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Developmental transcriptomics reveals stage-specific immune gene expression profiles in *Spodoptera frugiperda*

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The fall armyworm, *Spodoptera frugiperda*, a globally invasive pest, demonstrates distinct immune adaptations across developmental stages and sexes, which are critical for its survival and adaptability. Using high-throughput RNA sequencing, this study systematically profiled 56 immune-related gene families, identifying 157 genes involved in Toll and Imd signaling pathways, and 185 genes associated with cellular immunity. Dynamic expression patterns were observed, with humoral immunity indices peaking during the third (L3) and fifth (L5) instars and diminishing in the pupal (P) and egg stages. In contrast, cellular immunity indices were highest in pupae and adult females, while the sixth instar (L6) and adult males exhibited the lowest immune capacity. Female adults displayed superior immune potential compared to males, reflecting evolutionary pressures tied to reproductive fitness. Notably, larval stages exhibited heightened immune gene expression, which aligns with their vulnerability to pathogens. Validation via qRT-PCR confirmed these transcriptomic trends, highlighting the modulation of immunity throughout development. These findings offer novel insights into the interplay between developmental progression and immune regulation in *S. frugiperda*. By elucidating these stage-specific immune responses, this study provides a robust framework for developing targeted pest management strategies aimed at mitigating the impact of this destructive pest.

Keywords Cellular immunity, Development, Humoral immunity, *Spodoptera frugiperda*, Transcriptomics

The immune system plays a pivotal role in safeguarding organisms from challenging environmental conditions and microbial threats. In insects, host defense mechanisms are centered on the innate immune system, which counters a wide array of pathogens. This system is broadly categorized into humoral and cellular immunity. Humoral immunity functions through evolutionarily conserved signaling pathways, culminating in the production of effector molecules such as antimicrobial peptides. In contrast, cellular immunity involves hemocyte-mediated processes, including phagocytosis, nodulation, encapsulation, and coagulation[1]. Several intrinsic and extrinsic factors, including sex, mating status, social interactions, feeding behavior, and locomotion, influence immune responses in insects[2,3]. In vertebrates, immune competence is closely tied to age[4]. Similarly, in holometabolous insects, which undergo complete metamorphosis through egg, larval, pupal, and adult stages, susceptibility to pathogens fluctuates across developmental stages[5].

Insect immunity involves highly specialized mechanisms to recognize and eliminate pathogens, preventing their spread within the host. Key to this process are pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs) on invading microorganisms. These PRRs, categorized as either secreted or membrane-bound proteins, trigger conserved signaling pathways essential to the immune response. The Toll pathway primarily targets fungi and Gram-positive bacteria, while the Immune Deficiency (Imd) pathway

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is specialized for defense against Gram-negative bacteria. These signaling cascades drive the production of antimicrobial peptides and other effector molecules that neutralize pathogens[6]. On the cellular front, hemocyte-mediated responses, including hemolymph coagulation, clot formation, and pathogen encapsulation, are critical for immediate defense. Hemocytes rapidly accumulate at invasion sites, forming barriers to contain the pathogen and collaborating with humoral defenses to clear infections effectively. This interplay between cellular and humoral components ensures a robust and adaptive innate immune response in insects[7].

Insect immunity research often focuses on the differential expression of immune genes at various stages of the infection process[8,9]. However, recent findings indicate that the developmental stage of insects also plays a significant role in regulating immune capacity[10]. For example, the effects of *Beauveria bassiana* on *Chilo suppressalis* larvae have been investigated previously[11]. The authors found significant differences in LT_{50} values across instars, with a clear trend of increasing LT_{50} as larval developmental stage advanced. Similarly, another study evaluated the efficacy of *Metarhizium rileyi* against 2nd and 4th instar larvae of *Spodoptera frugiperda*. The results showed a median survival time of approximately 7.5 days for 2nd instar larvae, whereas the cumulative mortality of 4th instar larvae remained below 50%[12]. Furthermore, other studies have shown that fluctuation of phenoloxidase enzyme activity in female insects forms is a strategic adaptation to extend survival and maximize offspring production. In beetles, RNA interference (RNAi)-mediated silencing of phenoloxidase has been shown to prolong survival[13]. Immune responses can also vary within the same larval stage. In *Manduca sexta*, fifth instar larvae on day five exhibit fewer hemocytes and reduced capacities for nodulation, phagocytosis, and phenoloxidase activity compared to first-day fifth instar larvae[3]. Similarly, aging *Apis mellifera* honey bees demonstrate a decreased ability to resist pathogens and experience a higher intensity of infection[14]. This suggests a complex interrelationship between development and immunity.

Spodoptera frugiperda (J.E. Smith) (Insecta: Lepidoptera: Noctuidae), commonly known as the fall armyworm, is a highly destructive noctuid pest native to the Americas. Known for its great adaptability and resistance to control measures, this pest has become a serious threat to cultivated crops worldwide. While previous studies have identified and characterized genes involved in its immune response, they have primarily focused on specific developmental stages without providing a comprehensive analysis of immune gene expression across its entire life cycle[15]. Furthermore, the interplay between immune gene expression and developmental progression remains insufficiently explored.

The current study employed high-throughput RNA sequencing (RNA-Seq) to profile the expression of immune-related genes across all developmental stages of *S. frugiperda*, including eggs, first to sixth instar larvae, and adult males and females. The results aim to provide a robust theoretical framework for understanding insect immune mechanisms within the context of development. These findings could provide a basis for improving biological control methods against this pest, which is invasive and a global threat.

Materials and methods

Sample preparation, RNA extraction, and sequencing

Fall armyworms, *S. frugiperda*, were collected from the cornfield in Kunming, Yunnan Province, China. They were reared individually in plastic boxes containing fresh corn leaves. The colonies were maintained at 28 ± 3 °C with 70–75% relative humidity and a photoperiod of 16:8 h (L: D). Adults of *S. frugiperda* were kept in 30 cm × 40 cm mesh cages, provided with fresh 10% honey water and corn seedlings for egg laying. Eggs were collected within 24 h after they were laid. The larvae of the 1st to 6th instars were all collected on the second day after molting and were of the same size. Three-day-old pupae and unmated adult insects were also collected. For each experimental replicate, RNA was meticulously extracted from 300 eggs, alongside whole-body samples from 200 first-instar larvae, 100 s-instar larvae, 50 third-instar larvae, 10 fourth-instar larvae, 5 fifth-instar larvae, and 5 sixth-instar larvae. Furthermore, RNA was obtained from 5 pupae, 5 adult females, and 5 adult males, ensuring comprehensive coverage across all developmental stages. Samples from all stages were collected in triplicate.

Trizol Reagent (Invitrogen, Waltham, MA, USA) was used for total RNA extraction following the manufacturer's instructions. Genomic DNA was extracted using Turbo DNase (Thermo Fisher, Waltham, MA, USA). Gel extraction was performed, and the quality and quantity of the RNA samples were assessed using a Nanodrop ND-1000 spectrophotometer (LabTech, Hopkinton, MA, USA). RNA libraries were constructed and sequenced as previously described[16]. RNA-seq was conducted by a commercial company (Lianchuan, Hangzhou, China) using a next-generation sequencing platform (Illumina NovaSeq™ 6000). Expressed genes across all developmental stages were analyzed using the DESeq R package (version 1.10.1)[17].

Transcriptome assembly, annotation, and analysis

Low-quality reads, defined as those with more than 20% of bases having a quality score less than 10, reads containing adapters, and reads with more than 5% unknown bases (N bases), were removed using FastQC v.0.12.0 and Trimmomatic V0.32 (http://www.usadellab.org/cms/?page=trimmomatic). Paired-end clean reads were mapped to the *S. frugiperda* genome (http://v2.insect-genome.com/Organism/715) using the HiSat2 aligner. The assembled unigene sequences of *S. frugiperda* (Supplemental Table S3)were then compared to several databases, including Nr (non-redundant protein databases), SwissProt, COG (Cluster of Orthologous Groups), and KEGG (Kyoto Encyclopedia of Genes and Genomes)[18]using the BLASTx algorithm with a cut-off E-value of 10^{-5} (Supplemental Table S2). The RNA-Seq data were submitted to the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra).

Gene expression analyses were conducted using the DEGSeq R package, with P-values adjusted by the Benjamini-Hochberg procedure. Significant differential expression was determined with a P-value threshold of 0.05 and a log2(fold change) of 1[19,20]. Putative immunity-related genes were analyzed by searching the annotation and then manually screened with other identified immune-related genes through blastP and conserved domain analysis. The Toll and Imd pathway-related genes were identified and analyzed using the map04624: Toll and

Imd signaling pathway from the KEGG database[21]. The heatmaps of IRG in each stage were generated with log2(IRG_FPKM).

Quantitative real-time PCR (RT-qPCR)

Beacon Designer Software (Palo Alto, CA, USA) was utilized to design primers (Supplemental Table S1), with *RPL32* (Ribosomal Protein L32) serving as the reference gene. Q-RT-PCR experiments were conducted following the Minimum Information Required for Publication of Quantitative Real-Time PCR guidelines. A Premix Ex Taq™ II (Tli RNaseH Plus) Kit (Takara, Shiga, Japan) was used as the reagent for qRT-PCR analysis. The reaction conditions were set as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Reactions were performed in triplicate, with dissociation analysis conducted using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All developmental stages and sexes were examined with three separate biological replicates. The data were analyzed using the 2−ΔΔCT method [ΔΔCt = ΔCt (test) − ΔCt (calibrator)][16]. The following genes were selected for the q-RT-PCR assay as representatives specifically expressed at different developmental stages: *GNBP_Sfru123480*, *PGRP_Sfru122870*, *Toll_Sfru073570*, *SPZ_Sfru073580*, *DEF_Sfru018720*, *Cec_Sfru063640*, *AAMP_Sfru123110*, *LP_Sfru056250*, *dSR-CI_Sfru104410*, *and Integrin_Sfru080300.*

Results

Overview of immune related genes and their expression trends

A total of 157 genes related to the Toll and Imd signaling pathways and 185 genes associated with cellular immunity were differentially expressed between developmental stages or sexes in *S. frugiperda* ($p < 0.05$). To reveal the overall humoral immune capacity in each developmental stage and sex of *S. frugiperda*, the sum of Log2(FPKM) involves all humoral IRGs in various classifications, including recognition, modulation (serine protease and serine protease inhibitor), Toll and Imd transduction, and effector, which were defined as the age-dependent humoral immune index. The results showed that the total humoral immunity indices for L3 and L5 were the highest, reaching 233.64 and 210.14, respectively (Fig. 1A). These were followed by 4th instar (L4), 2nd instar (L2), and adult females (AF) with 186.28, 172.35, and 163.93, respectively (Fig. 1A). The overall humoral immunity index for 1st instar (L1), 6th instar (L6), and adult males (AM) was similar, 131.04 ~ 155.89, whereas for pupal stage (P) was 2.32, and for eggs was the lowest (− 304.07) (Fig. 1A).

Similarly, the sum of all related genes in these functional categories was calculated to reveal the development-dependent cellular immunity index. The total index showed a progressive upward trend from the egg stage to the L4 stage, with the number increasing from 23.0 to 145.3, with a minimum of − 77 at the L6 stage, followed by peaks at the pupal (295.7) and adult female stages (271.0) (Fig. 1B). Notably, the total cellular immunity index was very low in adult males (− 7.1) compared to adult females (Fig. 1B). The trend of the hemocyte-mediated immunity index was similar to the total cellular immunity index, but the values were much lower, ranging from − 121.4 to 45.3 (Fig. 1B).

Humoral immunity

Recognition

Pathogen recognition relevant immune genes index, the sum of Log2(FPKM) of five identified Gram-negative bacteria-binding proteins (*GNBPs*) and eight identified peptidoglycan recognition protein (*PGRP*), showed a gradual increase from egg to adult stages, with the egg stage being the smallest (-45.24) and the adult stage being the highest (62.21) (Fig. 2A). The overall expression level of all genes at each developmental stage showed that *GNBP_Sfru123480* had the highest expression and *PGRP_Sfru122870* had the lowest expression (Fig. 2B). The *GNBP* family was commonly expressed throughout all developmental stages and sexes. The expression levels

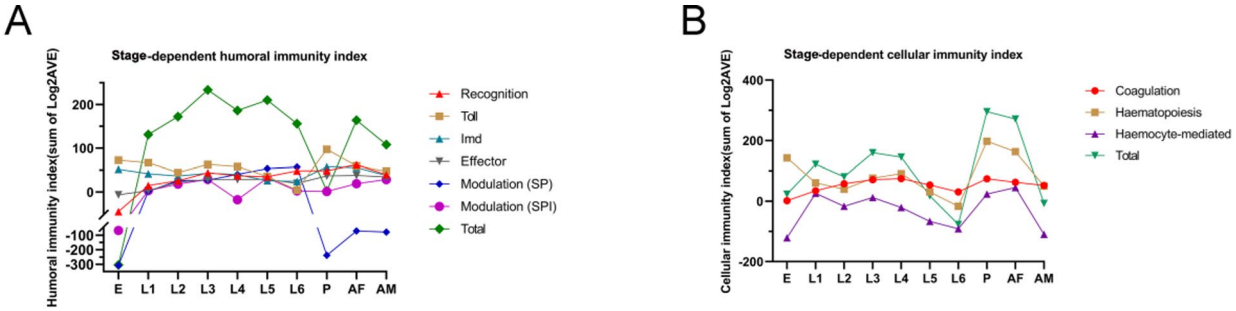

Fig. 1. Trends in the total gene expression of humoral and cellular immune index and their individual components index at different developmental stages in *Spodoptera frugiperda*. (**A**) Diagram of total humoral immune gene expression, recognition-related genes, Toll pathway-related genes, Imd pathway-related genes, effector genes, serine protease genes (SP), and serine protease inhibitors (SPI) in different developmental stages of *S. frugiperda* (the sum of Log2FPKM is used as the index calculation method). (**B**) Diagram of cellular immune gene expression index of *S. frugiperda* of developmental ages (the sum of Log2FPKM is used as the index calculation method). E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

