (Wybouw *et al.* 2016; Huang *et al.* 2024; Hu *et al.* 2025), it is important to perform a comparative analysis of planthoppers feeding on rice and other hosts. Recently, the salivary composition of another planthopper, *Epeurysa nawaii* Matsumura, has been characterized (Wang *et al.* 2024). Unlike the rice-feeding species, *E. nawaii* primarily infests and damages bamboo. A comparative analysis of salivary proteins between planthoppers with distinct feeding habits could offer new insights into their host adaptation mechanisms.

In this study, we compared salivary proteins between *E. nawaii* and three rice planthoppers, identifying a specific orthogroup OG0006935. Functional analysis revealed that OG0006935 genes are essential for insect survival, with RNAi silencing causing severe lethality across all three rice planthopper species. This lethal phenotype arose not only from disrupted plant defense suppression but also from impaired metabolic and cuticle-related pathways. Our findings demonstrate that a key orthogroup that plays a dual role in both insect physiology and host adaptation, providing new insights into the evolutionary arms race between Hemipteran pests and their host plants.

2. Materials and methods

2.1. Insects and plants

The *N. lugens* and *S. furcifera* strain were initially obtained from rice field located on the Huajiachi Campus of Zhejiang University in Hangzhou, China. The *L. striatellus* strain was sourced from a rice field in Ningbo, China. All insects and rice plants, including the resistant cultivar ASD7 and the susceptible cultivar Nipponbare, were reared in a controlled climate chamber set to (25±1)°C, 70–80% relative humidity, and a 16 h light/8 h dark cycle. Additionally, *Nicotiana benthamiana* plants were maintained under similar photoperiod conditions at (23±1)°C.

2.2. Bioinformatic analysis of salivary proteins

For orthogroup identification, protein-coding sequences from three planthopper species were obtained from previously published genome assemblies (Ye *et al.* 2021, 2022; Hu *et al.* 2022). Homologous relationships among proteins were inferred using an all-against-all BLASTp search, and reciprocal best hits were extracted as candidate orthologs. The BLAST results were subsequently subjected to clustering analysis with OrthoFinder with default parameters (Emms

and Kelly 2019).

The Delphacidae-specific salivary proteins were identified according to the previous described method (Huang *et al.* 2023). Briefly, salivary protein sequences identified from the three planthopper species were first compared against the predicted proteomes of *Acyrthosiphon pisum*, *Bemisia tabaci*, *Riptortus pedestris*, and *Drosophila melanogaster*, using BLASTp, with an E-value threshold of 10^{-5}. Candidate genes that did not yield significant matches in these species were subsequently queried against the NCBI non-redundant (nr) protein database to exclude homologs present in other taxa. Proteins that lacked detectable similarity outside of the three rice planthoppers were considered to be Delphacidae-specific. To investigate the evolutionary conservation of OG0006935, we carried out BLASTp searches using its protein sequence as the query against the predicted proteomes of 20 representative insect species, following the procedure outlined in our earlier work (Wang *et al.* 2023). Sequence hits with an E-value lower than 10^{-5} were considered significant and retained for downstream analyses.

Our previous work has performed the high-throughput transcriptomic sequencing of salivary glands, gut and residual parts from *E. nawaii* (Wang *et al.* 2024). The interested orthogroups in three rice planthoppers were searched against that assembled transcriptome-derived contigs to find the potential homologous genes in *E. nawaii*, with an E-value threshold of 10^{-5}. To further confirm the genomic presence of these target genes, we generated a draft genome assembly of *E. nawaii*. Briefly, genomic DNA was extracted and used for constructing a paired-end sequencing library with an average insert size of 350 bp. The library was sequenced on the Illumina NovaSeq 6000 platform to produce high-quality paired-end reads. The output data were then subjected to *de novo* assembly using Trinity v. 2.8.5 with default parameters (Grabherr *et al.* 2011). The raw sequence data have been deposited in the National Genomics Data Center under accession numbers CRA029439.

Signal peptide sequences and their corresponding cleavage sites were predicted using the SignalP 6.0 web server (http://www.cbs.dtu.dk/services/SignalP/). Putative transmembrane helices were analyzed with the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). In addition, the theoretical molecular weight (MW) and isoelectric point (pI) of the proteins were calculated with EditSeq software v. 7.1.0.

2.3. Quantitative real-time PCR (qRT-PCR) analysis

To obtain various tissue samples from *N. lugens*, fifth instar nymphs were dissected in phosphate-buffered saline (PBS; 137 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 8.1 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4) using fine forceps (Ideal-Tek, Switzerland). From these, carcasses (n=10), fat bodies (n=20), guts (n=30), and salivary glands (n=40) were collected. Additionally, testes (n=20) and ovaries (n=10) were isolated from adult males and females, respectively. Samples representing different developmental stages included eggs (n=100), nymphs (n=10), and adults (males: n=10; females: n=10). All sample sizes are indicated in parentheses. Each sample was homogenized in TRIzol reagent (#9109; TaKaRa, Dalian, China), and total RNA was extracted according to the manufacturer's instructions. First-strand cDNA synthesis was performed with HiScript II Q RT SuperMix (#R212-01; Vazyme, Nanjing, China). qRT-PCR was carried out on a Roche LightCycler® 480 system using SYBR Green Supermix (Yeasen, Shanghai, China; #11202ES08). The amplification program consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Primers were designed with Primer Premier v. 6.0 (Appendix A). *N. lugens actin* served as the reference gene, and relative transcript levels were calculated with the $2^{-\Delta\Delta Ct}$ method. Each assay included three independent biological replicates, with two technical repeats for each.

2.4. RNA interference

Target gene fragments were first amplified with specific primers (Appendix A) and inserted into the pClone007 vector (#TSV-007, Tsingke, Beijing, China). PCR products carrying T7 promoter sequences served as templates for *in vitro* transcription, and double-stranded RNAs (dsRNAs) were synthesized using the T7 High Yield RNA Transcription Kit (#TR101-01, Vazyme, Nanjing, China). RNAi treatment was carried out following a modified protocol (Xu *et al.* 2015). Briefly, insects were briefly immobilized with CO_2 (5–10 s) before microinjection. The dsRNA was delivered into the mesothorax with a FemtoJet injector (Eppendorf-Netheler-Hinz, Hamburg, Germany). Following injection, the insects were transferred onto rice seedlings at the 4–5-leaf stage and maintained for 24 h. Surviving individuals were subsequently collected for downstream analyses. Gene knockdown efficiency was evaluated on the fourth day post-injection by qRT-PCR, using the same procedures described in Section 2.3.

2.5. Insect bioassays

For survivorship analysis, a group of 30–40 third-instar nymphs treated with dsRNA was maintained on 4–5 leaf-stage rice seedlings under controlled climate conditions. Mortality was recorded daily over a twelve-day period. The experiment included three independent biological replicates.

