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Genomics, transcriptomics, and peptidomics of *Spodoptera frugiperda* (Lepidoptera, Noctuidae) neuropeptides

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Abstract

Neuropeptides control many physiological and behavioral processes, and so they are functionally important classes of cell-to-cell signaling molecules. Nowadays, the fall armyworm, *Spodoptera frugiperda*, is one of the most destructive agricultural pests in the world. In this study, we mined the publicly accessible genome assembly data for *S. frugiperda*, and the transcriptomic and proteomic data of the larval central nervous system (CNS) for putative neuropeptide-encoding, and subsequently we used these to anticipate a peptidome for this species. In essence, we could identify 57 orthologs of insect neuropeptides, including Allatotropin, CCHamide, Corazonin, pheromone biosynthesis activating neuropeptide, short neuropeptide F, Trissin, and Natalisin. Interesting features for *S. frugiperda* were the absence of genes coding for CNMamide, Elevein, and the differential evolution of ancestral neuropeptide genes such as adipokinetic corazonin-related peptide, adipokinetic hormone, Tachykinin, and Natalisin. In conclusion, our study provides the most complete neuropeptide description for the important pest *S. frugiperda* as a foundation to study the factors regulating insect growth, reproduction, and behavior. Second, we confirm that a comprehensive multi-omics analysis is necessary for the identification of neuropeptides. Finally, our

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data provide a reliable reference for other comparative studies in other insects beyond the supermodel insect of *Drosophila melanogaster* and the finding of potential candidates as selective for pests versus beneficial insects.

KEYWORDS

genomic, neuropeptide identification, proteomic, *Spodoptera frugiperda*, transcriptomic

1 | INTRODUCTION

The fall armyworm *Spodoptera frugiperda* is a noctuid polyphagous pest that is native to subtropical and tropical regions of the Americas (Nagoshi et al., 2017). *S. frugiperda* is a major pest of maize, and it has a vast host range of more than 80 plant species. Therefore, it can cause serious economic damage to agriculture and pasture production (Gouin et al., 2017). In early May 2018, this pest was first discovered in India, and in January 2019, it was found in China; since then, it has immigrated northward to Hebei Province, which is near Beijing (Sun et al., 2019). Currently, *S. frugiperda* is confirmed to be present in most eastern and southern Asian countries and is also strongly invasive into East-Africa.

Neuropeptides play an important role in regulating the reproduction, development, feeding, behavior, courtship, and many other physiological behavior processes in animals (Ons, 2017). Hence, neuropeptide signaling systems are considered as an ideal prospective target for breeding peculiar insect control agents (Altstein, 2001). In recent years, with the rapid development of genomics and proteomics, insect neuropeptides have been studied in more detail. Roughly, 50 identified insect neuropeptide families have been identified, especially those in the model organisms *Drosophila melanogaster*, *Bombyx mori*, *Acyrthosiphon pisum*, *Tribolium castaneum* (Huybrechts et al., 2010; Li et al., 2008; Nassel & Winther, 2010; Pandit et al., 2019; Roller et al., 2008; The International Aphid Genomics Consortium, 2010; Yeoh et al., 2017). Hence, as the genomes and transcriptomes of some important agricultural pests have also been sequenced, the neuropeptides of these pests have also been identified, such as *Bactrocera dorsalis*, *Spodoptera exigua*, *Cydia pomonella*, *Chilo suppressalis*, *Nilaparvata lugens*, *Spodoptera litura* (Cheng et al., 2017; Garczynski et al., 2019; Gui et al., 2017; Llopis-Giménez et al., 2019; Tanaka et al., 2014; Xu et al., 2016).

In this study, we mined the publicly accessible genome assembly data for *S. frugiperda* (Accession No. GCA_011064685.1; Gouin et al., 2017; Xiao et al., 2020), and the transcriptomic and proteomic data of the larval central nervous system (CNS) for putative neuropeptide-encoding, and subsequently we used these to anticipate a peptidome for this species. Totally, 57 neuropeptide precursors were annotated, and our data furnish a foundation for determining neuropeptide signaling systems to the regulation of development and behavior for the important pest *S. frugiperda*. Finally, they can provide a reliable reference for comparative studies and the finding of potential candidates as selective for pests versus beneficial insects in the development of bio-safe insect control agents.