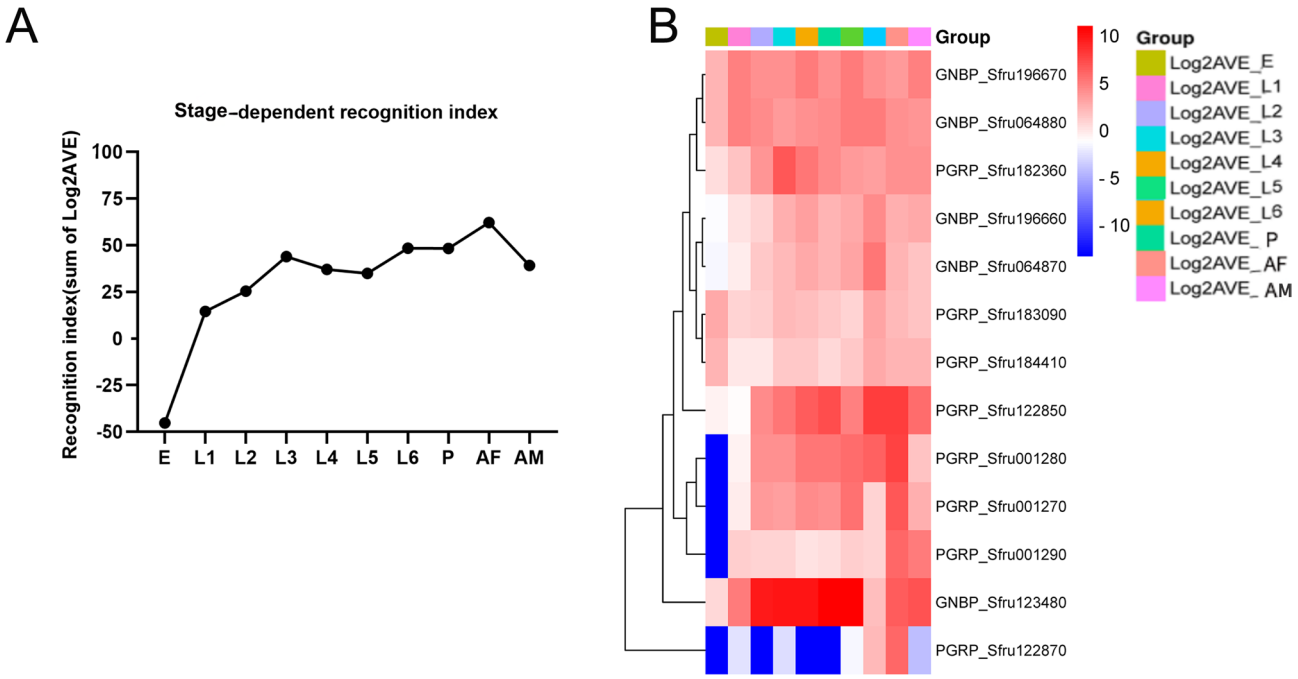

Fig. 2. Immune recognition-related gene expression profiles at different developmental stages of *Spodoptera frugiperda*. (**A**) Trends in total immune recognition gene expression (*GNBPs* and *PGRPs*) (the sum of Log2FPKM) at different developmental stages of *S. frugiperda*. (**B**) Heatmap of individual immune recognition gene expression profile (Log2FPKM) in humoral immunity at various developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

of two β-1,3-glucan-binding protein-like genes (*GNBP_Sfru064880* and *GNBP_Sfru196670*) were shown to be moderate at all developmental stages (Fig. 2B). The other two β-1,3-glucan-binding protein-like genes (*GNBP_Sfru064870* and *GNBP_Sfru196660*) were expressed at lower levels in egg, L1, and L2 stages than in the other developmental stages, and their expression levels peaked at the pupal stage (Fig. 2B). In contrast, the *PGRP* family contains four genes (*PGRP_Sfru001280*, *PGRP_Sfru001270*, *PGRP_Sfru001290*, and *PGRP_Sfru122870*), which were expressed at a lower level in the egg stage, but they were widely expressed at the other developmental stages (Fig. 2B). The expression patterns of the four PGRPs differed among developmental stages. Among them, the *PGRP*-like isoform X2 gene (*Sfru182360*) and the *PGRP*-like gene (*Sfru122850*) showed very high expression levels that extend from the L2 larval stage to the adult stage (Fig. 2B). The former gene showed low expression levels in the egg stage and moderate levels in the L1 stage, while the latter gene showed low transcript abundance in both the egg and L1 stages (Fig. 2B). The expression level of the *PGRP* LB-like gene (*Sfru001280*) was very high in the L2 and up to the female adult stages and moderate in the male adult stage (Fig. 2B). However, it was expressed at low levels in the egg and L1 stages. The transcript abundance of the *PGRP* LB-like gene (*Sfru001270*) was high from L2 to the adult stage of both males and females and low in the egg and L1 stages, except for the pupal stage (Fig. 2B). Another *PGRP* LB-like gene (*Sfru001290*) was highly expressed in female and male adults but had moderate to low expression in other stages, with the lowest transcript abundance in eggs (Fig. 2B). The *PGRP*-like isoform X2 (*Sfru122870*) showed a high expression pattern in nymphs and female adults and low expression levels in the other eight stages (Fig. 2B). In contrast to the first six genes of the *PGRP* gene family, which showed very low expression levels during the egg stage, the transcript abundance of the remaining two *PGRP* genes, *PGRP SB2*-like isoform X2 (*Sfru184410*) and *PGRP LC*-like isoform X1 (*Sfru183090*), was high during the egg stage and differed in all the other stages (Fig. 2B).

Toll signaling genes

Twenty-eight differentially expressed genes involved in Toll signaling were identified, representing 8 gene families. The total expression of genes in the Toll signaling pathway showed a gradual decrease from the egg and L1-L6 stages, reaching a minimum value of 4.14 at the L6 stage, and then fluctuated from the L6 stage to the adult stage, eventually reaching a maximum value of 97.37 at the pupal stage (Fig. 3A). A total of 14 Toll receptor-like genes were identified in *S. frugiperda*, most of which showed similar expression patterns in the egg, L1, L6, and pupal stages. Seven protein *Toll* genes (*Toll_Sfru156630*, *Sfru078860*, *Sfru078650*, *Sfru078750*, *Sfru191410*, *Sfru156680*, *and Sfru087470*) were more expressed in the egg and pupal stages than in the other eight stages, while the opposite was true for genes *Sfru052470*, *Sfru191290*, *Sfru078770*, and *Sfru073570* (Fig. 3B). High expression levels were observed in all developmental stages except for egg and L6. The remaining three Toll genes had high transcript abundance in the L2-L5, pupal, and adult stages, with different expression patterns in the remaining stages (Fig. 3B). Two genes coding for Toll-interacting proteins (*TOLLIP_Sfru194190*, *Sfru085820*)

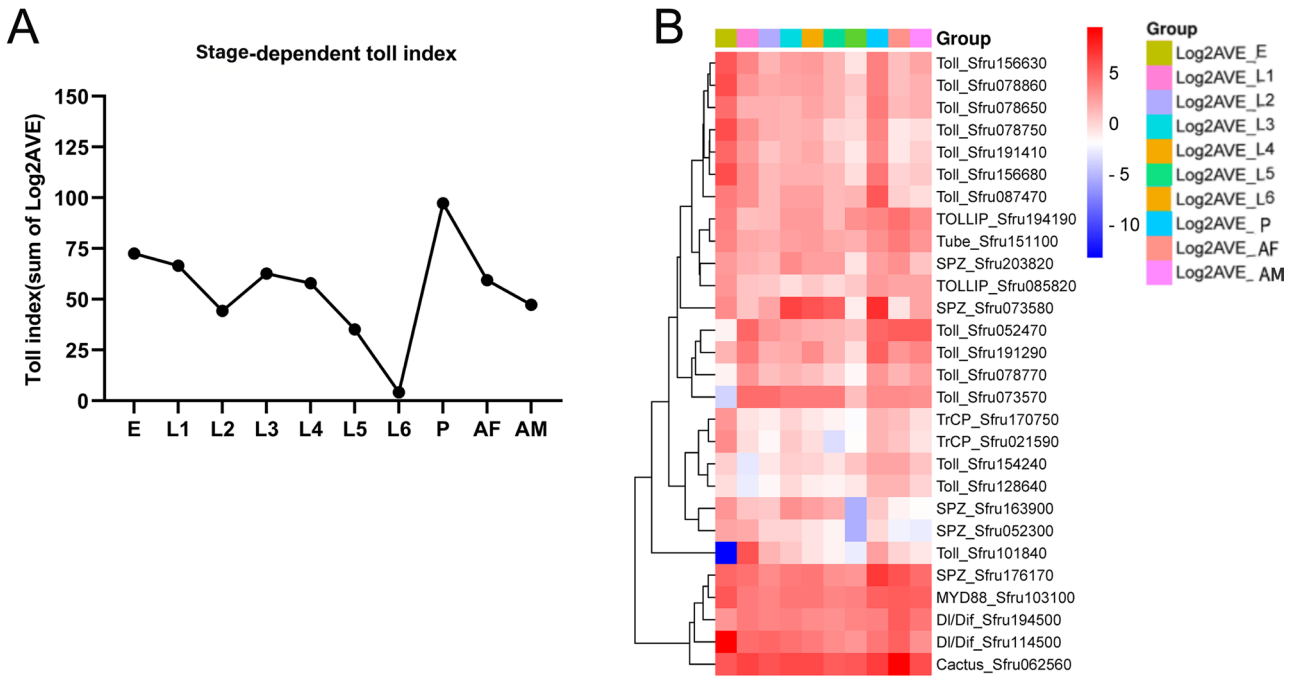

Fig. 3. Toll pathway-related gene expression profiles at different developmental stages of *Spodoptera frugiperda*. The gene annotations and pathway mapping were based on KEGG (https://www.kegg.jp/entry/map04624). (**A**) Trends in total Toll pathway gene expression (the sum of Log2FPKM) at different developmental stages of *S. frugiperda*. (**B**) Heatmap of individual Toll pathway gene expression profiles (Log2FPKM) in humoral immunity at developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

and one interleukin-1 receptor-associated kinase (*Tube_Sfru151100*) had similar expression profiles and were widely expressed in 10 stages (Fig. 3B). A total of five *Spaetzle* (*Spz*) genes were differentially expressed during development. One of the *Spz* genes (*Sfru176170*) was highly expressed throughout the developmental stages (Fig. 3B). All five *Spz* genes were highly expressed at the pupal stage, but four of them (*Sfru052300*, *Sfru163900*, *Sfru203820*, and *Sfru073580*) were expressed at low levels at the L6 stage (Fig. 3B). Five *Spz* genes had different expression patterns at the other eight stages. The death domain containing myeloid differentiation factor 88 (*MyD88_Sfru103100*), NF-kappa-B inhibitor alpha (*cactus_Sfru062560*), and two c-Rel proto-oncogene proteins (*DI/Dif_Sfru194500 and Sfru114500*) had similar expression profiles with high transcript abundance at all developmental stages (Fig. 3B). Two TrCPs (F-box and WD-40 domain protein) (*Sfru170750* and *Sfru021590*) were expressed at moderate to high levels in egg, pupal, and female adult stages and at low levels in the other stages (Fig. 3B).

Imd signaling genes

Seventeen differentially expressed genes involved in Imd signaling were identified, representing 10 gene families. The total expression of genes in the Imd pathway decreases during development from the egg to the L6 stage, then gradually increases, peaking at the pupal stage (57.15) and the adult female stage (55.94) (Fig. 4A). A similar expression pattern was found in the ubiquitin-binding enzyme *E2 variant 2* (*Sfru208330*), fas-related death domain gene (*FADD_Sfru102350*), *Ird5* (*Sfru098380*), and transforming growth factor *β-activated kinase-1* (*TAK1_Sfru107230*) genes, which were moderately expressed at all 10 developmental stages (Fig. 4B). Two of the three transcription factor *AP (Jra)* genes had the opposite expression, with one *Jra* (*Sfru028390*) showing high levels at all 10 stages and the other (*Sfru199080*) showing low levels throughout (Fig. 4B). The third *Jra* (*Sfru212830*) gene, showed moderate to high expression levels in most developmental stages except L5 (Fig. 4B). *Ankyrin-1-like isoform X5* (*Sfru165290*) showed low levels before the pupal stage and high expression in the pupal and adult stages (Fig. 4B). Three *FAS*-related factor genes (*Sfru023860*, *Sfru082950*, and *Sfru174210*) showed similar expression profiles, with high levels in 9 of the 10 developmental stages (except the L5 stage) and highest expression levels in the egg stage (Fig. 4B). *MEKK1* (*Sfru128270*) was expressed at high levels in the egg, pupal, and adult stages but low in the other stages (Fig. 4B). The cyclic adenosine-dependent transcription factor *ATF-6α isoform X2* (*Sfru058210*) showed moderate to high expression levels throughout development (Fig. 4B). The *dual oxidase isoform X1* (*Sfru190250*) showed high expression in the L3, L4, L5 larval, pupal, and female adult stages and relatively low expression in the other stages (Fig. 4B).