For the artificial diet feeding assay, one day post-injection, dsRNA-treated insects were transferred to an artificial diet adapted from previous reports (Fu *et al.* 2001), which was kindly provided by Prof. Jinli Zhang at the China National Rice Research Institute (Hangzhou, China). Thirty insects were housed in a plastic container featuring a metal net on one side and a double-layered Parafilm membrane enclosing the artificial diet (D-97) on the other. The diet was refreshed daily, and mortality was recorded over eight days. Three independent replicates were conducted.

For fecundity assay, newly emerged adults were treated with dsRNA and, after 24 h, paired for mating and oviposition over a 10-day period. The number of hatched offspring was recorded for each pair. A minimum of seven replicates were performed per treatment.

For honeydew measurement, fifth-instar nymphs were confined in a Parafilm sachet (Bemis NA, Neenah, WI, USA)

attached to rice stems. After 24 h of feeding, honeydew production was quantified by weighing the sachet before and after the assay using an electronic balance (Sartorius, Beijing, China; accuracy: 0.001 g). Each treatment included at least ten replicates.

For host choice assay, rice seedlings at the 4–5-leaf stage were individually confined in plastic cups (6 cm diameter, 10 cm height) equipped with a central release chamber. Fifteen *N. lugens* nymphs were introduced into the chamber, and the number of insects settling on each plant was recorded at 1, 3, 12, 24, and 48 h post-release. Each experiment consisted of at least ten replicates.

2.6. Scanning electron microscopy

The treated insects four days post-injection were transferred to a plastic container. After a 24-h feeding period on an artificial diet, sections of Parafilm carrying salivary sheaths were carefully excised and gently rinsed with PBS. The samples were then mounted on specimen stubs and subjected to vacuum desiccation for thorough drying. Subsequently, a thin layer of gold was applied to the specimens via sputter coating. Observations and imaging were performed using a Hitachi TM4000 II Plus scanning electron microscope (Tokyo, Japan). The length of each salivary sheath was determined by measuring the vertical distance from its apex to the base on the Parafilm substrate.

2.7. *Agrobacterium*-mediated plant transformation

The target gene fragments were inserted into the indicated vectors. These recombinant constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 (#TSC-A01, Tsingke, Beijing, China) using the heat-shock transformation approach. Transformed colonies were selected on LB agar supplemented with kanamycin (50 μg mL^{-1}) and rifampicin (10 μg mL^{-1}) and incubated at 28°C for about 60 h. Single colonies harboring the recombinant plasmids were cultured in liquid LB medium and collected by centrifugation at 2,400×g for 2 min. The bacterial pellets were then resuspended in induction buffer (10 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} MES, pH 5.6, and 200 μmol L^{-1} acetosyringone) and adjusted to an OD_{600} of 1.0, followed by incubation for 2 h. The prepared suspensions were infiltrated into leaves of *N. benthamiana* plants at approximately six weeks of age. Leaf samples were subsequently observed with a Leica SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) to capture fluorescence signals.

2.8. Diaminobenzidine (DAB) staining

Accumulation of H_2O_2 in *N. benthamiana* leaves was visualized by DAB staining. Briefly, leaf segments were excised and incubated in 1 mg mL^{-1} 3,3′-diaminobenzidine tetrahydrochloride (DAB-HCl; #D8001, Sigma, St. Louis, MO, USA) solution, adjusted to pH 3.8, for 6 h. Following incubation, the staining solution was discarded and tissues were immersed in absolute ethanol to remove chlorophyll at 65°C overnight. The decolorized leaves were then imaged using a digital camera.

2.9. Generation of transgenic plants

The coding region of NISP6935 without its predicted N-terminal signal peptide (NISP6935^{-sp}) was amplified by PCR and subcloned into an LIC-flag binary vector. The resulting construct was subsequently introduced into *A. tumefaciens* strain EHA105 through a heat-shock transformation procedure. For rice transformation, *Agrobacterium*-mediated gene transfer was performed by BioRun Biosciences (Wuhan, China). Briefly, embryogenic calli derived from rice seeds were co-cultivated with the recombinant *Agrobacterium* strain, followed by selection on antibiotic-containing medium and regeneration of resistant seedlings under controlled culture conditions. Transformed plants were transferred to soil and maintained in a greenhouse with a 16 h/8 h light–dark photoperiod at (28±2)°C. Molecular identification of transgenic lines was carried out using RT-PCR to confirm transgene transcription, and Western blotting to detect protein expression. Two independent T2 homozygous lines were chosen for subsequent functional characterization.

2.10. Transcriptomic analysis

To assess the effect of dsRNA treatment on *N. lugens*, fifth-instar nymphs were collected three days post injection, the time when no significant lethal effect or morphology change was observed. The collected insects were immediately homogenized in TRIzol reagent (TaKaRa) for total RNA extraction. For analysis of plant gene expression in response to insect feeding, rice seedlings at the 4–5-leaf stage were exposed to groups of five ds*GFP*- or ds*NISP6935*-treated *N. lugens* for a continuous 24 h feeding period. For assessing gene expression patterns in transgenic plants overexpressing EV and NISP6935^{-sp}, 4–5-leaf-stage rice plants without treatment were used. Total RNA was extracted following the manufacturer's protocol, and the RNA samples were submitted to Novogene Co., Ltd. (Beijing, China) for transcriptome sequencing. For library construction, poly(A)- RNA was enriched from approximately 20 μg of pooled total RNA using oligo(dT)-conjugated magnetic beads. The purified mRNA was subsequently fragmented at 94°C for 5 min in the presence of divalent cations to generate short templates suitable for cDNA synthesis. After library construction, sequencing was performed on the Illumina NovaSeq™ X Plus platform, generating paired-end reads for downstream bioinformatic analyses. All raw sequence data have been deposited in the National Genomics Data Center under accession numbers PRJCA045459, PRJCA043787 and PRJCA043788.

Raw sequencing reads were initially processed using Novogene in-house pipeline to remove adaptor sequences, reads with ambiguous bases, and low-quality sequences. The resulting clean reads from each cDNA library were then mapped to the reference genomes of *N. lugens* and *O. sativa* with HISAT2 v. 2.1.0 (Kim *et al.* 2015). Alignments with low mapping quality were further discarded using SAMtools v. 1.7 (Li *et al.* 2009). Transcript abundance was quantified as transcripts per million (TPM) with Cufflinks v. 2.2.1 (Trapnell *et al.* 2012). Differentially expressed genes (DEGs) were identified using DESeq2 v. 2.2.1 (Wang *et al.* 2010), with thresholds set at $|\log_2(\text{fold change})|>1$ and adjusted

P-value<0.05. To explore global transcriptomic variation, principal component analysis (PCA) was carried out using the plotPCA function in R (Huang *et al.* 2023). Functional enrichment of DEGs was conducted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, implemented through TBtools v. 1.0697 (Chen *et al.* 2020).

3. Results

3.1. Identification of salivary proteins that potentially associated with planthopper feeding on rice hosts

Species-specific salivary proteins are critical for insects adapting on specific host plants (Huang *et al*. 2025). To reveal the salivary proteins that restricted to rice-feeding planthoppers but not the bamboo-feeding planthoppers, we re-analyzed the salivary proteome data published previously (Huang *et al*. 2018; Wang *et al*. 2024). The Delphacidae-specific salivary proteins in three planthoppers, which can be grouped into 19 orthogroups, were used as queries to search their homologies in the assembled transcriptomic data of *E. nawaii*, respectively (Appendix B). The results showed that 16 orthogroups have homologous genes in *E. nawaii*, while 3 orthogroups (OG0013347, OG0011977, and OG0006935) were specific to rice planthoppers but not *E. nawaii* (Appendix B).