2 | MATERIALS AND METHODS

2.1 | Insect

S. frugiperda larvae were collected on maize from Dehong City, Yunnan Province, China in January 2019, and then reared in an incubator kept at 25 ± 2°C with a relative humidity of 65 ± 10% and a photoperiod with 14 h of light

and 10 h of dark. Honey water (10%) was provided as food for adults, and larvae were fed with a synthetic diet, as described by Jiang et al. (1999).

2.2 | Sample collection and RNA sequencing

The CNS of 5th-instar larvae of *S. frugiperda* was dissected in chilled 0.01 M phosphate-buffered saline (PBS). Totally, 200 CNS tissues were used and stowed in 300 μl of TRIzol reagent (Invitrogen). The total RNA was refined using TRIzol reagent, and a second purification step was applied using an RNeasy Mini Kit (Promega). Purified RNA was eluted with Milli-Q water, and the RNA integrity verified on a 1.5% agarose gel. The RNA concentration and quantity were measured in a NanoDrop 2000 (Thermo Fisher Scientific). The purified RNA was used for the RNA-seq using a paired-end (PE) approach in an Illumina HiSeq 2000 (Illumina) at BioMarker Technology. PE reads were filtered, processed, and assembled using Trinity software using a standard pipeline and default parameters (Haas et al., 2014).

2.3 | Protein extraction and determination

We chose 200 5th-instar larvae for dissection, CNS were removed carefully under the stereoscopic microscope and stored in 0.01 M PBS. Then, CNS were ground with liquid nitrogen. Later, we added 1 ml of 0.25% acetic acid solution and the appropriate amount of protease inhibitor, and sonicated in ice bath for 2 min. The contents of the tube were centrifuged at 12,000*g* at 4°C and the supernatant transferred to a 10 KD ultrafiltration centrifuge tube. The supernatant was centrifuged at 12,000*g* at 4°C by adding 100 μl of 0.25% acetic acid solution and again centrifuged at 12,000*g* for 10 min. The process was repeated twice for collection and filtration and later dried by adding an appropriate amount of 0.1% Trifluoroacetic acid solution and using a C18 Cartridge for desalination.

The peptides were chromatographed using the Easy nLC 1200 chromatography system (Thermo Fisher Scientific) with a nanoliter flow rate. The sample was injected into a Trap Column (100 μm x 20 mm, 5 μm, C18; Dr. Maisch GmbH) and then passed through a chromatographic analysis column (75 μm x 150 mm, 3 μm, C18; Dr. Maisch GmbH) for gradient separation at a flow rate of 300 nl/min. After the separation of peptides, DDA (data-dependent acquisition) was performed with Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) for mass spectrometry (MS) analysis. The chromatographic dissociation time was 70 min and with a MS scanning range of (*m*/*z*) 300—1800.

The MS database search software MaxQuant 1.6.1.0 was used for this project. The enzyme was set to unspecific by the following criteria; precursor tolerance (main search) was 4.5 ppm; precursor tolerance (first search) was 20 ppm; MS/MS Tolerance 20 ppm; fixed modifications were carbamidomethyl (C); variable modifications were oxidation (M), acetyl (protein N-term); PSM FDR and PSM FDR were both set to 0.01.

2.4 | Gene predictions and sequence alignment

The similarity search was performed using the local BLAST Search Tool. The settings for BLAST were predetermined and unchangeable. For a long query, only the expectation value (*e*-value) was increased. For short queries (short peptide), the *e*-value was raised to 10,000, the word size decreased to 1, the gap penalty reduced to 10, and the filter was turned off. For searching genomic data, we used the FGENESH (www.softberry.com) gene prediction programs to anticipate genes for the potential BLAST scaffold. The workflow cited above was used for predicting the structures of mature peptides. In particular, each deduced precursor protein appraised for the existence of a signal peptide by the usage of the online site SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011), and the neuropeptide cleavage sites determined was based on similarities with publicly neuropeptides by using the findings in Veenstra (2000). ClustalX software was used to accomplish multiple sequence alignments, by applying the default

weight matrix. Sequence logos of manually aligned homologous neuropeptide precursors were created by using the online tool WebLogo (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004).