Effectors

Five differentially expressed antimicrobial peptide-coding genes across developmental stages have been identified in *S. frugiperda*. The sum of gene expression was similar throughout development except for L6 (19.93), in which

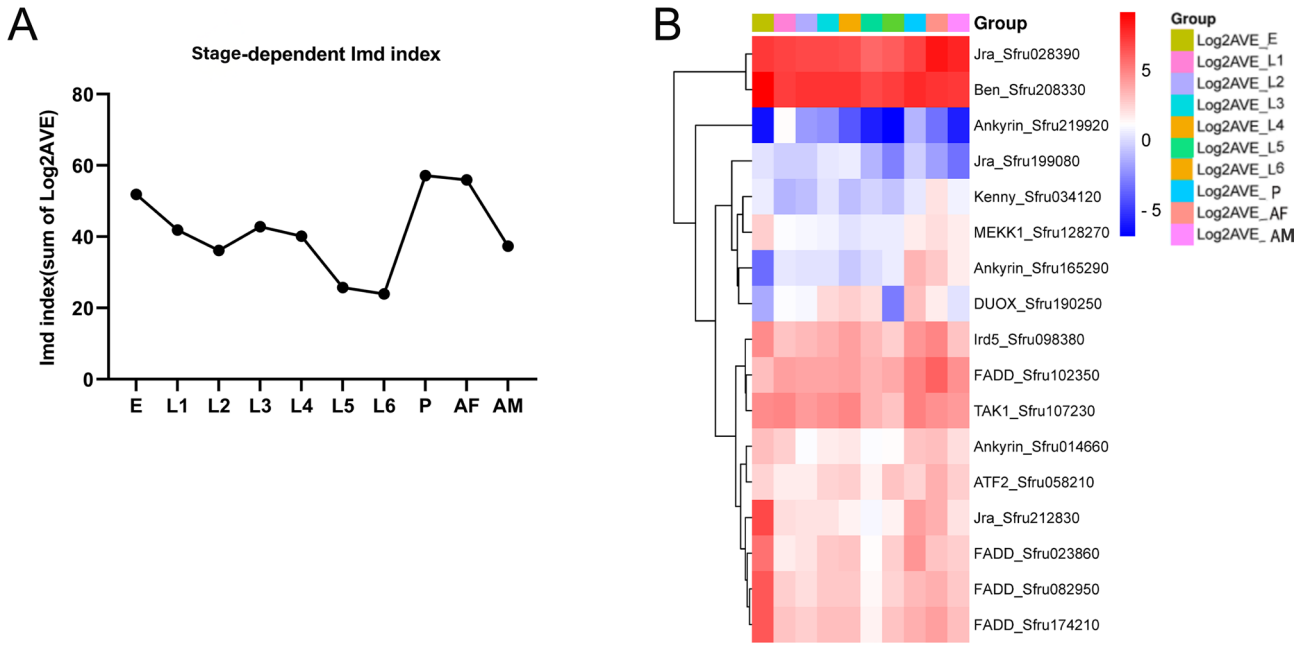

Fig. 4. Imd pathway-related gene expression profiles at different developmental stages of *Spodoptera frugiperda*. The gene annotations and pathway mapping were based on KEGG (https://www.kegg.jp/entry/map04624). (**A**) Trends in total Imd pathway gene expression (sum of Log2FPKM) at different stages of development of *S. frugiperda*. (**B**) Heatmap of individual Imd pathway gene expression profiles (Log2FPKM) in humoral immunity at developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

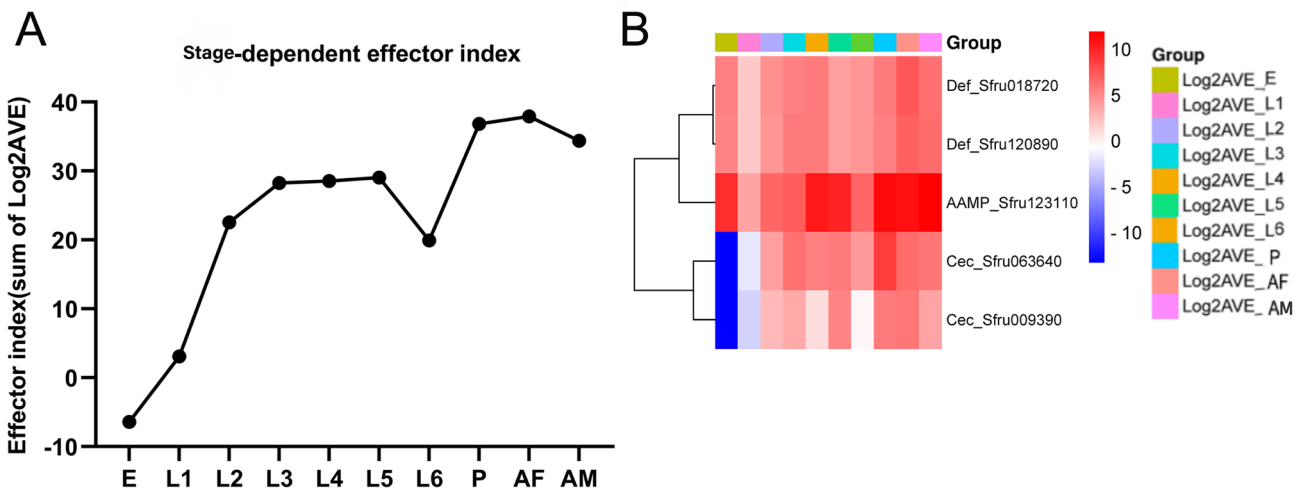

Fig. 5. Effector gene expression profiles at different developmental stages of *Spodoptera frugiperda*. (**A**) Trends in total immune effector gene expression (sum of Log2FPKM) in *S. frugiperda* at different developmental stages. (**B**) Heatmap of individual effector gene expression profiles (Log2FPKM) in humoral immunity at developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

gene expression showed a gradual increase across developmental stages, reaching a maximum in the pupal stage (36.85) and the female adult stage (37.95) (Fig. 5A). Two *defensin precursor* genes (*Sfru120890* and *Sfru018720*) and the *anionic antimicrobial peptide 2-like* gene (*Sfru123110*) showed high expression levels throughout the developmental stages, except for a relatively lower level at the L1 stage (Fig. 5B). One *cecropin A2* (*Sfru063640*) presented a low expression level in the egg and L1 stages and higher levels in the other 8 stages (Fig. 5B). The transcript abundance of another *cecropin A2* (*Sfru009390*) was low in the egg, L1 and L6 stages but high in the other 7 stages (Fig. 5B).

Serine proteases

A total of 70 serine protease (*SP*) genes were expressed. The total serine protease gene expression, measured as the Log2FPKM sum, demonstrates distinct patterns across the developmental stages of *S. frugiperda* (Fig. 6A). Eleven *SP* genes (*Sfru157760*, *Sfru002110*, *Sfru183050*, *Sfru139950*, *Sfru147560*, *Sfru224700*, *Sfru094840*, *Sfru054250*, *Sfru149890*, *Sfru149310*, and *Sfru085130*) had similar expression profiles, with moderate to high levels of expression in all 10 developmental stages and higher expression in the egg stage (Fig. 6B). In contrast, 11 *SP* genes (*Sfru124810*, *Sfru147650*, *Sfru105980*, *Sfru064550*, *Sfru140070*, *Sfru064580*, *Sfru026440*, *Sfru106420*, *Sfru199970*, *Sfru159340*, *and Sfru213980*) were expressed at lower levels in the egg stage compared to the other nine developmental stages (Fig. 6B). Gene *Sfru029070* was expressed at low levels in the egg, pupal, and adult stages and at moderate levels in the other six stages (Fig. 6B). In the four *SP* genes (*Sfru147840*, *Sfru139640*, *Sfru147830*, and *Sfru139800*), *Sfru147840* and *Sfru139640* exhibited low expression levels at the L3 and L5 larval stages, with higher expression levels in the other stages (Fig. 6B). *Sfru147830* showed low expression levels at the L2, L5, L6, and adult stages, with higher expression levels in the remaining five stages (Fig. 6B). *Sfru139800*, except for high expression levels in L1, L2, and male adults, exhibited lower expression levels in the other developmental stages (Fig. 6B). Seven *SP* genes (*Sfru044380*, *Sfru001750*, *Sfru153450*, *Sfru057200*, *Sfru045930*, *Sfru219640*, and

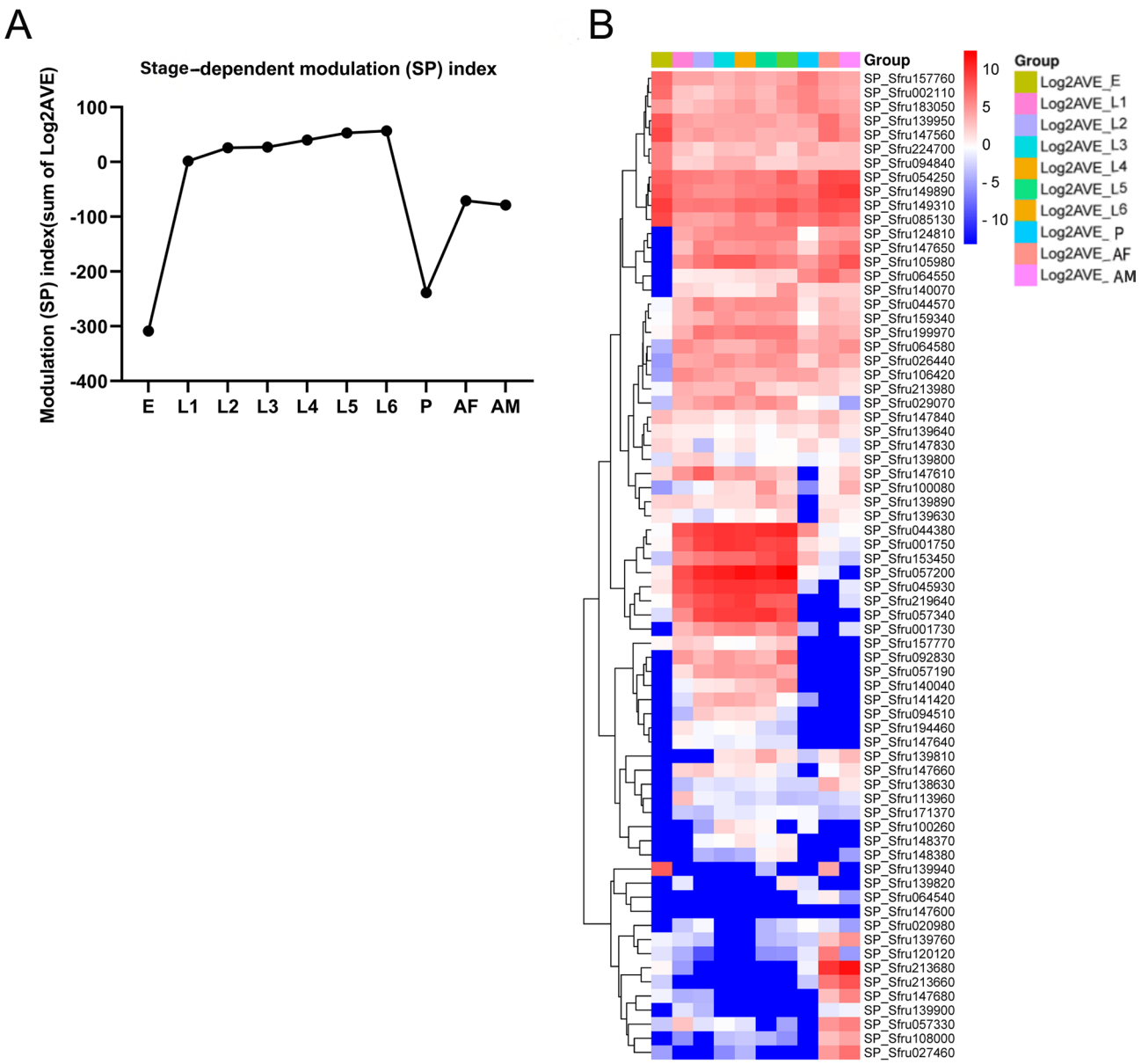

Fig. 6. Serine proteases gene expression profiles at different developmental stages of *Spodoptera frugiperda*. (**A**) Trends in total immune serine protease gene expression (the sum of Log2FPKM) at different developmental stages of *S. frugiperda*. (**B**) Heatmap of individual serine protease gene expression profiles (Log2FPKM) at developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

Sfru057340) had similar expression profiles, with high transcript abundance in the L1-L6 larval stages and low transcript abundance in the egg, pupal, and adult stages (Fig. 6B). Similarly, nine *SP* genes (*Sfru7Sfru001730*, *Sfru157770*, *Sfru092830*, *Sfru057190*, *Sfru140040*, *Sfru141420*, *Sfru094510*, *Sfru194460*, and *Sfru147640*) showed low transcript abundance in the egg, pupal, and adult stages and moderate transcript abundance in the remaining six stages (Fig. 6B). Seven *SP* genes (*Sfru147660*, *Sfru113960*, *Sfru171370*, *Sfru139820*, *Sfru064540*, *Sfru147600*, and *Sfru020980*) were mostly expressed at low levels throughout development, with very few periods of higher expression (Fig. 6B). Additionally, *Sfru113960* showed high expression levels in L1 stage, while *Sfru139820* exhibited high expression levels in L5 stage (Fig. 6B). Eight *SP* genes (*Sfru139760*, *Sfru120120*, *Sfru213680*, *Sfru213660*, *Sfru147680*, *Sfru057330*, *Sfru108000*, and *Sfru027460*) were highly expressed in the adult stage, except for genes *Sfru120120* and *Sfru057330*, which were expressed at higher levels in the male adult stage and L1 stage, respectively (Fig. 6B). Finally, gene *Sfru139940* was highly expressed in the egg stage and the female adult stage and at lower levels in the remaining eight developmental stages (Fig. 6B).