The orthogroup OG0013347, which annotated as annexin-like protein 5, was restricted to *N. lugens*, while the orthogroup OG0011977 and OG0006935 were conserved across three rice planthoppers (Appendix B). Previous work demonstrated that most salivary proteins exhibited salivary gland (SG)-biased expression (van Bel and Will 2016). Therefore, we preliminary analyzed their expression in different tissues *via* the transcriptomic data. The results showed that all genes in orthogroup OG0011977 were expressed in all analyzed tissues, and did not show SG-biased expression (Appendix C). In contrast, all genes in orthogroup OG0006935 showed typical SG-biased expression patterns (Appendix C), and were selected for further analysis.

3.2. NISP6935 is critical for *N. lugens* survivor and reproduction

In previous study, OG0006935 member from *N. lugens* (tentatively annotated as NISP6935) was validated to be secreted into diets (Liu *et al.* 2016), with 7 unique peptides detected by LC-MS/MS (Appendix D). The open reading frame of NISP6935 is 789 bp in length, and potentially encodes a 262 amino acid residue peptide with a predicted molecular weight of 29.5 kDa and an isoelectric point of 10.2 (Fig. 1-A). With the exception of the predicted signal peptide, no other transmembrane domain was detected, indicating the secretory property of this protein. To investigate the presence of NISP6935 homologs, NISP6935 was used as a query to perform BLAST searches against the assembled genomes and predicted coding sequences of 20 insect species. The results showed that NISP6935 homologs were only present in three rice planthopper species, but not in other analyzed insects (Appendix E). Neither NISP6935 nor its homologous genes in other planthopper species have been functionally studied before. In addition, we performed whole-genome sequencing

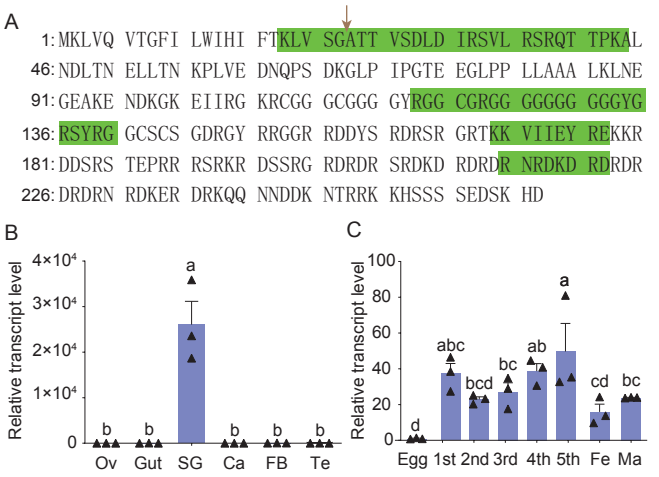

Fig. 1 Characteristics of NISP6935. A, deduced amino acid sequence of NISP6935. Arrow indicates the signal peptide cleavage site. Green shading indicates identified peptides according to LC-MS/MS results (Liu *et al.* 2016). B and C, expression patterns of *NISP6935* in different tissues (B) and at different developmental stages (C) quantified by qRT-PCR. Ov, ovary; SG, salivary glands; Ca, carcass; FB, fat body; Te, testis; Fe, female; Ma, male. Data are presented as mean value±SEM (*n*=3). Different lowercase letters indicate statistically significant differences at *P*<0.05 level according to one-way ANOVA test followed by Tukey's multiple comparisons test.

of *E. nawaii* using the Illumina NovaSeq6000 platform, and the generated raw data (accession no. CRA029439) were preliminarily assembled using Trinity software. The results showed that no homologous sequence of NISP6935 was detected in the *E. nawaii* genome (Appendix E), further confirming its absence.

qRT-PCR analysis demonstrated that *NISP6935* was almost exclusively expressed in the salivary glands and is primarily expressed during the nymph and adult stages, with negligible expression in the egg stage (Fig. 1-B and C). To investigate the potential function of NISP6935, RNA interference (RNAi) was employed. The silencing efficiency was confirmed by RT-qPCR, which showed that ds*NISP6935* effectively suppressed the transcription of the target gene (Appendix F). BPH nymphs were injected with ds*NISP6935* and reared on the resistant rice variety ASD7. Following gene silencing, 92.5% of individuals survived 4 days post-injection (Fig. 2-A). However, survival declined sharply thereafter, with 81.2% survival on day 5, 42.8% on day 7, and nearly complete mortality by day 12. These results were significantly different from those of the ds*GFP*-treated control group (Fig. 2-A). For the morphological observation, the ds*NISP6935*-treated individuals were smaller than that of the ds*GFP* control (Fig. 2-B and C). To determine whether the lethal effects were due to rice resistance, ds*NISP6935*-treated *N. lugens* were also reared on the susceptible rice variety Nipponbare and artificial diets. The results showed that *NISP6935* silencing was lethal regardless of food source (Fig. 2-D; Appendix F), indicating that this gene plays a vital role independent of host plant resistance.

Salivary proteins typically do not induce such severe lethality, except for those involved in salivary sheath formation (Huang *et al.* 2015; Will and Vilcinskas 2015). Therefore,

we examined the salivary sheaths secreted by ds*NISP6935*-treated insects. However, no morphological differences were observed between treated and control groups (Fig. 2-E and F), which contrasts with previously reported sheath alterations caused by silencing *NISHP*, *NIMLP*, or *NISalivap3* (Huang *et al.* 2015, 2016, 2017). Honeydew production is an indicator of feeding activity in planthoppers. We quantified honeydew excretion in dsRNA-treated insects. *NISP6935*-silenced *N. lugens* excreted significantly less honeydew than controls (Fig. 2-G). This reduction may reflect either a weakened physiological status that indirectly impairs feeding behavior or a direct effect of enhanced host plant defenses.

We also treated the newly emerged female *N. lugens* with dsRNA. The results showed that ds*NISP6935*-treated *N. lugens* produced significantly less offspring than that of the control, with the majority of treated insects being unfertilized (Fig. 2-H). We examined the ovaries of treated insects. The results revealed that the ovaries of ds*NISP6935*-treated female adults were underdeveloped at five days post-emergence, containing fewer banana-shaped eggs compared to the ds*GFP*-treated control (Fig. 2-I and J).

3.3. Conserved function of genes from orthogroup OG0006935

Genes in orthogroup OG0006935 is a single copy gene, with each species harboring one gene. Sequence alignment demonstrated that OG0006935 members from *N. lugens*, *L. striatellus* (LsSP6935), and *S. furcifera* (SfSP6935) were conserved at the nuclear and amino acid levels (Appendix G).