3 | RESULTS

3.1 | CNS transcriptome assembly and annotation

A total of 7.95 Gb clean data was obtained, and the Q30 length percentage of each sample was not less than 94.00%. The 40,910 unigenes obtained after sequence assembly using Trinity and the N50 of unigene was 943. For annotations, unigene sequences were compared with NR, Swiss-Prot, GO (Gene Ontology), COG (Clusters of Orthologous Groups), KOG (Karyotic Orthologous Groups), eggNOG4.5 and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases by using BLAST software, and the KEGG Orthology results of unigene in KEGG were obtained using KOBAS 2.0. Totally, 21,896 (53.52%) unigenes were annotated using the NCBI-Nr database; 9584 (23.42%) by Swiss-Prot; 10,294 (25.16%) by KEGG; 4860 (11.87%) by COG; 10,836 (26.48%) by GO, which covered 22,587 (55.21%) of the total unigenes. As shown in Figure S1, for GO terms, 7471, 14,123, and 10,649 transcripts were assigned to the cellular component, molecular function, and biological processes, respectively.

3.2 | Omics identification analysis

At first, the BLAST search was performed using precursor sequences deduced from other species in the assembly of the *S. frugiperda* transcriptome. In the CNS transcriptome data of *S. frugiperda* larvae, we identified 44 neuropeptide precursor genes. In contrast, in the genome, we found 57 neuropeptide precursor genes. Allatostatin B, Calcitonin-B1, ETH, ITG, IMFamide, LQDVamide, Natalisin (NTL), Neuroparsin, NPLP, NPF 2, RYamide, Tachykinin (TK), and Trissin-1 were identified in the genome-based search only (Figure 1 and Table S2). In the peptidome analysis, we found a total of 278 peptides belonging to 37 neuropeptide precursors (Table S1). Furthermore, the peptidome analysis of CNS confirmed the predicted sequence of ETH, NTL, Neuroparsin, NPLP, NPF2, RYamide, and TK, which had not been predicted in the transcriptome (Figure 1 and Table S2). Through the total data (Figure 1), we report here that all neuropeptide genes can be identified from the genome, but in the absence of the genome, the analysis of the combined transcriptome and the peptidome can also identify most neuropeptides.

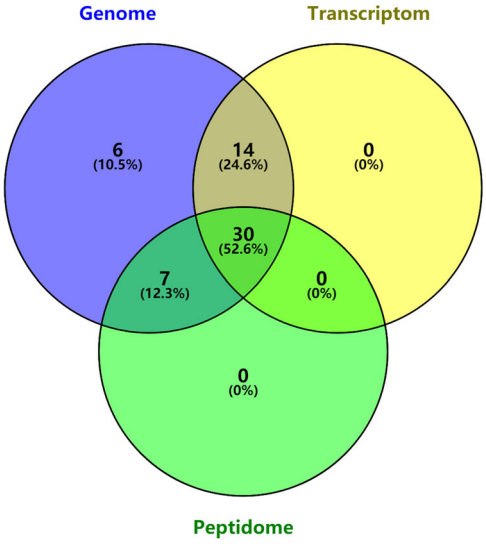

FIGURE 1 Neuropeptide identification results for genomes, transcriptomes, and peptidome, and overlap between databases

4 | DISCUSSION

In this study, we conducted a quality evaluation of the neuropeptides of *S. frugiperda* from genome, transcriptome, and peptidome databases, followed by a multidirectional analysis of gene loss and a comprehensive analysis of multi-omics. Finally, we compared the structural differences between the neuropeptides of *S. frugiperda*, *B. mori*, *Z. nevadensis*, *T. castaneum*, *A. pisum*, and *L. migratoria*, and conducted a detailed difference analysis. With regard to habitat, the larvae of *S. frugiperda* and *B. mori* live in a certain water content condition (Hussain et al., 2011; Johnson, 1987), while *Z. nevadensis* and *T. castaneum* live in a relatively dry and higher temperature condition (Abe et al., 2000; White, 1987). For species with very different living conditions, many neuropeptides would require better water and ion regulation especially in water loss, and this may be related to the presence or absence of certain neuropeptides such as Diuretic hormone, ITG, IMFamide, and Kinin as has also been reported by Zandawala (2012) and Cohen (2013).