Serine protease inhibitors

There are 17 genes that may be related to immune modulation (*serine protease inhibitor*, *SPI*), and 7 serpin genes were identified to be differentially expressed between stages. The expression of serine protease inhibitor genes, summarized by the Log2FPKM values, reveals developmental stage-specific variations in *S. frugiperda* (Fig. 7A). Among the *SPI* genes, seven (*Sfru118470*, *Sfru064460*, *Sfru118730*, *Sfru064440*, *Sfru105540*, *Sfru055080*, and *Sfru155870*) were expressed at high levels throughout the developmental stages, except for the last three genes, which were expressed at low levels in the egg stage (Fig. 7B). Two *SPI* genes (*Sfru105400* and *Sfru055210*) were also expressed at low levels in the egg and L1 stages and then highly expressed in the remaining eight stages (Fig. 7B). One *SPI* gene (*Sfru064470*) was expressed at low levels in the pupal stage and moderately expressed throughout development, while gene *Sfru094100* was expressed at the highest level in the pupal stage and the lowest level in the L6 stage (Fig. 7B). Five *SPI* genes (*Sfru034470*, *Sfru108630*, *Sfru055090*, *Sfru185160*, and *Sfru105520*) had similar expression profiles, and genes *Sfru055090*, *Sfru185160*, and *Sfru105520* were all moderately-highly expressed in the adult male and female stages and lowly expressed in all other stages (Fig. 7B). Three serpin genes (*Sfru205920*, *Sfru212840*, and *Sfru155860*) were moderately expressed in the egg stage and moderately-highly expressed in all other stages, while three other serpin genes (*Sfru009670*, *Sfru098660*, and *Sfru009680*) showed medium expression in L1, L2, L3, L5, and L6 stages and low expression in the remaining five stages (Fig. 7B). The expression of another serpin gene (*Sfru009640*) peaked during the egg stage and then decreased during the L4 larval stage (Fig. 7B).

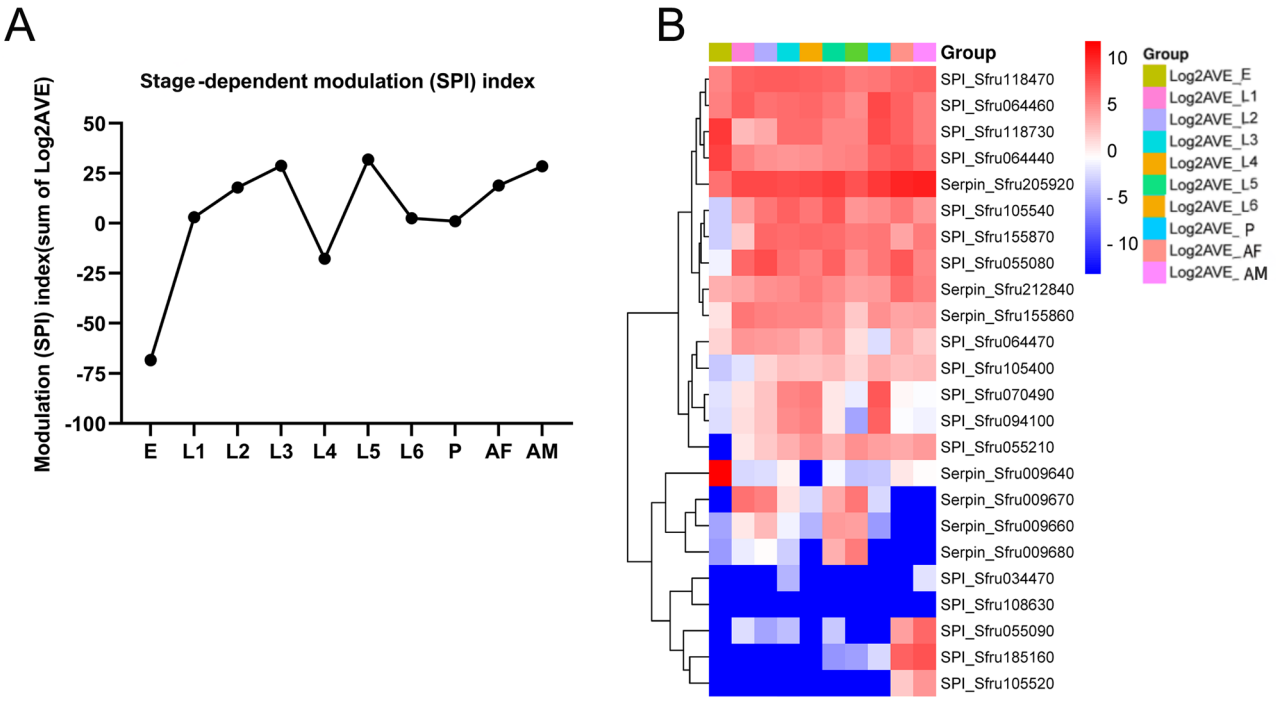

Fig. 7. Serine protease inhibitor gene expression profiles at different developmental stages of *Spodoptera frugiperda*. (**A**) Trends in total serine protease inhibitor gene expression (sum of Log2FPKM) in *S. frugiperda* at different developmental stages of *S. frugiperda*. (**B**) Heatmap of individual serine protease inhibitor gene expression profiles (Log2FPKM) in humoral immunity at various developmental stages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

Cellular immune gene expression patterns: hemolymph coagulation, clot formation and wound healing

Regarding the expression of hemolymph clotting-related genes, the total index gradually increased from the egg stage to the L4 larval stage and then decreased from L4-L6 before it gradually increased again, reaching the maximum values in the L4 and pupal stages, which were 94.97 and 94.06, respectively, while the lowest value was 30.95 in the L6 stage (Fig. 8A). Clot formation and wound healing are represented by 22 genes belonging to three gene families. Two *ApoLp*, six gelsolins, and 14 *LP* genes were expressed in different developmental stages, and both sexes and genes from the same gene family were expressed at various levels in the same developmental stage (Fig. 8B). Two *ApoLp* genes (*Sfru104770* and *Sfru014290*) were moderately expressed in the L1 stage and at high levels in the rest of the developmental stages (Fig. 8B). Four genes (*Sfru215420*, *Sfru086370*, *Sfru086380*, and *Sfru131920*) were expressed at intermediate levels throughout development (Fig. 8B). Two genes (*Sfru154490* and *Sfru151730*) were expressed at higher levels in the L2-L5 stage than in the remaining six stages (Fig. 8B). Expression of 14 *LP* genes varied widely throughout development, with five genes (*Sfru192180*, *Sfr u103510*, *Sfru101150*, *Sfru215410*, and *Sfru101140*) having low transcript abundance in the egg stage and higher in the other nine stages (Fig. 8B). Gene Sfru056250 was highly expressed in the L2-L4 stage and showed the lowest expression level in the male adult stage (Fig. 8B). Five *LP* genes (*Sfru216220*, *Sfru216200*, *Sfru216230*, *Sfru164010*, and *Sfru116370*) showed low or no expression levels throughout development, with higher expression in the pupal stage than in the other stages (Fig. 8B). Genes *Sfru164010* and *Sfru116370* were expressed at higher levels in the egg and pupal stages, respectively, and showed low or no expression in the other developmental stages (Fig. 8B). Gene *Sfru156990* had low transcript abundance in all developmental stages compared to the other 13 genes in the LP gene family (Fig. 8B).

Hematopoiesis

As for the hematopoiesis-related genes in the 10 developmental stages tested, the immunity index was higher in the egg, pupal, and female adult stages, with a downward trend from the egg stage to the lowest value in the L6 larval stage (−16.77) (Fig. 9A). There were 79 differentially expressed genes, representing 12 gene families. Among them, 27 *Notch* genes (*Sfru025660*, *Sfru063940*, *Sfru059120*, *Sfru046440*, *Sfru015630*, *Sfru209530*, *Sfru095710*, *Sfru059830*, *sfru210130*, *sfru116070*, *sfru185390*, *sfru120730*, *sfru183570*, *sfru091010*, *sfru104240*, *sfru067090*, *sfru096690*, *sfru030500*, *sfru104290*, *Sfru091000*, *Sfru033630*, *Sfru085450*, *Sfru033640*, *Sfru162900*, *Sfru218740*, *Sfru162060*, and *Sfru058540*) had similar expression profiles, and they were widely expressed throughout development (Fig. 9B). These genes were expressed at higher levels in the egg, pupal, and adult stages. Two genes (*Sfru210920* and *Sfru210940)*, in contrast, were expressed at low levels in the egg and adult stages and

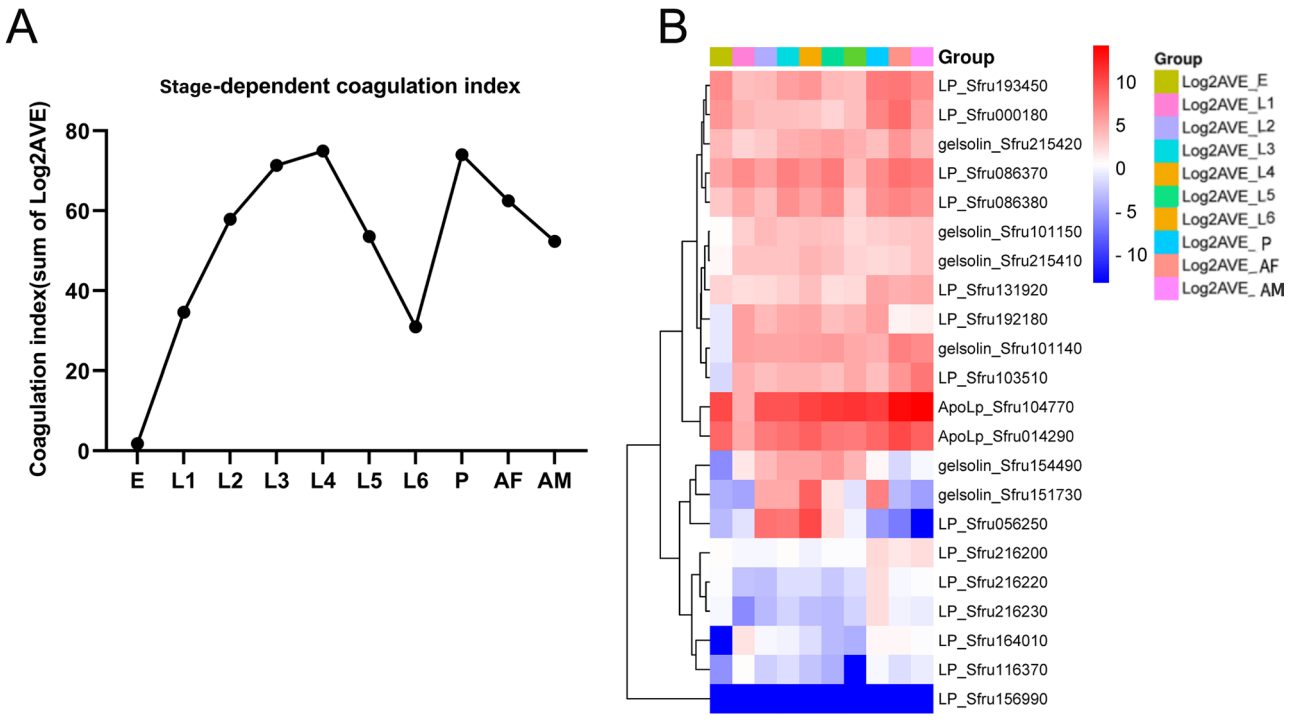

Fig. 8. Hemolymph coagulation-, clot formation-, and wound healing-related gene expression profiles at different developmental stages of *Spodoptera frugiperda.* (**A**) Trends in total immune hemolymph coagulation, clot formation, and wound healing gene expression (sum of Log2FPKM) at different developmental stages of *S. frugiperda*. (**B**) Heatmap of individual hemolymph coagulation, clot formation, and wound healing gene expression profiles (Log2FPKM) at various developmental stages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