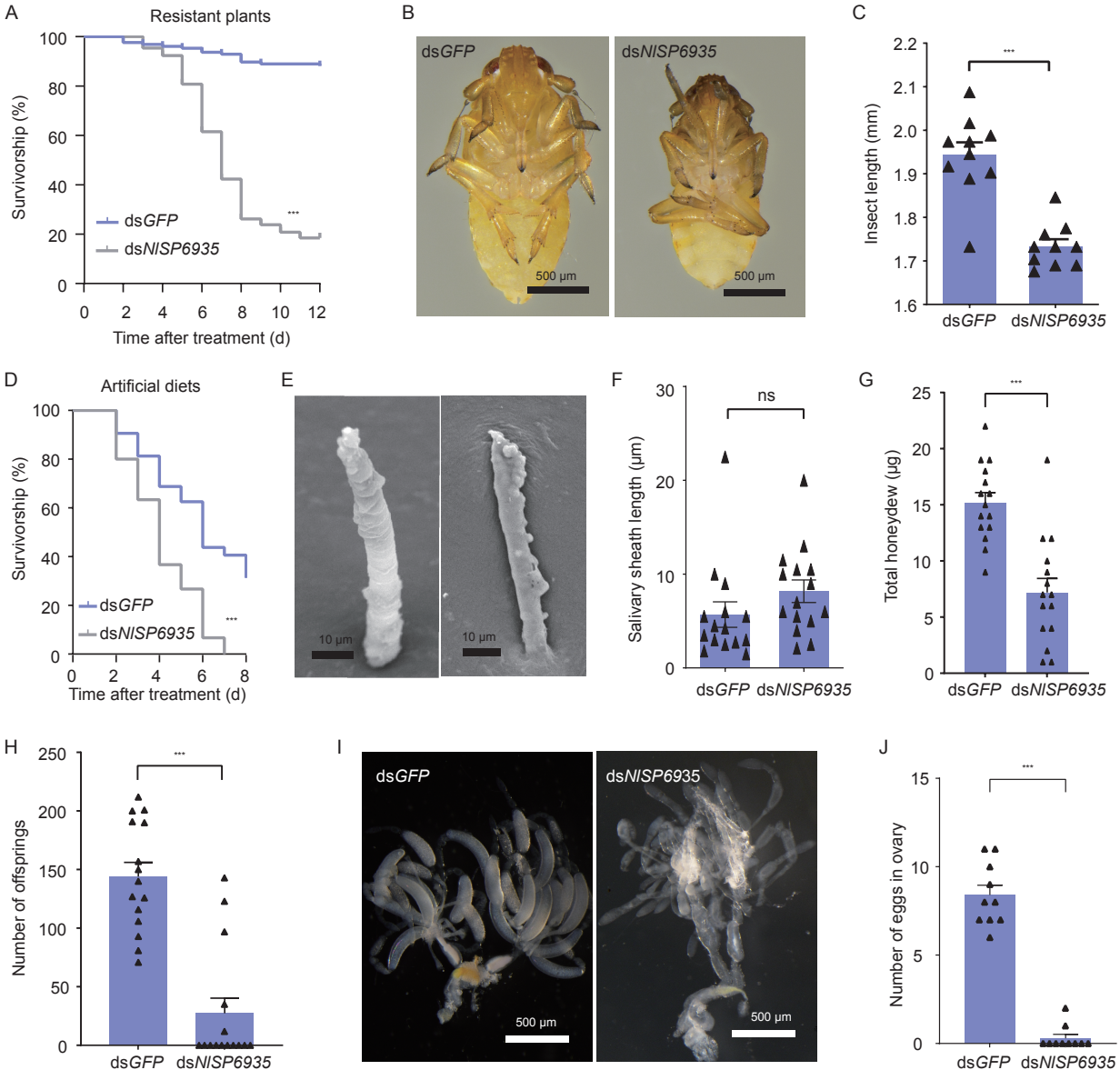

Fig. 2 Effects of *NISP6935* silencing on *Nilaparvata lugens*. A–C, effects of *NISP6935* knockdown on insect survivorship and size when feeding on resistant plant ASD7. D, survivorship of ds*NISP6935*- and ds*GFP*-treated *N. lugens* on artificial diets. E–G, effects of *NISP6935* knockdown on salivary sheath formation and honeydew excretion. H–J, effects of *NISP6935* knockdown on adult reproduction and ovary development. Data are presented as mean value±SEM. *P*-values were determined by log-rank test in A and D, while by two-tailed unpaired Student's *t*-test in other studies. ***, $P<0.001$; ns, not significant.

RNAi was used to investigate the function of LsSP6935 and SfSP6935 (Fig. 3). The results showed that ds*LsSP6935* and ds*SfSP6935* treatments caused severe death phenotype in *L. striatellus* and *S. furcifera* feeding on rice plants and artificial diets, respectively (Fig. 3-B, C, H, and I). Similar with ds*NlSP6935* treatment, the ds*LsSP6935*-/ds*SfSP6935*-treated individuals were significantly smaller than that of the ds*GFP* control (Fig. 3-D, E, J, and K). Additionally, the ovaries of ds*LsSP6935*-/ds*SfSP6935*-treated individuals were underdeveloped at five days post-emergence (Fig. 3-F and L). These results suggest that the functions of genes from orthogroup OG0006935 are conserved across three rice planthoppers.

3.4. Silencing NISP6935 inhibits metabolism- and cuticle-associated genes

To investigate the potential roles of NISP6935 in insect physiology, transcriptomic comparison was performed on ds*GFP*- and ds*NlSP6935*-treated *N. lugens*. Fifth instar nymphs at three days post dsRNA treatments were selected, the time when no significant lethal effect or morphology change was observed.

PCA showed that samples from the biological replicates of each treatment can be well clustered (Fig. 4-A), suggesting the non-negatable influence of NISP6935 on insect physiology. A total of 566 differentially expressed genes (DEGs) were detected, including 493 downregulated DEGs and 73 upregulated DEGs (Appendix H). The *NlSP6935* was the most significantly downregulated, followed by a few cuticular proteins and uncharacterized proteins. In contrast, phospholipase B1, regucalcin, cytochrome P450 4C1-like, and matrix metalloproteinase were most significantly upregulated (Appendix H). KEGG enrichment analysis demonstrated that DEGs associated with metabolisms