4.1 | Multi-omics comprehensive identification of neuropeptide precursors

We predicted and annotated a total of 57 neuropeptide precursors (57 with genome, 44 with transcriptome, and 37 with peptidome), which epitomize the comprehensive analysis of neuropeptide precursors in the fall armyworm *S. frugiperda* (Figure 2; Supporting Information Data S1). Compared with the neuropeptides of other species, IMFamide, ITG, Kinin, LQDVamide, and PBAN have only been identified in *S. frugiperda* and *B. mori*. But also, some neuropeptides are missing in some species. With the sequencing of the *T. castaneum* genome (Richards et al., 2008), Li et al. (2008) and Pandit et al. (2019) found that the common neuropeptide genes Corazonin and Allatostatin A are not present in beetle insects. Veenstra (2014) completed the identification work of the termite *Z. nevadensis* neuropeptides. As the termite is a relatively ancient species, the results showed that it retains more neuropeptides than the better known holometabolous species (Veenstra, 2014). With the use of the termite neuropeptide data, we found that Trissin-1 is not present in *B. mori*. The neuropeptide Trissin was originally discovered and highly expressed in the CNS of *Drosophila* (Ida et al., 2011); however, its specific function is still unknown. In *S. frugiperda*, we have identified Trissin-2 in the genome, transcriptome, and peptidome, but only found the presence of Trissin-1 in the genome (Table S2). Trissin peptides have conservative mature peptides, which contain a CxxCxxxCxxxCxxxxxxxCC structure in all insects (Figure 3). No signal peptide was predicted due to the 5′ deletion of Trissin-1 in *S. frugiperda*.

4.2 | Gene loss in omics analysis

In *S. exigua*, Llopis-Giménez et al. (2019) could identify 64 neuropeptides. Although it is a very closely related species to *S. frugiperda* in the same genus, some neuropeptides such as CNMamide, Elevein, DH 44, Proctolin, have not been identified in *S. frugiperda*, and we think this can be due to a variety of reasons. At first, it may be that SNP genes are often ignored by automatic annotation programs. For example, Veenstra (2019) showed that the Elevein precursor is poorly conserved in different species, so its identification is difficult by the BLAST program. Second, when a gene does not exist in the transcriptome, it may be that its expression level is low. If a gene does not exist in the genome assembly, it may be located in a part of the genome that has not been transformed into the genome assembly. Finally, it should also be remarked here that although some neuropeptide genes are missing in the genome, their function may not be missing as it may be replaced by some new neuropeptides or related functional peptides.

4.3 | Diversity in neuropeptide precursor structure

A neuropeptide is a signaling molecule involved in the regulation of many physiological functions of insects. In this study, there are more than 50 neuropeptide genes in insect species and they have different sequence structures.