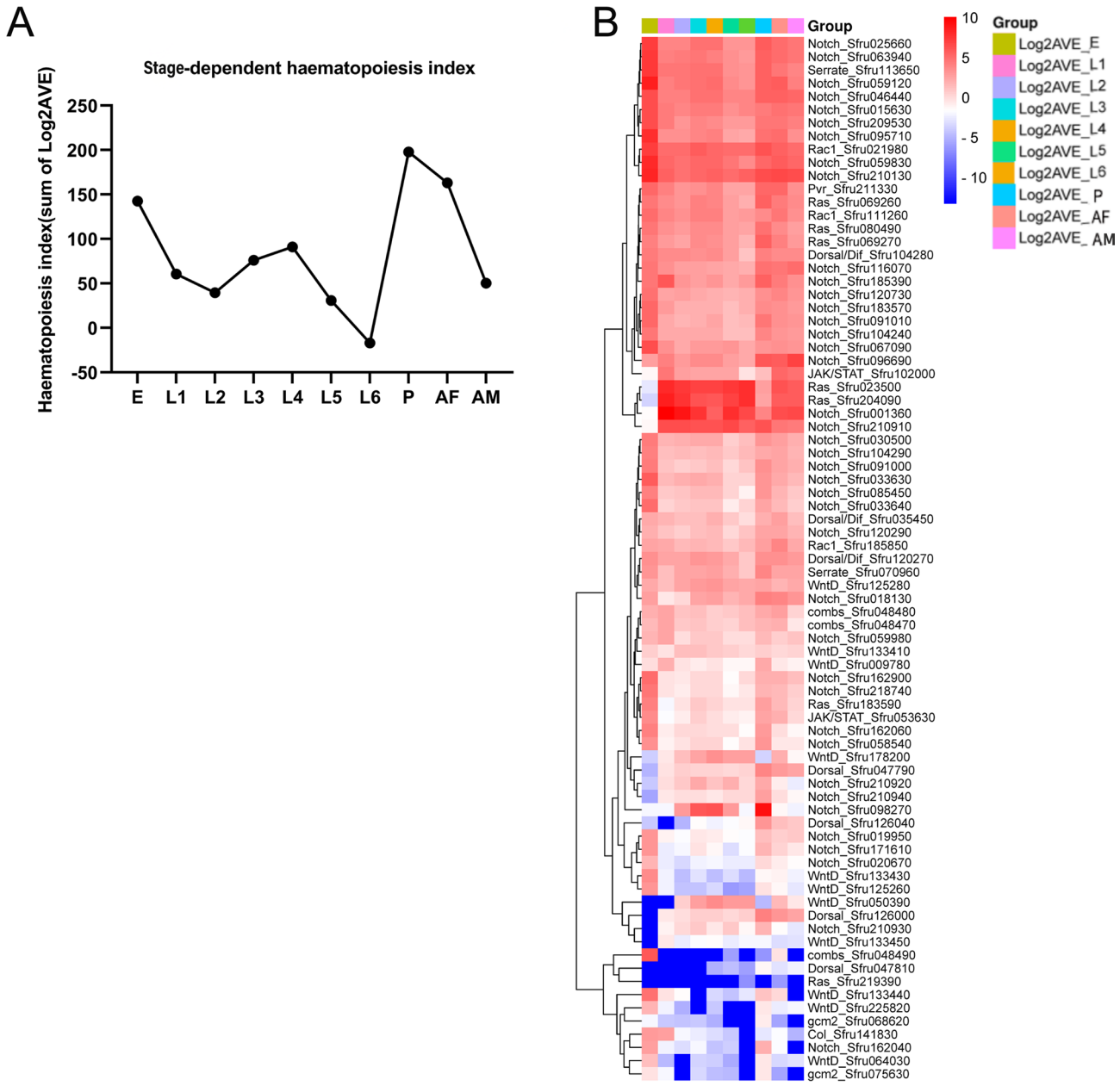

Fig. 9. Hematopoiesis-related gene expression profiles at different developmental stages of *Spodoptera frugiperda*. (**A**) Trends in total immune hematopoiesis gene expression (sum of Log2FPKM) at various developmental stages of *S. frugiperda*. (**B**) Heatmap of individual hematopoiesis gene expression profiles (Log2FPKM) in cellular immunity at various developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

at high levels in the remaining developmental stages (Fig. 9B). Gene *sfru098270* was widely expressed in most stages, with medium expression in the L2-L5 larval stages, high expression in the pupal stage, and low expression in the other stages (Fig. 9B). Two *Notch* genes (*Sfru019950* and *Sfru020670*) exhibited high expression levels only in the egg stage, with relatively low expression levels in the other nine developmental stages (Fig. 9B). Among eight genes (*Serrate_Sfru113650*, *Serrate_Sfru070960*, *Rac1_Sfru021980*, *Rac1_Sfru111260*, *Rac1_Sfru185850*, *Ras_Sfru069260*, *Ras_Sfru023500*, and *Ras_Sfru204090*), except for *Ras_Sfru023500* and *Ras_Sfru204090*, where two genes had low expression levels in the egg stage, the other six genes exhibited high expression levels at each developmental stage (Fig. 9B). The three *Dorsal/Dif* genes (*Sfru104280*, *Sfru035450*, and *Sfru120270*) were moderately to highly expressed in all stages (Fig. 9B). Two *JAK/STAT* genes (*Sfru102000* and *Sfru053630*) had high expression levels in the former except for the egg and L6 stages, with the highest expression in the adult stage, while the latter had low expression in the L1-L6 stages and high expression in all other stages (Fig. 9B). Genes encoding *wntD* (*Sfru125280*, *Sfru133410*, and *Sfru009780*) were expressed throughout (Fig. 9B). *Sfru178200* was expressed at low levels in the egg and pupal stages, while expression was high in the rest of the stages (Fig. 9B).

In contrast, genes (*Sfru133430* and *Sfru125260*) were expressed at low levels in the L1-L6 larval stage and at high levels in all the other stages. Genes *Sfru178200* and *Sfru050390* were highly expressed in the L2-L6 stages and at low levels in the egg, L1, and pupal stages (Fig. 9B). Four genes (*Sfru133450*, *Sfru133440*, *Sfru225820*, and *Sfru064030*) and the *Dorsal* gene (*Sfru047810*) were transcriptionally abundant in most developmental stages (Fig. 9B). The two *Combs* genes (*Sfru048480* and *Sfru048470*) were expressed at intermediate levels throughout development, while gene *Sfru048490* was highly expressed in the egg and adult female stages, with the remaining eight periods showing low expression (Fig. 9B). *Gcm2* genes (*Sfru068620* and *Sfru075630*) and *Col* genes (*Sfru141830*) showed low expression levels in eight developmental stages except for the egg and pupal stages, in which they were expressed at high levels (Fig. 9B).

Hemocyte-mediated immune responses

During the *S. frugiperda* development, the total immune index fluctuated, showing low values in the egg, L6, and male adult stages and high values in the female adult (45.31) (Fig. 10A). A total of 84 genes, representing 15 gene families, were involved in the hemocyte-mediated immune response. Eight of the *immunoglobulin-like*

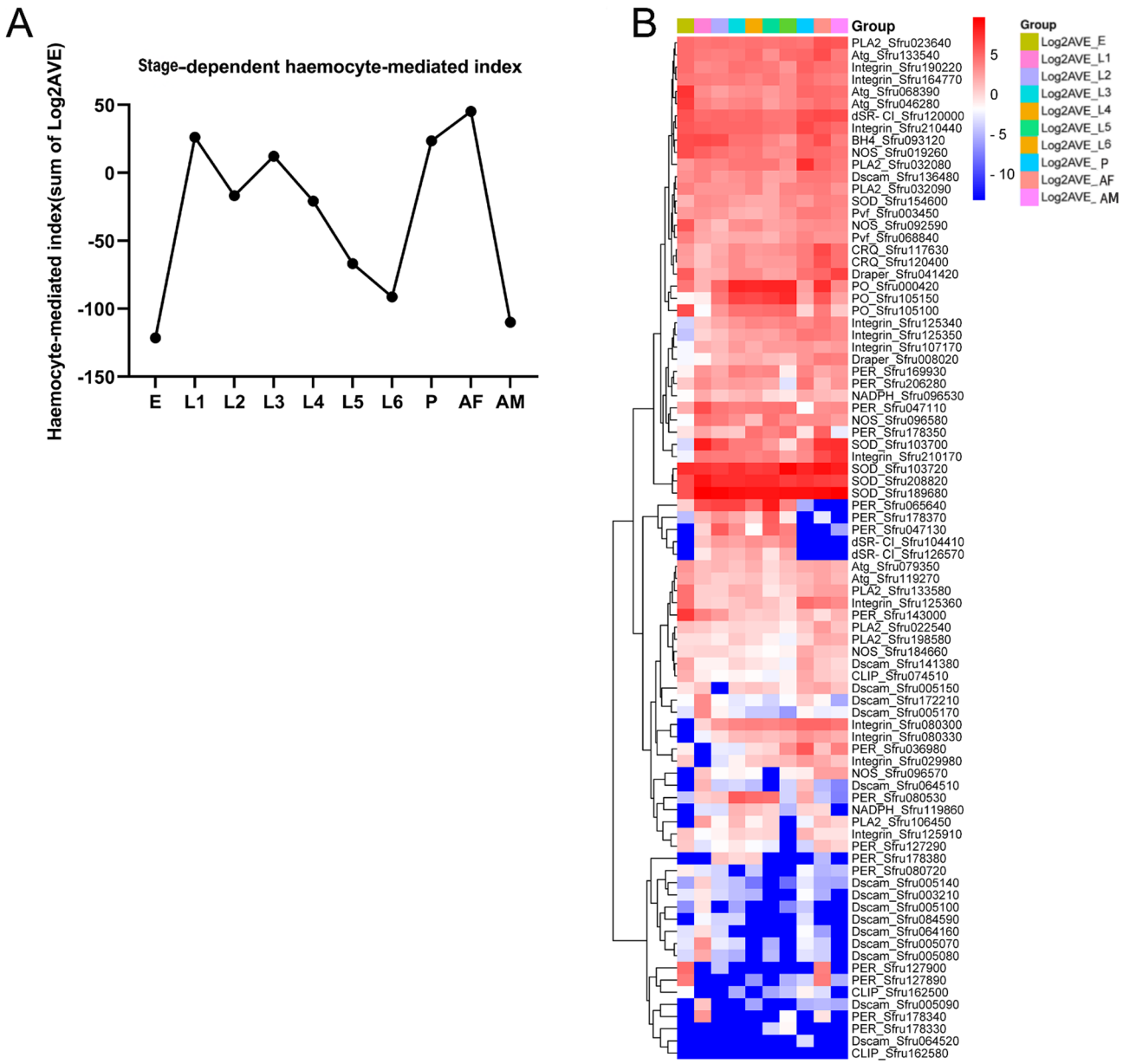

Fig. 10. Hemocyte-mediated immune response-related gene expression profiles at different developmental stages of *Spodoptera frugiperda*. (**A**) Trends in total hemocyte-mediated immune gene expression at different developmental stages of *S. frugiperda*. (**B**) Heatmap of individual hemocyte-mediated immune gene expression profiles (Log2FPKM) at various developmental ages. E: Egg; L1: 1st instar; L2: 2nd instar; L3: 3rd instar; L4: 4th instar; L5: 5th instar; L6: 6th instar; P: Pupa; AF: Adult female; AM: Adult male.

cell adhesion molecules (*Sfru005140*, *Sfru003210*, *Sfru005100*, *Sfru084590*, *Sfru064160*, *Sfru005070*, *Sfru005080*, and *Sfru005090*) had similar expression patterns, with higher expression in the L1 and pupal stages and lower expression in the rest of the stages (Fig. 10B). Among them, genes *Sfru141380*, *Sfru005150*, *Sfru172210*, *Sfru005170*, and *Sfru064510*, as well as *Dscam_Sfru064520*, had the lowest transcript abundance in all stages (Fig. 10B). *PLA2* genes (*Sfru023640*, *Sfru023080*, and *Sfru032090*) were widely expressed throughout all developmental stages (Fig. 10B). Genes *Sfru133580*, *Sfru022540*, and *Sfru198580* were lowly expressed in all stages (Fig. 10B). Three (*Sfru133540*, *Sfru068390*, and *Sfru046280*) and two (*Sfru079350*, and *Sfru119270*) *Atg* genes showed medium-high and medium expression levels, respectively, throughout all developmental stages (Fig. 10B). Three *integrin* genes (*Sfru190220*, *Sfru164770*, and *Sfru210440*) showed medium-high expression levels during development, and three *Integrin* genes (*Sfru125340*, *Sfru125350*, and *Sfru107170*) showed low expression in the egg stage and medium expression in all the other stages (Fig. 10B). One *Integrin* gene (*Sfru210170*) was highly expressed in all stages, except for the egg stage, with the highest expression in the adult stage (Fig. 10B). In contrast, one *Integrin* gene (*Sfru125360*) was expressed at low levels in all 10 stages (Fig. 10B). Two *Integrin* genes (*Sfru080300* and *Sfru080330*) were expressed at low levels in the egg stage and at high levels in the remaining stages (Fig. 10B). One *Integrin* gene (*sfru029980*) was expressed at low levels in the egg and L1 stages and then highly expressed throughout development (Fig. 10B). One *Integrin* gene (*sfru125910*) was not expressed in the L6 stage and was expressed at low levels in all the other stages (Fig. 10B). Two *DSR-CI genes* (*Sfru104410* and *Sfru126570*) were lowly expressed in the L1-L6 larval stages with higher transcript abundance than in the remaining four stages (Fig. 10B). One *DSR-CI* (*Sfru120000*) and one *BH4* (*Sfru093120*), as well as three *NOS synthase* genes (*Sfru019260*, *Sfru092590*, and *Sfru096580*), showed moderate-high transcript abundance throughout all stages, except for *sfru096580*, which was expressed at a lower level in the egg stage than in the rest of the stages (Fig. 10B). Four *SOD* genes (*Sfru154600*, *Sfru103720*, *Sfru208820*, and *Sfru189680*) were widely expressed at high levels throughout the growth stages (Fig. 10B). One *SOD* gene (*Sfru103700*) was expressed at high levels except for the egg stage, with the highest expression in the L1 and adult stages (Fig. 10B). The *Pvf* genes (*Sfru003450* and *Sfru068840*) had similar moderate expression profiles, and *CRQ* (*Sfru117630* and *Sfru120400*) showed moderate to high expression throughout development, with the highest expression in female adults (Fig. 10B). Two *PO* genes (*Sfru000420* and *Sfru105150*) were highly expressed in the L3-L6 and adult female stages (Fig. 10B). Two *PER* genes (*Sfru169930* and *Sfru206280*) showed medium expression in most developmental stages except for egg and L6, and gene *Sfru047110* showed the lowest expression in the pupal stage (Fig. 10B). Gene *PER_sfru178350* showed high expression in all stages, except for male adults, where it was expressed at low levels (Fig. 10B). Three *PER* genes (*Sfru065640*, *Sfru178370*, and *Sfru047130*) had medium-high expression in the L1-L6 stages and the lowest expression in the pupal and adult stages (Fig. 10B). Gene *PER_sfru143000* had high expression in the egg stage, and gene *PER_Sfru080530* had high expression in the L1-L5 stage and low expression in the egg, L6, and adult stages (Fig. 10B). Gene *PER_Sfru127290* showed lower expression in L1, L5, L6, and pupal stages than the rest of the stages (Fig. 10B). Gene *PER_Sfu178380* showed higher expression in the L2-L4 stages and lower expression in the other stages, and gene *PER_Sfru080720* showed higher expression in the egg and pupal stages (Fig. 10B). Two PER genes (*Sfru127900* and *Sfru12890*) had similar expression profiles, being mainly expressed in the egg and female adult stages and at low levels in the remaining stages (Fig. 10B). The expression profile of *PER* genes *Sfru178340* and *Sfru1783300* was similar in all developmental stages except for the former, where transcript abundance was moderate-high in the L1 stage (Fig. 10B). A *NADPH* gene *Sfru096530* was expressed at moderate levels everywhere apart from the egg stage, whereas *NADPH_sfru119860* was expressed in the egg, L1, L2, L6, male and female adult stages and at low levels in the rest (Fig. 10B). Two clip domain serine proteases (*CLIPs*) genes (*Sfru162500* and *Sfru162580*) showed low expression throughout the whole period (Fig. 10B).