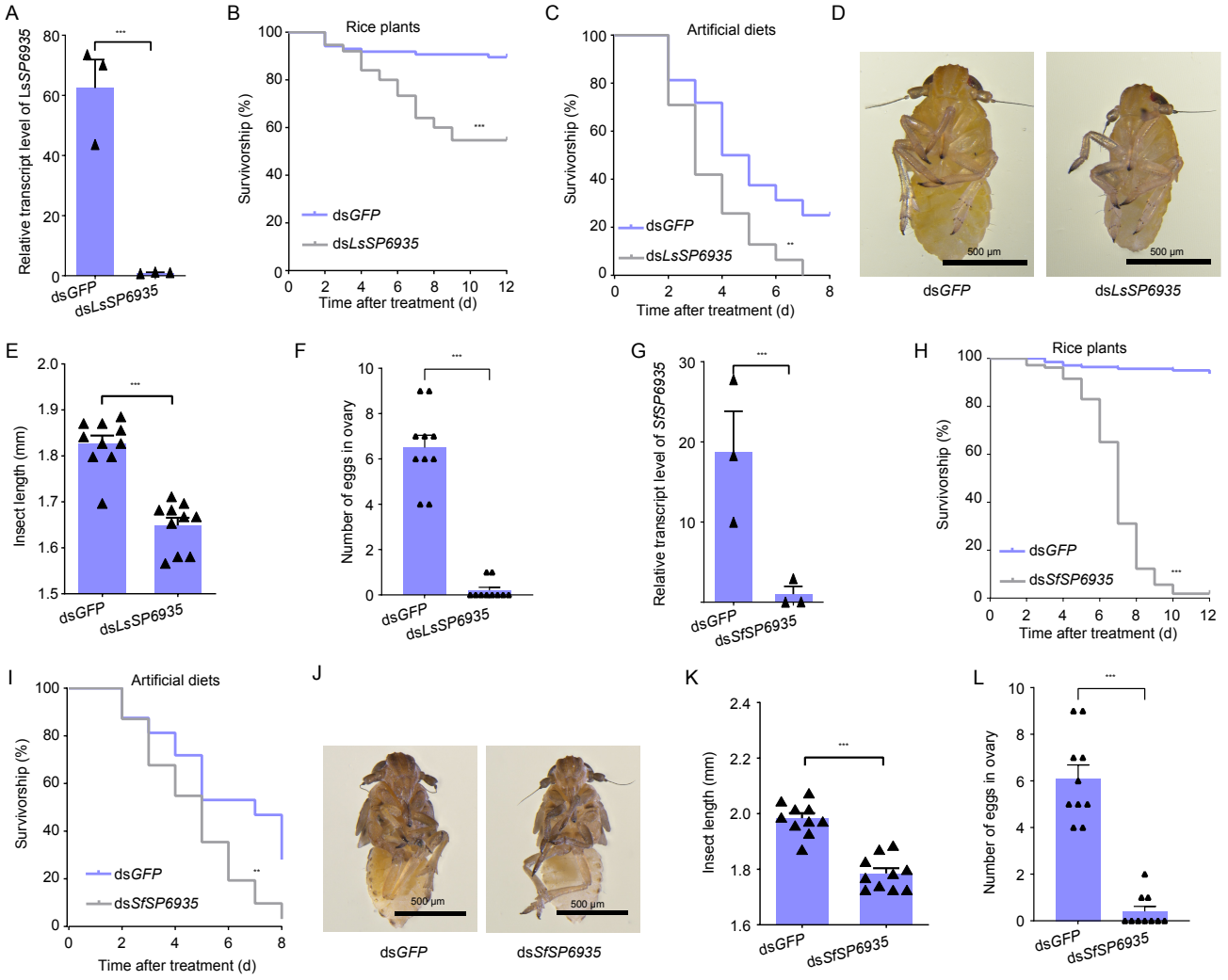

Fig. 3 Effects of *LsSP6935* and *SfSP6935* silencing on *Laodelphax striatellus* and *Sogatella furcifera*. A, the transcript level of *LsSP6935* after dsRNA treatment. B and C, the survivorship of treated *L. striatellus* when reared on rice plants (B) and artificial diets (C). D and E, comparison of *L. striatellus* size after ds*LsSP6935* and ds*GFP* treatments. F, the number of eggs in ovary after ds*LsSP6935* and ds*GFP* treatment. G, the transcript level of *SfSP6935* after dsRNA treatment. H and I, the survivorship of treated *S. furcifera* when reared on rice plants (H) and artificial diets (I). J and K, comparison of *S. furcifera* size after ds*SfSP6935* and ds*GFP* treatments. L, the number of eggs in ovary after ds*SfSP6935* and ds*GFP* treatment. Data in A, E, F, G, K, and L are presented as mean value±SEM, and statistically analyzed by two-tailed unpaired Student's *t*-test. The log-rank test was used to determine the difference in survivorship. ***, $P<0.001$; **, $P<0.01$.

were significantly enriched, including lipid metabolism, carbohydrate metabolism, and amino sugar/nucleotide sugar metabolism (Fig. 4-B). Interestingly, all DEGs in lipid metabolism and amino sugar/nucleotide sugar metabolism were downregulated upon ds*NISP6935* treatments (Fig. 4-C and D). Additionally, there were as much as 49 DEGs annotated as cuticular proteins, with most of them dramatically downregulated (Appendix I). The observed disruptions in metabolic processes and cuticular protein expression may contribute to the lethal phenotype observed in ds*NISP6935*-treated insects. However, we cannot rule out the possibility that the altered expression of metabolism- and cuticle-related genes in *NISP6935*-silenced insects represents an indirect effect of suppressed growth, given that ds*NISP6935*-treated *N. lugens* showed reduced survival and honeydew excretion, which deserved further investigation.

3.5. Transient expression of NISP6935 suppress the H_2O_2 accumulation in *N. benthamiana*

Given that NISP6935 was secreted during insect feeding, the role of NISP6935 on plant immune responses was firstly investigated using *N. benthamiana* transient expression system. As the signal peptide was cleaved before NISP6935 secreted into plants, the truncated NISP6935 without signal peptide (NISP6935^{-sp}) was used. First, we infiltered agrobacterium harboring GFP-fused NISP6935 (NISP6935^{-sp}-GFP) into *N. benthamiana*, with agrobacterium harboring GFP served as a negative control while *Phytophthora infestans* elicitor INF1-GFP and *N. lugens* elicitor NIMLP-GFP served as positive controls. The H_2O_2 accumulation was visualized by DAB staining, which showed that NISP6935-GFP exert no significant effect on H_2O_2 accumulation, which is significantly different from INF1-GFP and NIMLP-GFP (Fig. 5-A and B).

Then, we co-expressed NISP6935-GFP with INF1-GFP and NIMLP-GFP, respectively. The results showed that INF1-GFP- or NIMLP-GFP- induced H_2O_2 accumulation can be successfully inhibited by NISP6935^{-sp}-GFP, but not the GFP control (Fig. 5-C and D). The successful expression of target proteins was visualized by fluorescence signal. It was interestingly to find that NISP6935^{-sp}-GFP was exclusively expressed in plant nucleus, which was significantly different from the other controls (Fig. 5-E). These results suggest that NISP6935 is capable of suppressing the H_2O_2 accumulation triggered by salivary or other elicitors.

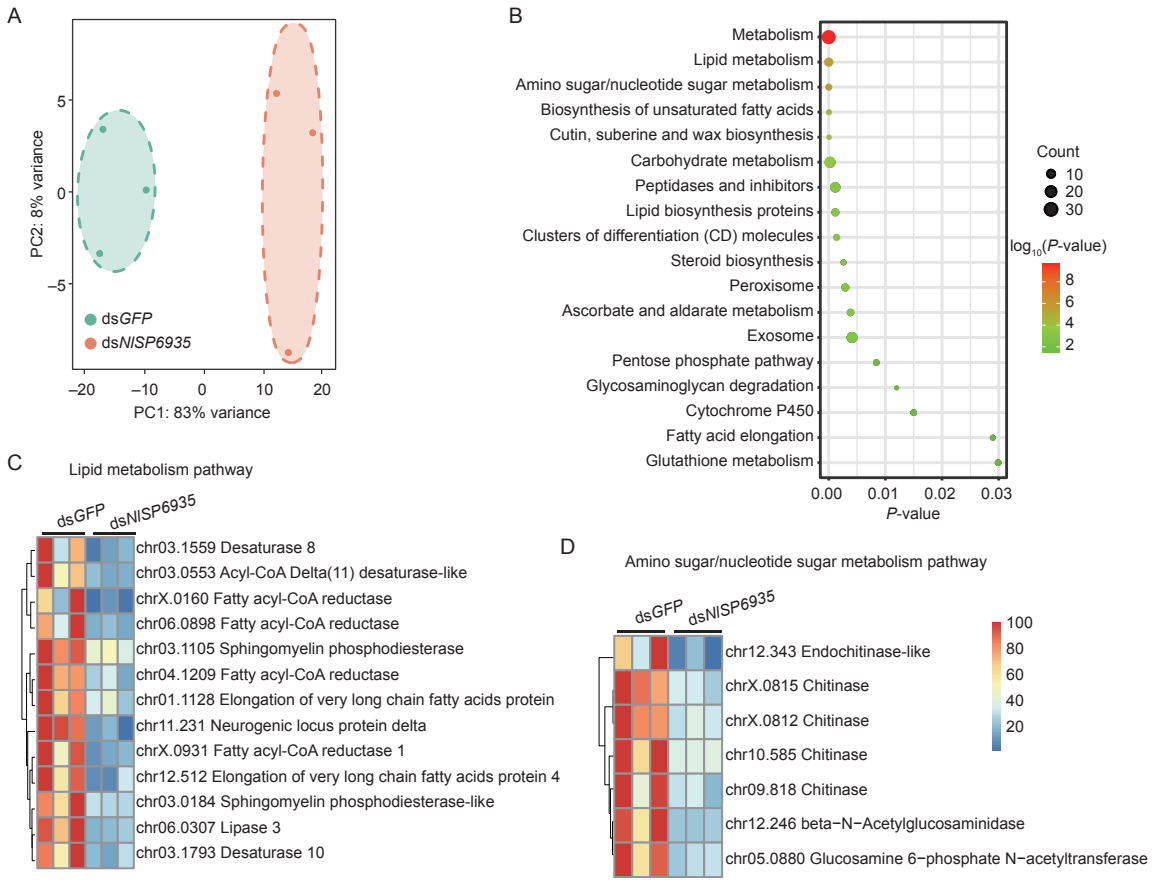

Fig. 4 Gene expression changes of *Nilaparvata lugens* in response to *NISP6935* silencing. A, principal component analysis of gene expression patterns in ds*GFP*- and ds*NISP6935*-treated *N. lugens*. The first two principal components (PC1 and PC2) based on transcriptomic results are shown. B, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes (DEGs) between ds*GFP*- and ds*NISP6935*-treated *N. lugens*. Enriched *P*-values were calculated according to one-sided hypergeometric test. C and D, expression patterns of DEGs associated with lipid metabolism (C) and amino sugar/nucleotide sugar metabolism (D). Data are illustrated by a heatmap based on the transcripts per million (TPM) expression values. The max TPM value of each gene was set as 100.

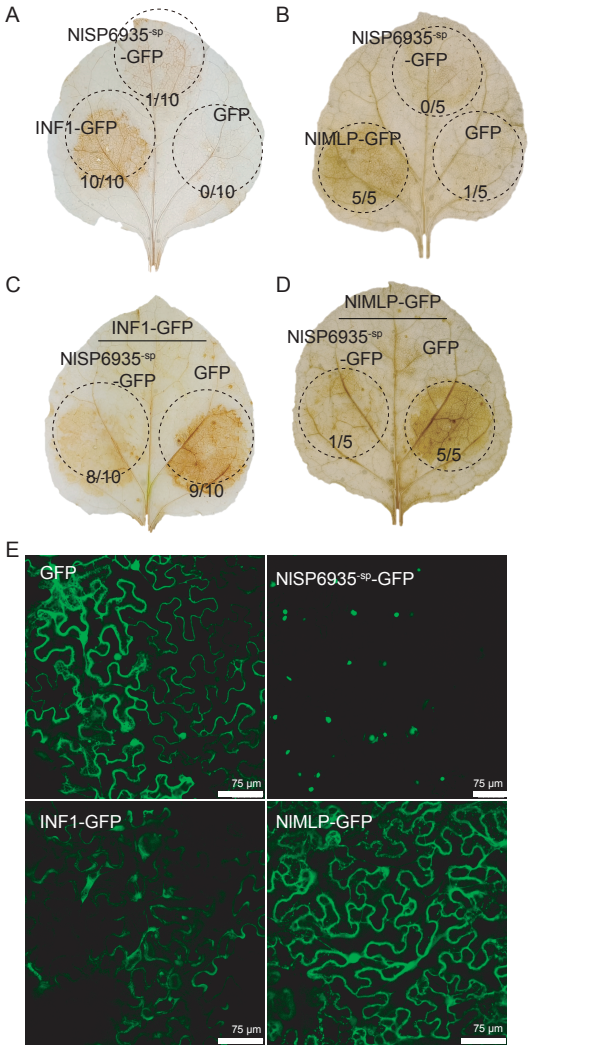

Fig. 5 Effects of NISP6935 overexpression on H_2O_2 accumulation in *Nicotiana benthamiana*. A and B, H_2O_2 accumulation in *N. benthamiana* transiently expressing NISP6935^{-sp}-GFP. Leaves overexpressing GFP served as the negative control, while *Phytophthora infestans* elicitor INF1-GFP and *Nilaparvata lugens* elicitor NIMLP-GFP were used as positive controls. C and D, Influence of NISP6935^{-sp}-GFP and GFP on the H_2O_2 accumulation in *N. benthamiana* expressing INF1-GFP- or NIMLP-GFP. The ratios in the circle represent the time of H_2O_2 positive relative to the total time of the experiments. E, the successful expression of GFP-fused target proteins was visualized by the fluorescence signal.

3.6. Overexpressing NISP6935 benefit insect feeding but cannot rescue the negative effect caused by NISP6935-silencing in *N. lugens*

Previous studies have demonstrated that a deficiency in salivary protein secretion can affect plant physiology (Huang *et al.* 2023; Zhang *et al.* 2024). To this end, we conducted a transcriptomic analysis of rice plants infested by either ds*GFP*-treated or ds*NISP6935*-treated *N. lugens*. PCA revealed clear clustering among biological replicates within each treatment group (Appendix J), underscoring the significance of NISP6935 secretion in modulating plant physiological responses. Comparative analysis identified 161 upregulated and 251 downregulated DEGs in plants infested with ds*NISP6935*-treated insects compared to those infested with ds*GFP*-treated controls (Appendix K). KEGG enrichment analysis indicated that the upregulated DEGs were significantly associated with plant hormone signal transduction, transcription factors, plant–pathogen interaction, and MAPK signaling pathways (Appendix J). Conversely, the downregulated DEGs were predominantly enriched in metabolic pathways, including the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, amino acid metabolism, carbohydrate metabolism, and terpenoid backbone biosynthesis (Appendix J). These findings suggest that impaired secretion of NISP6935 influences both plant defense regulation and metabolic processes.