qRT-PCR gene validation

To validate the results of RNA-seq, RT-qPCR was performed to test the expression levels of some humoral and cellular immune-related genes at different developmental stages of *Spodoptera frugiperda*. Ten key immune-related genes were selected for validation: *GNBP_Sfru123480*, *PGRP_Sfru122870*, *Toll_Sfru073570*, *SPZ_Sfru073580*, *DEF_Sfru018720*, *Cec_Sfru063640*, *AAMP_Sfru123110*, *LP_Sfru056250*, *dSR-CI_Sfru104410*, and *Integrin_Sfru080300* (Fig. 11).

Results showed that most genes demonstrated similar trends of expression level at all developmental stages, and there was strong concordance between RNA-seq data and RT-qPCR. Notably, the expression of *GNBP_Sfru123480*, a gene involved in pathogen recognition, showed progressive increase from egg to L6 instar larvae. Moreover, its expression in the adult stage was marginally higher in females compared to males, consistent with RNA-seq observations. Another recognition-related gene, *PGRP_Sfru122870*, was highly upregulated in adult females, suggesting that it is involved in higher immune preparedness at this stage. Genes associated with the Toll signaling pathway, like *Toll_Sfru073570* and *SPZ_Sfru073580*, showed different expression profiles. *Toll_Sfru073570* was expressed mainly in early developmental stages, indicating that it might be involved in the immune response of early developmental stages. In contrast, *SPZ_Sfru073580*, a ligand of the Toll receptor, peaked at the pupal stage, indicating a critical role in modulating immunity during metamorphosis. Effector genes, such as *DEF_Sfru018720* and *Cec_Sfru063640*, showed stage-specific expression. *DEF_Sfru018720* was constantly expressed through the larval stages but strongly upregulated during pupal development. On the other hand, *Cec_Sfru063640* showed a relatively higher expression in late larval stages. *AAMP_Sfru123110*, a gene associated with anionic antimicrobial peptide activity, showed high-level expression in both pupae and adult stages. *LP_Sfru056250* exhibited a peak in early larval stages for cellular immunity. The expression levels of a cellular immunity-related gene, *dSR-CI_Sfru104410*, increased in late larval stages. Finally, *Integrin_Sfru080300* exhibited a trend in steady upward from early larval to adult stages. Taken together, these results confirm the

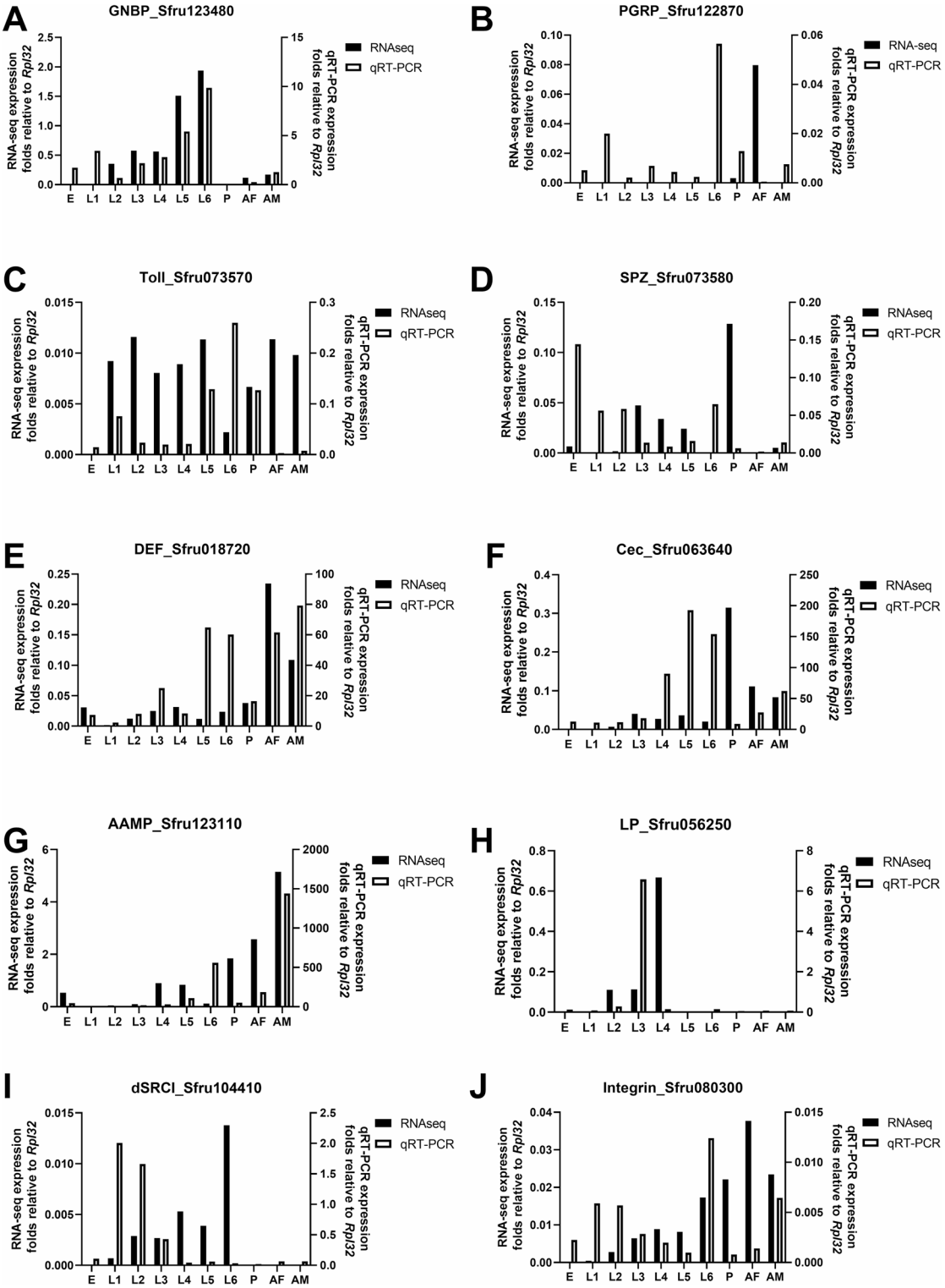

Fig. 11. RT-qPCR expression validation of immune-related genes in *Spodoptera frugiperda*. Expression levels of GNBP_Sfru123480 (**A**), PGRP_Sfru122870 (**B**), Toll_Sfru073570 (**C**), SPZ_Sfru073580 (**D**), DEF_Sfru018720 (**E**), Cec_Sfru063640 (**F**), AAMP_Sfru123110 (**G**), LP_Sfru056250 (**H**), dSR-CI_Sfru104410 (**I**), and Integrin_Sfru080300 (**J**) relative to the housekeeping gene (*RpL32*) by RNAseq and qRT-PCR across different developmental stages of *S. frugiperda*.

reliability of the RNA-seq data and point out the dynamic modulation of immune-related genes in response to developmental and environmental pressures in *S. frugiperda*.

Discussion

This study represents a high-throughput transcriptomic analysis of humoral and cellular immune-related genes in *S. frugiperda* across development stages and sexes. In general, the expression profiles of Toll, Imd, and Recognition pathway genes displayed obvious stage-specific and sex-specific expression profiles, especially with highly noticeable regulation in larvae, pupae, and adult females. These immune components have dynamic responses to developmental and environmental pressures, thus giving insight into their functional roles and evolutionary adaptations in immunity.

We divided humoral immune-related genes into six categories according to the specific steps of this type of immune response (Recognition, Toll, Imd, Effector, SP, and SPI) to perform transcriptomic analysis of different developmental stages and sexes. The line chart of the humoral immune index showed that Toll and Imd pathways were expressed in all developmental stages, and the expression levels decreased gradually from the egg to the larval stage, while the expression levels were higher in the pupal and adult stages. The expression levels of genes in the Recognition, Effector, SP, and SPI categories were low in the egg stage. In particular, the expression level of SP genes was substantially low in the egg, pupal, and adult stages, indicating that the function of humoral immunity in different stages of *S. frugiperda* varies significantly. The total humoral immunity index revealed that the expression of humoral immunity genes was the lowest in the egg stage, followed by the pupal stage, and higher in the larval and adult stages. The expression of humoral immunity genes first increased in the early larval stages and then decreased in the late larval stages, which indicates that humoral immune genes can be regulated within a single developmental stage. Humoral immune genes were expressed at higher levels in females than in males, possibly because the life-history strategies of male and female individuals vary[2,22]. In particular, the mating success of males differs from that of females, leading to an evolutionary preference for reproduction in males and immune function in females[23].

In the pathogen recognition category, the index increased gradually from the egg stage to the adult stage and reached the highest value in female adults. The progressive increase (augmentation) in the pathogen recognition immune gene index from egg to adult stages likely reflects developmental increases in immune preparedness. There is minimal immune response during the egg stage, possibly due to the protective effect of the serosa, which may be a physical barrier to microbial invasion[24]. As larvae emerge and engage in active feeding, they are exposed more to pathogens and, therefore, upregulate the expression of recognition genes such as peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs). The high expression in the adult stage, particularly in the female, may be due to a need for increased immunity to support reproductive activity, which is energy costly and exposes them to infection at higher levels. This pattern would be paralleled in other insects where immunity in reproducing females is strengthened for protecting offspring. Two important gene families participate in microbial recognition: peptidoglycan recognition proteins (*PGRPs*) and Gram-negative binding proteins (*GNBPs*). *PGRPs* are involved in the recognition of Gram-positive bacteria, which bind to bacterial peptidoglycan to trigger conserved signaling immune pathways[25]. *GNBPs* were originally identified in the silkworm *Bombyx mori*, and they bind to Gram-negative bacteria. Most *GNBPs* and *PGRPs* are highly expressed in the adult stage except for *GNBP_Sfru064870* and *GNBP_Sfru196660*. Two *PGRPs* (*Sfru001290* and *Sfru122870*) are even more specifically highly expressed in the adult stage, whereas *PGRP_Sfru183090* and *PGRP_Sfru184410* are higher expressed in female adults than males. Two putative pattern recognition receptors, *PGRP-SA* and *GNBP1*, have been identified to be required in *D. melanogaster* adults for detecting the presence of Gram-positive bacteria[26]. Except for *PGRP_Sfru183090*, other genes were differentially expressed in the larval stage. In particular, *GNBP_Sfru123480* and *PGRP_Sfru182360* were highly expressed in the larval stage, suggesting that the function of these two genes may be related to humoral immune recognition targeting in the larval stage of *S. frugiperda*[27].

Antimicrobial peptide genes are regulated by the Toll and Imd signaling pathways. The Toll pathway is activated by Gram-positive bacteria, yeast, or fungi, while the Imd pathway is mainly triggered by most Gram-negative bacteria and some Gram-positive bacteria containing DAP-type PGN[27]. Most of the Toll and Imd genes were highly expressed in the egg and pupal stages, which was consistent with previous results in *P. xylostella* indicating that these genes are involved in the innate immune response of both eggs and pupae of *S. frugiperda*[28]. It is worth mentioning that certain genes of the Toll pathway (*TOLLIP_Sfru194190*, *Cactus_Sfru062560*, *Dl/Dif_Sfru194500*) and certain genes of the Imd pathway (*FADD_Sfru102350*, *Jra_Sfru212830*, *Ird5_Sfru098380*) were highly expressed at the female adult stage, which may be related to the immune response after mating. In *D. melanogaster*, for example, sex peptides activate the immune function in female flies after mating by stimulating the Toll and Imd pathways[29].

Insect Toll pathway contains a number of gene families like Toll receptors, Spaetzle (SPZ) ligands, adaptors (MyD88, Tube, Pelle), and transcription factors (Dorsal/Dif)[30–32]. The multigene family composition allows for subtle immune responses against various pathogens. There are many family members, and this enhances functional redundancy, thus ensuring immune robustness and flexibility even if some components are inhibited or downregulated. Additionally, variations in Toll gene expression during development suggest stage-specific immune activity, which could reflect that the insect host deals with various types of microbial challenges at each stage. The Imd pathway, responsible primarily for defense against Gram-negative bacteria, has a biphasic expression pattern: decreasing from egg to L6, and increasing at the pupal and female adult stages. This pattern is likely to reflect shifting immune priorities. High pathogen levels are experienced during the early larval stages through feeding and demand strong Imd activation. Immune suppression during later larval stages could be due to developmental trade-offs since energy is redirected towards molting. Increased pupal and female adult production signals re-activation of immunity for the defense of metamorphic stages and reproductive

investment. Regulation may include hormonal control (e.g., ecdysteroids), feedback inhibition, and interaction with other immune mechanisms.