We also construct transgenic rice plants overexpressing flag-fused NISP6935^{-sp}. Two independent homozygous lines (oxSP#1 and oxSP#2) were selected. The empty vector (EV) transgenic plant was used as a negative control (Fig. 6-A). Three bioassay experiments were conducted, including the insect fecundity, plant attraction to *N. lugens*, and insect developmental duration. No significant differences were observed in *N. lugens* fecundity between EV and oxSP plants (Fig. 6-B). However, oxSP plants were more attractive to tested insects than that of EV plants (Fig. 6-C and D), and *N. lugens* feeding on oxSP plants exhibited accelerated development (Fig. 6-E). These results suggest that NISP6935 overexpression in rice benefits *N. lugens*.

To determine whether NISP6935 overexpression in rice could counteract the negative effects of NISP6935 silencing, we reared ds*NISP6935*-treated *N. lugens* on EV and oxSP plants. The results showed that ds*NISP6935*-treated insects still exhibited lethal effects on both oxSP lines, though the severity was slightly reduced (Fig. 6-F). This suggests that NISP6935 overexpression cannot rescue the negative effect caused by NISP6935-silencing.

To investigate the potential role of NISP6935^{-sp} after secreted into host plants, transcriptomic analysis was performed on EV, oxSP#1, and oxSP#2 transgenic plants. PCA showed that samples from oxSP#1 and oxSP#2 clustered together, which can be well separated from that of the EV plants (Fig. 6-G), suggesting that NISP6935^{-sp} affects plant physiology when overexpressed. A total of 255 and 1,175 DEGs were detected in oxSP#1 *vs*. EV and oxSP#2 *vs*. EV, respectively (Fig. 6-H). The co-regulated genes in oxSP#1 and oxSP#2, including 102 co-downregulated and 80 co-upregulated DEGs, were selected to investigate the specific role of NISP6935^{-sp} on plants. DEGs such as terpene synthase, iso-kaurene synthase, pathogenesis-related (PR) protein osmotin/1/4/5, were dramatically downregulated in oxSP plants, while ankyrin-1-like, protodermal factor 1, ATP binding cassette subfamily B19, and lipid transfer protein were dramatically upregulated (Appendix L). KEGG enrichment analysis demonstrated that the downregulated DEGs were mainly associated with diterpenoid biosynthesis, metabolism of terpenoids and polyketides, and phenylpropanoid biosynthesis (Appendix M). In contrast, most upregulated DEGs were associated with lipid metabolism, carbohydrate metabolism, glycerophospholipid metabolism, and linoleic acid metabolism (Appendix M). Noteworthily, a total of 7 DEGs involved in terpenoid metabolism, all of which

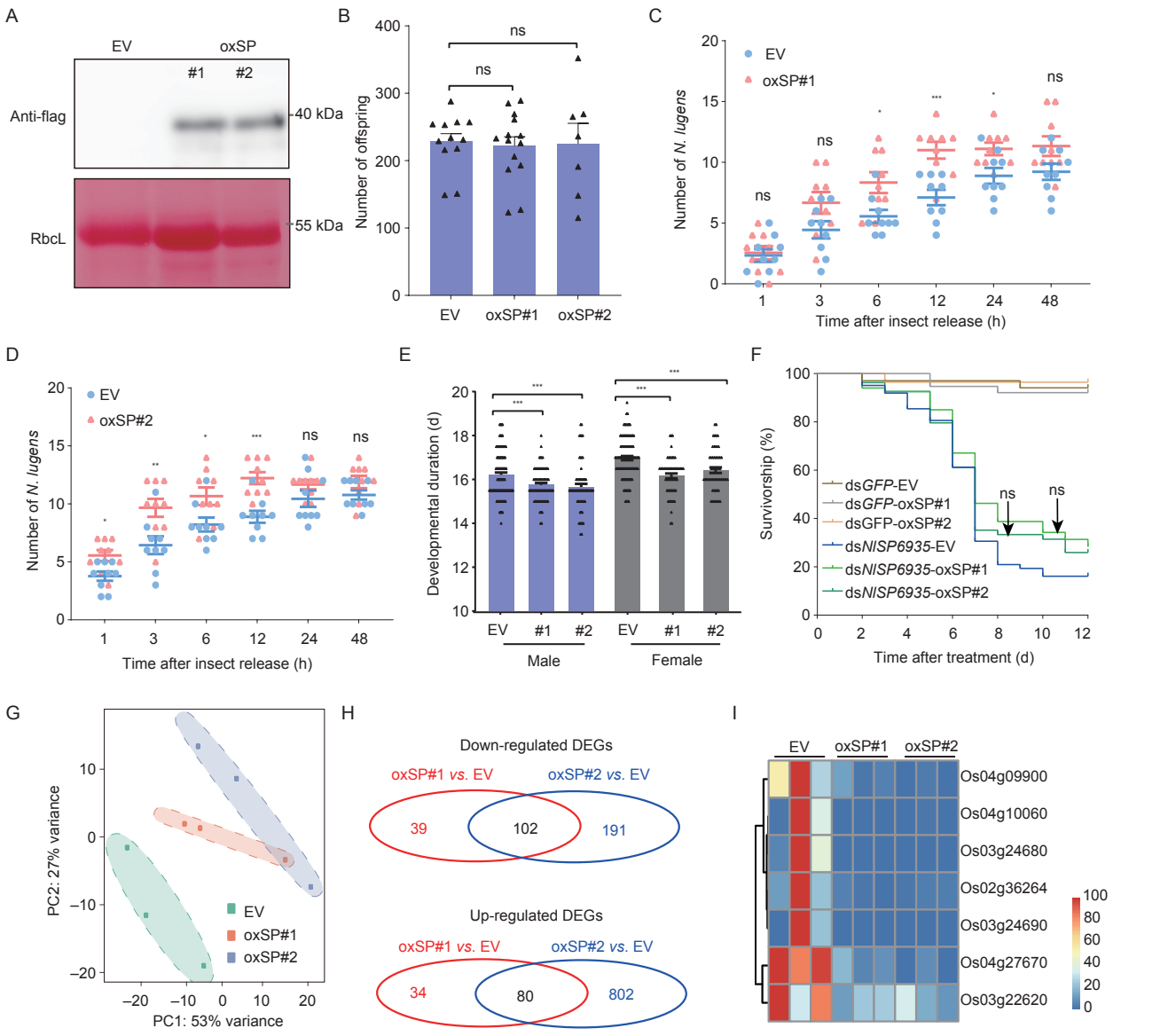

Fig. 6 Effects of NISP6935 overexpression on transgenic rice plants. A, protein levels of NISP6935 in two independent transgenic rice plants overexpressing NISP6935^{-sp}-flag (oxSP). B–E, effects of oxSP plants on insect reproduction (B), feeding preferences (C and D), and developmental duration (E). F, survivorship of dsRNA-treated *Nilaparvata lugens* feeding on EV and oxSP plants. Data in B to E are presented as mean value±SEM, and statistically analyzed by two-tailed unpaired Student's *t*-test. The log-rank test was used to determine the difference in survivorship. ***, P<0.001; **, P<0.01; *, P<0.05; ns, not significant. G, principal component analysis of gene expression patterns in EV, oxSP#1, and oxSP#2 plants. The first two principal components (PC1 and PC2) based on transcriptomic results are shown. H, analysis of differentially expressed genes (DEGs) in oxSP#1 *vs*. EV and oxSP#2 *vs*. EV. The co-downregulated and co-upregulated DEGs are displayed in Venn diagram, respectively. I, expression patterns of DEGs associated with terpenoid metabolism. Data are illustrated by a heatmap based on the transcripts per million (TPM) expression values. The max TPM value of each gene was set as 100.

were dramatically downregulated in oxSP plants (Fig. 6-I), suggesting the inhibition of defense-associated secondary metabolites.

4. Discussion

Herbivorous insects have evolved sophisticated feeding strategies, with their saliva playing a pivotal role in this process. A deeper understanding of plant–insect interactions can offer valuable insights into the evolution of life. In this study, we investigated salivary proteins uniquely identified in rice planthoppers, and revealed that the rice planthopper-specific orthogroup OG0006935 was critical for insect survival, as it suppressed plant defenses and regulated the insect's intrinsic physiology. These findings expand our understanding of salivary proteins, highlighting their dual role in modulating plant defenses and influencing insect physiology.

The orthogroup OG0006935 was exclusively identified in three rice planthopper species. All genes within this orthogroup were single-copy and exhibited high sequence and functional conservation across these planthoppers. Notably, despite the evolutionary proximity of *E. nawaii* to rice

planthoppers, no homologous sequences were detected in *E. nawaii*, even after thorough searches of its transcriptomic and genomic data. This absence suggests two possible evolutionary scenarios: either OG0006935 was lost in bamboo-feeding planthoppers or it was newly acquired in the rice-feeding lineage. Previous studies have demonstrated that host adaptation in Hemiptera frequently involves salivary protein gene family expansions or new gene acquisition (Wybouw *et al.* 2016; Huang *et al.* 2024; Hu *et al.* 2025). The strict conservation of OG0006935 among the three rice planthoppers implies that its acquisition likely predated their speciation, possibly coinciding with their adaptation to rice hosts. However, since orthogroup classification relies solely on sequence similarity, we must consider that functionally analogous proteins in *E. nawaii* may exist despite lacking detectable homology (Altenhoff *et al.* 2019). Indeed, some fungal effectors with divergent sequences but conserved structural features perform comparable roles (Le Naour-Vernet *et al.* 2025). Thus, while no OG0006935 homologs were identified in *E. nawaii*, we cannot exclude the possibility of functional convergence by other proteins in this species.

OG0006935 members were indispensable for planthopper survival, as RNAi-mediated silencing resulted in severe lethality across all three rice planthopper species (Figs. 2 and 3). Given the high conservation of OG0006935 members at the nuclear acid level (Appendix G), SP6935 might be a promising target for developing transgenic RNAi rice or RNA-based pesticides to control planthopper populations. To date, most characterized insect salivary proteins function primarily in suppressing plant defenses, with minimal or no lethal effects on the insects themselves (Liu *et al.* 2024; Hu *et al.* 2025; Shang *et al.* 2025). In a few cases, the fitness costs associated with salivary gene knockdown could be rescued by overexpressing the corresponding protein in host plants (Huang *et al.* 2023; Fu *et al.* 2025). Our study revealed that *NISP6935* silencing induced high mortality even on susceptible rice plants, artificial diets, and NISP6935 overexpressing plants, suggesting that NISP6935 exerts role beyond plant defense regulation and nutrient acquisition. Transcriptomic analysis further demonstrated that *NISP6935* knockdown disrupted critical metabolic pathways (e.g., lipid and carbohydrate metabolism) and significantly downregulated cuticular proteins (Fig. 4). This metabolic dysregulation, coupled with impaired cuticle formation, likely underlies the rapid mortality post-RNAi, particularly given that several affected pathways include known targets for pest control (Xi *et al.* 2015; Zhao *et al.* 2025). However, an alternative interpretation is that the transcriptomic changes reflect secondary consequences of growth impairment in ds*NISP6935*-treated *N. lugens*. Previous studies have shown that knockout of other salivary genes, such as *NISP5* and *NISP7*, significantly prolonged nymphal development time in *N. lugens* (Liu *et al.* 2024). Thus, the mechanistic link between NISP6935 and core metabolic/cuticular processes remains unclear, presenting a key question for future research.

Plants have evolved specific receptors to recognize insect salivary elicitors or wound-derived signals, activating pattern-triggered immunity, including a burst of reactive oxygen species (ROS) and the activation of defense genes (Wu and Baldwin 2009; Guiguet *et al.* 2016; Jiang *et al.* 2019). In response, insects and pathogens secrete effector proteins to disrupt these defense mechanisms. Salivary effector such as DcE1 from *Diaphorina citri*, Sm10 from *Sitobion miscanthi*, and AI106 from *Apolygus lucorum* have been widely reported to suppress the elicitor-induced H_2O_2 accumulation (Dong *et al.* 2022; Fu *et al.* 2023; Kuang *et al.* 2024). A few salivary proteins from planthoppers have been reported to trigger ROS burst, but the factors that counteract this elicitation remain limited (Shangguan *et al.* 2018; Qi *et al.* 2025a, b). By overexpressing NISP6935 in *N. benthamiana* and rice plants, our study confirmed that NISP6935 functions as a suppressor of plant defense responses, particularly by inhibiting H_2O_2 accumulation and modulating defense-related gene expression. Notably, NISP6935 overexpression significantly downregulated terpenoid biosynthesis genes (Fig. 6-I). Terpenoids, act as the most common group of secondary metabolites and volatile compounds, play a critical role in plant defense response to planthoppers (Zhan *et al.* 2022). The downregulated terpenoid metabolism might partially explain the enhanced attrition of oxSP plants to planthoppers (Fig. 6-C and D).

5. Conclusion

Our study identifies NISP6935 as a key salivary effector in rice planthoppers, essential for both insect survival and plant defense suppression. Silencing *NISP6935* disrupts insect metabolism and cuticle formation, while its expression in plants inhibits elicitor-induced H_2O_2 accumulation and terpenoid-based defenses. Crucially, transgenic NISP6935 rice only weakly rescues RNAi lethality, revealing its dual role in insect physiology and plant manipulation. These findings highlight a conserved adaptation mechanism in planthoppers and offer new targets for pest control.

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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 the writing process

During the preparation of this manuscript, the authors used ChatGPT 4.0 and DeepSeek to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

Ethical statements

All applicable international, national and institutional guidelines

for the care and use of animals were followed.

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