The Effector component includes the anionic antimicrobial peptide/protein (AMP) genes, which have been identified as an important component of the innate immune system of vertebrates, invertebrates, and plants[33,34]. These peptides are active against bacteria, fungi, and viruses. Here we found that the AMP *Sfru123110* was highly expressed in all developmental stages except for the first instar and reached the highest expression levels in the pupal and adult stages. This suggests that *AAMP_sfru123110* may be involved in the immune process against pathogens in the mid- and late stages of development. Interestingly, previous studies have shown that the bacterial pathogen *Campylobacter jejuni* can be transmitted through the *Musca domestica* life stages, and during pupal development the number of *C. jejuni* bacterial cells decreases substantially, which coincides with increased expression of AMP-encoding genes[35].

Serine proteases (SP) regulate the biological activity of target proteins through proteolytic cleavage. When these serine proteases are no longer needed, they are inactivated by Serine protease inhibitors (SPI), which not only play a key role in insect digestion, metamorphosis, and development but they participate in immune responses[36,37]. The expression pattern of SP genes was different in different developmental stages, with some genes being highly expressed in the larval stage and some being only highly expressed in egg stage or adult stage, indicating their potential involvement in immune regulation during development. The expression level of most SPI genes was higher in larval and adults but lower in the egg stage, indicating that SPI genes have lower functional specificity than SP genes and can function at different developmental stages. The dynamic interplay between SPs and SPIs highlights the requirement for immune homeostasis at various life stages to enable *S. frugiperda* to maintain efficient defense mechanisms without wasting energy on immune responses when they are not needed. In honeybees, the expression of some SP and Serpin genes increased significantly after the microbial treatment of the adult stage, indicating that they are involved in the immune response in the adults[38]. In our study, genes that are highly expressed in the adult stage (*SP_Sfru213680*, *SP_Sfru213660*, *SP_Sfru149890*, and *Serpin_Sfru205920*) may perform similar functions. The serine protease inhibitory activity in Lepidoptera was controlled by hormones and fluctuated greatly during development[39]. In *G. mellonella*, SPI activity in hemolymph is up-regulated during pupation, suggesting regulation of endogenous proteases associated with metamorphosis, which is similar to *SPI_Sfru094100*, *SPI_Sfru118730*, and *SPI_Sfru064460* in our study[40]. Previous studies have shown that RNAi-mediated serine protease inhibitor gene knockdown increases mortality in *P. xylostella* larvae in response to destruxin A, which is a mycotoxin that is secreted by entomopathogenic fungi, suggesting that SPI genes in larvae play an important role in the resistance to entomopathogenic fungi. In our study, there were also some SPI genes that were highly expressed in the larval stage, such as *SPI_Sfru070490*, *SPI_Sfru155870*, *SPI_Sfru105540*, and *SPI_Sfru055080*, which may participate in the resistance of *S. frugiperda* larvae to entomopathogenic fungi and can be used as promising control targets.

In hemolymph coagulation, clot formation, and wound healing, lipoproteins are responsible for lipid transport in insect hemolymph. Gelsolin regulates cell growth and apoptosis by modulating actin. Its expression increases with development and is responsible for age-related apoptosis. In our study, the *Gelsolin* gene family (*Sfru101140*, *Sfru101150*, *Sfru215410*, *and Sfru215420*) genes were expressed at low levels except for the egg stage, while *Sfru154490* and *Sfru151730* were expressed at low levels from the larval to the adult stage, suggesting that *S. frugiperda Gelsolin* genes may not be involved in regulating cell growth[41]. Apolp III (apolipophorin-III) is involved in the translocation of lipids, pathogen recognition, nodulation, and antimicrobial activity. The *Apolp III* gene of *S. frugiperda* was highly expressed throughout development, which is consistent with *Spodoptera exigua*[42].

Notch signaling regulates larval hematopoiesis and Serrate, the receptor for Notch, which is expressed in plasmatocytes. Notch and Serrate family genes are expressed at higher levels in *S. frugiperda* eggs than in other stages, and at higher levels in honeybee embryos. Serrate is expressed in plasma cells and is also necessary for crystal cell differentiation, with a pattern of expression consistent with Notch that is higher in the egg[43,44]. *Ras* (ras-specific guanine nucleotide-releasing factor) is involved in phagocytosis. In beet nocturnal moths, *Ras* genes are constitutively expressed from egg to adult. In the meadow greedy night moth, *Ras* genes are constitutively expressed at medium-high transcript levels, consistent with the trend of Ras genes in *S. exigua*, except for *Ras_Sfru219390*, which was expressed at low levels throughout development. *Pvr* (Vegfr receptor) is involved in the regulation of hemocyte proliferation and is abundantly expressed in many tissues in the embryo[44]. *Dorsal/Dif* (Dorsal-related immunity factor) has a role as an initiator of effectors (e.g., AMPs) and in the regulation of plasma cell production in hematopoiesis. *Dif* also regulates the generation of lamellipodia and *Col* (*Collier*), which regulates the specialization of lamellipodia. *Rac1* (ras-related protein Rac1) was studied in *S. exigua*, and its function is to regulate the cytoskeleton, encapsulation, and hemocyte recruitment. Constitutive expression of the genes *rac1_Sfru111260* and *Rac1_Sfru185850* in *S. frugiperda*[6,45]. Regarding the varying expression levels of the immunoglobulin-like cell adhesion molecules throughout the developmental stages, their upregulation in pupae and adult females indicates increased immune surveillance in metamorphosis and reproduction. Their downregulation in later larval stages (L6) may be a transient suppression of hemocyte-mediated immunity, possibly due to energy diversion into ecdysis.

WntD (Wnt inhibitor of Dorsal) is a cytokine that regulates the NF-κB pathways as well as other signaling pathways depending on the developmental stage[46]. *JAK/STAT* (Janus kinase/signal transducers and activators of transcription) controls hemocyte proliferation and limits tumor growth during trauma. *JAK/STAT_Sfru211330* is lowly expressed in the egg and L6, and hemocyte proliferation is rapid in the egg[47]. *Gcm2* (transcription factor, glial cells missing 2-like) genes *Gcm2_Sfru075630* and *Gcm2_Sfru068620* are expressed in the egg and pupal stages, where cell division is rapid and involves cell differentiation and rapid cell growth and development, and in the pupal stage for the completion of metamorphosis, which also involves cell growth and differentiation. Therefore, *Gcm2* is expressed at higher levels in the egg and pupal stages[48,49].

PLA2 (phospholipase A2) is involved in activating the Toll pathway and affecting the expression of immune genes such as *MyD88*, with the highest transcript levels observed mainly in the fat body and high expression in the egg and adult stages[50]. The expression of the *PLA2* genes was similar in different stages of the moth, except for *PLA2_Sfru022540*, *PLA2_Sfru198580*, *PLA2_Sfru106450* and *PLA2_Sfru133580*, which were differentially expressed[51].

Atg (Autophagy-related genes) exhibited constitutive medium-high expression at different developmental stages of the insect, which is consistent with the expression profile of *Atg* genes in *Tenebrio molitor*[52]. Integrin transmits signals to promote cell adhesion and spreading and regulates cellular immunity by regulating phagocytosis and encapsulation. Expression of most Integrin genes increases with growth in *S. frugiperda*[53].

NOS (nitric oxide synthase-like), which regulates NO synthesis, acts as a vasodilator by inducing immunity through the activation of signaling pathways; it also induces high expression of effectors and leads to an increase in *NOS* expression following pathogen infestation[54]. *SOD* (peroxidase-like) regulates the concentration and conversion of reactive oxygen species (ROS), which promotes defense against invading microbial pathogens, but ROS overproduction can also cause negative effects on the host. *SOD_Sfru103720*, *SOD_Sfru208820*, and *SOD_Sfru189680* are highly expressed throughout development. The high level of SOD gene expression during all developmental stages suggests that the modulation of oxidative stress is critical during *S. frugiperda* development. At larval stages, increased metabolic activity and feeding activity demand strict control of ROS to prevent self-destruction while mounting an effective immune response. In the adult stage, and especially in females, high expression of SOD may be to balance reproductive fitness with immune defense.

Insect PO (phenoloxidase) plays an immune role against entomopathogens and promotes melanin synthesis[55]. *PO* showed high expression in older larval stages and adult females. The high expression of *PO* in adult females may make them more resistant to pathogens, possibly due to increased survival for reproductive purposes. The CLIP-associating protein (*CLIP*) serine proteases decrease PO enzyme activity and reduce resistance to pathogens in mosquitoes. In our study, both *CLIP_Sfru162580* and *CLIP_Sfru162500* show no expression, while *CLIP_Sfru074510* has low expression in all developmental stages[56,57]. *Draper* is involved in the phagocytosis of apoptotic cells and is expressed in hemocytes, with the highest expression in the adult stage. The increase in *Draper* expression slows down with aging, a process that occurs throughout the life of the insect, and the expression trend of this gene varies widely among species, with *Draper_Sfru041420* being highly expressed in the pupal and adult stages relative to larvae[58]. *dSR-CI* (*Drosophila Scavenger receptor class C, type I*) is a PRR (pattern recognition receptor) that recognizes Gram-negative bacteria; *Dscam* (Down syndrome cell adhesion molecule-like protein) also recognizes pathogens and mediates phagocytosis, and the gene *Pvf* affects hemocyte proliferation, *CRQ* (protein croquemort-like), *PER* (peroxidase-like), BH4, and other genes have been less studied regarding immunity and development.

In comparison with other related Lepidopteran species, *S. frugiperda* has both conserved and species-specific immune gene expression[59]. For instance, Toll and Imd pathway gene expression profiles are generally comparable to each other in *B. mori*, with enhanced immune function in larvae and pupae but reduced immune investment in adult females in comparison to *S. frugiperda*[60]. In *Manduca sexta*, hemocyte-mediated immunity is highest earlier in larval life, whereas in *S. frugiperda*, comparable responses are elevated in later life stages, most notably in pupae and adult females[61]. Interestingly, *Plutella xylostella* AMP genes are constitutively expressed at early instars, while *S. frugiperda* AMP expression is optimized in the pupal and adult stages, with divergent life-history priorities for investment in immunity[28]. These trends are predicted as species-specific trade-offs with environmental and life-history environments. *S. frugiperda* is polyphagous and highly migratory as an insect pest, and this would have created a demand for enhanced long-term and sex-specific investment in immunity, particularly in the females, to enhance fecundity success and longevity. These evolutionary differences are important for understanding the modulation of immune control by environmental stress and can be applied for improving targeted control measures.

Although our work provides a complete transcriptomic profiling of immune gene expression in *S. frugiperda*, certain key areas of research still need to be addressed. Functional verification of the major immune genes through RNA interference (RNAi) would be required to determine their precise functions in host defense and resistance to pathogens[62,63]. Pathogen challenge assays against various life stages would also reveal how immune gene expression is translated into immune competency. Environmental factors, including diet quality, microbial exposure, and abiotic stressors, must also be tested for their effects on immune gene regulation. In addition, hormonal regulation, in the form of juvenile hormone and ecdysteroid action, can be explored to determine mechanistically how developmental and immune processes are coordinated. Comparative genome and transcriptomic analyses across diverse *Spodoptera* species would reveal lineage-specific as well as conserved immune responses that can be employed to illuminate evolutionary forces leading to immune system adaptations. Taken together, such research directions would not only deepen our knowledge regarding insect immunity but also guide more efficient biological control measures for *S. frugiperda*.

This comprehensive transcriptomic analysis reveals a nuanced understanding of the immune architecture in *S. frugiperda*, illustrating how immunity evolves across developmental stages and between sexes. The intricate regulation of humoral and cellular immunity, particularly the differential expression of key immune pathways such as Toll and Imd, aligns with the physiological and ecological demands of each stage. Enhanced immune gene expression in larval and adult female stages underscores the biological prioritization for survival and reproduction, respectively. These findings deepen our knowledge of insect immunity and highlight potential targets for biological control strategies that exploit vulnerabilities in specific life stages. By linking developmental biology with immune dynamics, this work sets a foundation for innovative pest management approaches tailored to the lifecycle of *S. frugiperda*.

Data availability

The raw sequencing data have been deposited at the National Center for Biotechnology Information (NCBI). The Bioproject accession number is PRJNA1145502. The transcriptome data are available under the following accession numbers and the letters in parentheses represent the corresponding sample name: SRR30233905 (AF_R1), SRR30233904 (AF_R2), SRR30233893 (AF_R3), SRR30233882 (AM_R1), SRR30233881 (AM_R2), SRR30233880 (AM_R3), SRR30233879 (E_R1), SRR30233878 (E_R2), SRR30233877 (E_R3), SRR30233876 (L1_R1), SRR30233903 (L1_R2), SRR30233902 (L1_R3), SRR30233901 (L2_R1), SRR30233900 (L2_R2), SRR30233899 (L2_R3), SRR30233898 (L3_R1), SRR30233897 (L3_R2), SRR30233896 (L3_R3), SRR30233895 (L4_R1), SRR30233894 (L4_R2), SRR30233892 (L4_R3), SRR30233891 (L5_R1), SRR30233890 (L5_R2), SRR30233889 (L5_R3), SRR30233888 (L6_R1), SRR30233887 (L6_R2), SRR30233886 (L6_R3), SRR30233885 (P_R1), SRR30233884 (P_R2), and SRR30233883 (P_R3), respectively.

Received: 17 December 2024; Accepted: 11 June 2025
Published online: 11 July 2025

References

1. Strand, M. R. The insect cellular immune response. *Insect Sci.* **15**, 1–14 (2008).
2. Schmid-Hempel, P. Variation in immune defence as a question of evolutionary ecology. *Proc. R Soc. Lond. B.* **270**, 357–366 (2003).
3. Eleftherianos, I., Baldwin, H., ffrench-Constant, R. H. & Reynolds, S. E. Developmental modulation of immunity: changes within the feeding period of the fifth larval stage in the defence reactions of *Manduca sexta* to infection by *Photorhabdus*. *J. Insect Physiol.* **54**, 309–318 (2008).
4. Gao, H. et al. Age-associated changes in innate and adaptive immunity: role of the gut microbiota. *Front. Immunol.* **15**, 1421062 (2024).
5. Keith, S. A. Steroid hormone regulation of innate immunity in *Drosophila melanogaster*. *PLoS Genet.* **19**, e1010782 (2023).
6. Eleftherianos, I. et al. Diversity of insect antimicrobial peptides and proteins - A functional perspective: A review. *Int. J. Macromol.* **191**, 277–287 (2021).
7. Eleftherianos, I. et al. Haemocyte-mediated immunity in insects: cells, processes and associated components in the fight against pathogens and parasites. *Immunology* **164**, 401–432 (2021).
8. Ferrandon, D., Imler, J. L., Hetru, C. & Hoffmann, J. A. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat. Rev. Immunol.* **7**, 862–874 (2007).
9. Mollah, M. M. I. & Kim, Y. HMGB1-like dorsal switch protein1 of the mealworm, *Tenebrio molitor*, acts as a damage-associated molecular pattern. *Arch. Insect Biochem. Physiol.* **107**, e21795 (2021).
10. Wang, L. et al. Gene expression differences between developmental stages of the fall armyworm (*Spodoptera frugiperda*). *DNA Cell. Biol.* **40**, 580–588 (2021).
11. Wang, P. et al. Performance of *Trichogramma Japonicum* as a vector of *Beauveria Bassiana* for parasitizing eggs of rice striped stem borer, *Chilo suppressalis*. *Entomol. Gen.* **41**, 147–155 (2021).
12. Gu, M. et al. Efficacy of *Metarhizium rileyi* granules for the control of *Spodoptera frugiperda* and its synergistic effects with chemical pesticide, sex pheromone and parasitoid. *Entomol. Gen.* **43**, 1211–1219 (2023).
13. Khan, I., Agashe, D. & Rolff, J. Early-life inflammation, immune response and ageing. *Proc. R Soc. B.* **284**, 20170125 (2017).
14. Roberts, K. E. & Hughes, W. O. H. Immunosenescence and resistance to parasite infection in the honey bee, *Apis mellifera*. *J. Invertebr Pathol.* **121**, 1–6 (2014).
15. Legeai, F. et al. Establishment and analysis of a reference transcriptome for *Spodoptera frugiperda*. *BMC Genom.* **15**, 704 (2014).
16. Zhang, W. et al. Comparative transcriptomic analysis of immune responses of the migratory locust, *Locusta migratoria*, to challenge by the fungal insect pathogen, *Metarhizium acridum*. *BMC Genom.* **16**, 867 (2015).
17. Jia, C. et al. Odorant-binding proteins and chemosensory proteins in *Spodoptera frugiperda*: From genome-wide identification and developmental stage-Related expression analysis to the perception of host plant odors, sex pheromones, and Insecticides. *Int. J. Mol. Sci.* **24**, 5595 (2023).
18. Xu, P. et al. Novel partiti-like viruses are conditional mutualistic symbionts in their normal lepidopteran host, African armyworm, but parasitic in a novel host, fall armyworm. *PLoS Pathog.* **16**, e1008467 (2020).
19. Gouin, A. et al. Two genomes of highly polyphagous lepidopteran pests (*Spodoptera frugiperda*, Noctuidae) with different host-plant ranges. *Sci. Rep.* **7**, 11816 (2017).
20. Huot, L. et al. Partner-specific induction of Spodoptera frugiperda immune genes in response to the entomopathogenic nematobacterial complex *Steinernema carpocapsae-Xenorhabdus nematophila*. *Dev. Comp. Immunol.* **108**, 103676 (2020).
21. Kanehisa, M. & Goto, S. K. E. G. G. Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
22. Sevgili, H. Sex differences in immunity in a natural population of bush-cricket (Orthoptera: Phaneropterinae). *Biologia* **74**, 501–508 (2019).
23. Ahtiainen, J. J., Alatalo, R. V., Kortet, R. & Rantala, M. J. Immune function, dominance and mating success in drumming male Wolf spiders *Hygrolycosa rubrofasciata*. *Behav. Ecol. Sociobiol.* **60**, 826–832 (2006).
24. Jacobs, C. G. C., Spaink, H. P. & Van Der Zee, M. The extraembryonic Serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. *eLife* **3**, e04111 (2014).
25. Yoshida, H., Kinoshita, K. & Ashida, M. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem.* **271**, 13854–13860 (1996).
26. Pili-Floury, S. et al. In vivo RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in Drosophila adults. *J. Biol. Chem.* **279**, 12848–12853 (2004).
27. Lin, J. et al. Gene expression profiling provides insights into the immune mechanism of *Plutella Xylostella* midgut to microbial infection. *Gene* **647**, 21–30 (2018).
28. Xia, X. et al. Genome-wide characterization and expression profiling of immune genes in the Diamondback moth, *Plutella Xylostella* (L). *Sci. Rep.* **5**, 9877 (2015).
29. Peng, J., Zipperlen, P. & Kubli, E. *Drosophila* sex-peptide stimulates female innate immune system after mating via the toll and Imd pathways. *Curr. Biol.* **15**, 1690–1694 (2005).
30. Yudai, N. et al. Functional crosstalk across IMD and Toll pathways: insight into the evolution of incomplete immune cascades. *Proc. R. Soc. B.* **286**, 20182207 (2019).
31. Lima, L. F., Torres, A. Q., Jardim, R., Mesquita, R. D. & Schama, R. Evolution of toll, spatzle, and MyD88 in insects: the problem of the Diptera bias. *BMC Genom.* **22**, 562 (2021).
32. Valanne, S., Vesala, L., Maasdorp, M. K., Salminen, T. S. & Rämet, M. The *Drosophila* toll pathway in innate immunity: from the core pathway toward effector functions. *J. Immunol.* **209**, 1817–1825 (2022).
33. Harris, F., Dennison, S. R. & Phoenix, D. A. Anionic antimicrobial peptides from eukaryotic organisms. *Curr. Protein Pept. Sci.* **10**, 585–606 (2009).

34. Mollah, M. M. I., Choi, H. W., Yeam, I., Lee, J. M. & Kim, Y. Salicylic acid, a plant hormone, suppresses phytophagous insect immune response by interrupting HMG-like DSP1. *Front. Physiol.* **12**, 744272 (2021).
35. Gill, C., Bahrndorff, S. & Lowenberger, C. *Campylobacter jejuni* in *Musca domestica*: an examination of survival and transmission potential in light of the innate immune responses of the house flies. *Insect Sci.* **24**, 584–598 (2017).
36. Tripathi, L. P. & Sowdhamini, R. Genome-wide survey of prokaryotic serine proteases: analysis of distribution and domain architectures of five Serine protease families in prokaryotes. *BMC Genom.* **9**, 549 (2008).
37. Ligoxygakis, P., Roth, S. & Reichhart, J. M. A serpin regulates dorsal-ventral axis formation in the Drosophila embryo. *Curr. Biol.* **13**, 2097–2102 (2003).
38. Zou, Z., Lopez, D. L., Kanost, M. R., Evans, J. D. & Jiang, H. Comparative analysis of Serine protease-related genes in the honey bee genome: possible involvement in embryonic development and innate immunity. *Insect Mol. Biol.* **15**, 603–614 (2006).
39. Eguchi, M., Matsui, Y. & Matsumoto, T. Developmental change and hormonal control of chymotrypsin inhibitors in the haemolymph of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **84**, 327–332 (1986).
40. Fröbius, A. C., Kanost, M. R., Götz, P. & Vilcinskas, A. Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth *Galleria Mellonella*. *Eur. J. Biochem.* **267**, 2046–2053 (2000).
41. Ahn, J. S. et al. Aging-associated increase of gelsolin for apoptosis resistance. *Biochem. Biophys. Res. Commun.* **312**, 1335–1341 (2003).
42. Son, Y., Hwang, J. & Kim, Y. Functional study of the gene encoding apolipophorin III in development and immune responses in the beet armyworm, *Spodoptera exigua*. *J. Asia Pac. Entomol.* **15**, 106–112 (2012).
43. Wilson, M. J., McKelvey, B. H., Van Der Heide, S. & Dearden, P. K. Notch signaling does not regulate segmentation in the honeybee, *Apis mellifera*. *Dev. Genes Evol.* **220**, 179–190 (2010).
44. Bachmann, A. & Knust, E. Positive and negative control of *Serrate* expression during early development of the *Drosophila* wing. *Mech. Dev.* **76**, 67–78 (1998).
45. Ip, Y. A dorsal-related gene that mediates an immune response in *Drosophila*. *Cell* **75**, 753–763 (1993). Dif.
46. Gordon, M., Dionne, M., Schneider, D. & Nusse, R. WntD is a feedback inhibitor of Dorsal/NF-κB in *Drosophila* development and immunity. *Nature* **437**, 746–749 (2005).
47. Zeidler, M. P., Bach, E. A. & Perrimon, N. The roles of the Drosophila JAK/STAT pathway. *Oncogene* **19**, 2598–2606 (2000).
48. Altenhein, B. et al. Expression profiling of glial genes during *Drosophila* embryogenesis. *Dev. Biol.* **296**, 545–560 (2006).
49. Alfonso, T. B. & Jones, B. W. *gcm2* promotes glial cell differentiation and is required with glial cells missing for macrophage development in *Drosophila*. *Dev. Biol.* **248**, 369–383 (2002).
50. Mollah, M. M. I., Ahmed, S. & Kim, Y. Immune mediation of HMG-like DSP1 via toll spatzle pathway and its specific Inhibition by Salicylic acid analogs. *PLoS Pathog.* **17**, e1009467 (2021).
51. Li, Q., Dong, X., Zheng, W. & Zhang, H. The PLA2 gene mediates the humoral immune responses in *Bactrocera dorsalis* (Hendel). *Dev. Comp. Immunol.* **67**, 293–299 (2017).
52. Tindwa, H. et al. Molecular cloning and characterization of autophagy-related gene *TmATG8* in *Listeria*-invaded hemocytes of *Tenebrio molitor*. *Dev. Comp. Immunol.* **51**, 88–98 (2015).
53. Zhang, K. et al. A novel granulocyte-specific α integrin is essential for cellular immunity in the silkworm *Bombyx mori*. *J. Insect Physiol.* **71**, 61–67 (2014).
54. Weiske, J. & Wiesner, A. Stimulation of NO synthase activity in the immune-competent lepidopteran *Estigmene acraea* hemocyte line. *Nitric Oxide*. **3**, 123–131 (1999).
55. Mollah, M. M. I. Spatzle processing enzyme is required to activate dorsal switch protein 1 induced toll immune signaling pathway in *Tenebrio molitor*. *PLoS ONE*. **18**, e0291976 (2023).
56. Barchuk, A. R., Figueiredo, V. L. C. & Simões, Z. L. P. Downregulation of ultraspiracle gene expression delays pupal development in honeybees. *J. Insect Physiol.* **54**, 1035–1040 (2008).
57. Ravaiano, S. V., Barbosa, W. F., Campos, L. A. & Martins, G. F. Variations in circulating hemocytes are affected by age and caste in the stingless bee *Melipona quadrifasciata*. *Sci. Nat.* **105**, 48 (2018).
58. Estévez-Lao, T. Y. & Hillyer, J. F. Involvement of the *Anopheles gambiae* Nimrod gene family in mosquito immune responses. *Insect Biochem. Mol. Biol.* **44**, 12–22 (2014).
59. Xiong, G. H. et al. High throughput profiling of the cotton bollworm *Helicoverpa armigera* immunotranscriptome during the fungal and bacterial infections. *BMC Genom.* **16**, 321 (2015).
60. Tanaka, H. et al. A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. *Insect Biochem. Mol. Biol.* **38**, 1087–1110 (2008).
61. von Bredow, Y. M. et al. Differential expression of immunity-related genes in larval *Manduca sexta* tissues in response to gut and systemic infection. *Front. Cell. Infect. Microbiol.* **13**, 1258142 (2023).
62. Vogel, E., Santos, D., Mingels, L., Verdonckt, T. W. & Broeck, J. V. RNA interference in insects: protecting beneficials and controlling pests. *Front. Physiol.* **9**, 1912 (2019).
63. Christiaens, O., Niu, J. & Taning, C. N. T. RNAi in insects: A revolution in fundamental research and pest control applications. *Insects* **11**, 415 (2020).

Acknowledgements

This research was supported by the National Natural Science Foundation of China (32160666, 32472644) and the Guizhou Province Science and Technology Support Project [2022] General 239.

Author contributions

GW, JR, CJ, LZ, JRPM, RKAA prepared the samples, performed the transcriptomic experiment, analyzed the results, validated the data with qRT-PCR, and constructed the figures. AM, WZ, RKAA, NOK, IE supervised the project, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-06939-1.

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