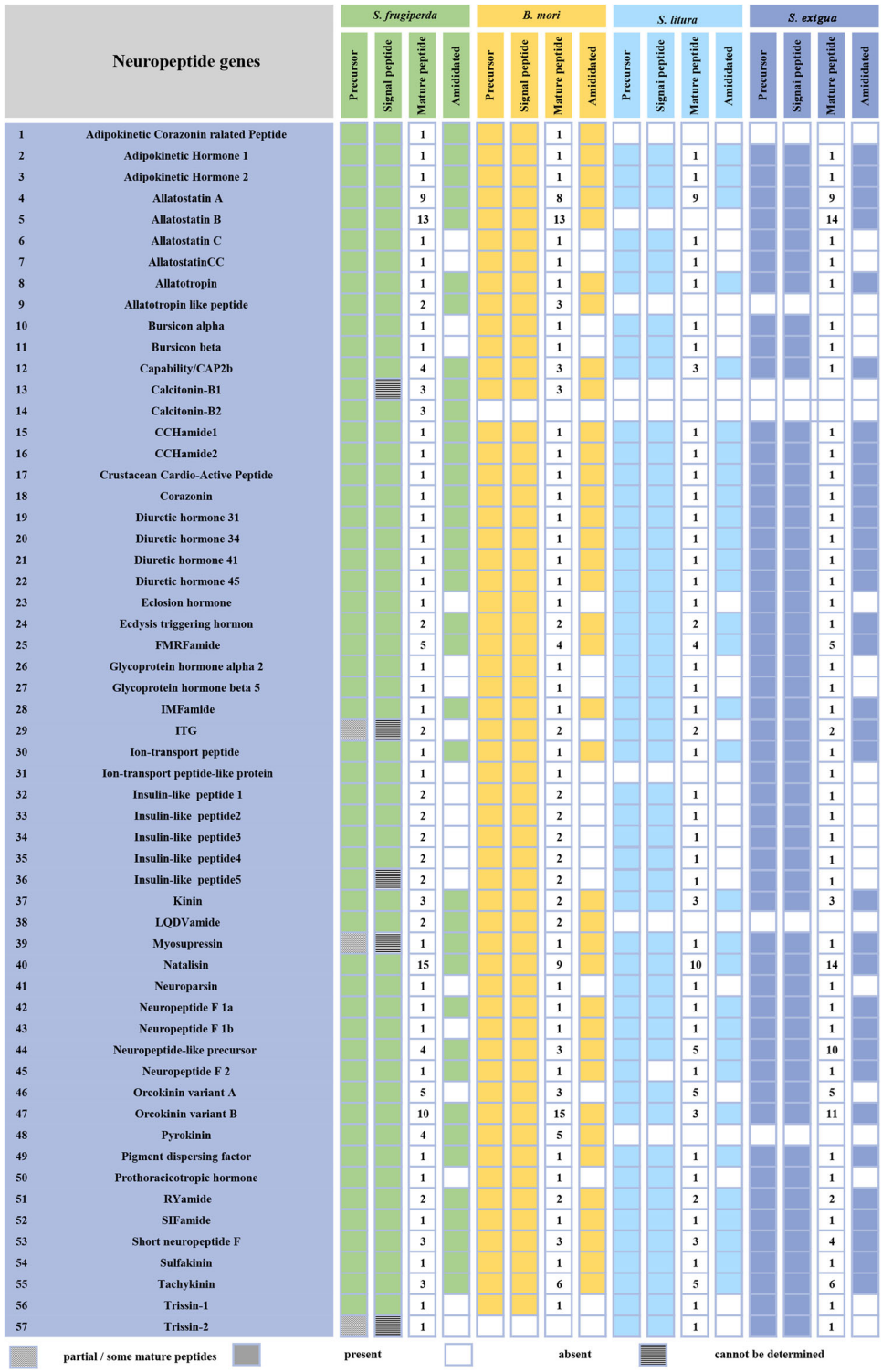

FIGURE 2 Summary of neuropeptide precursors identified in *Spodopterafrugiperda*, *Bombyx mori*, *Spodoptera exigua*, and *Spodoptera litura*. The number of mature peptides and the presence of amidation and signal peptides derived from each precursor

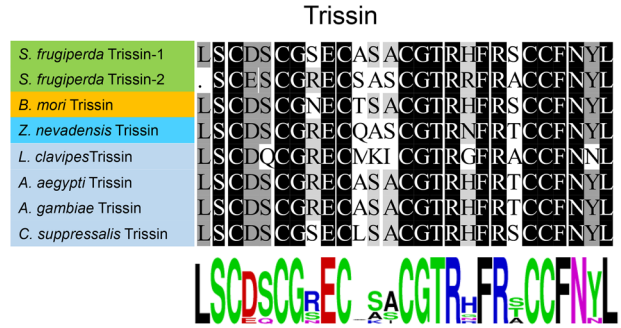

FIGURE 3 Multiple sequence alignments of Trissin mature peptides

Interestingly, some neuropeptide sequences have similar conservative structures, but there are many differences in physiological functions and tissue distribution, especially neuropeptides with the same evolutionary origin, so it is necessary to explore the relationship and differences between them.

By comparing the AKHs of *S. frugiperda*, *B. mori*, *T. castaneum*, *Z. nevadensis*, *L. migratoria*, *A. pisum*, and other species, the mature peptide of AKH1 contains nine amino acid residues and is more conservative. The mature peptide with AKH3/4 contains eight amino acid residues. All AKHs peptides have a C-terminal amide (Figure 4a–c).

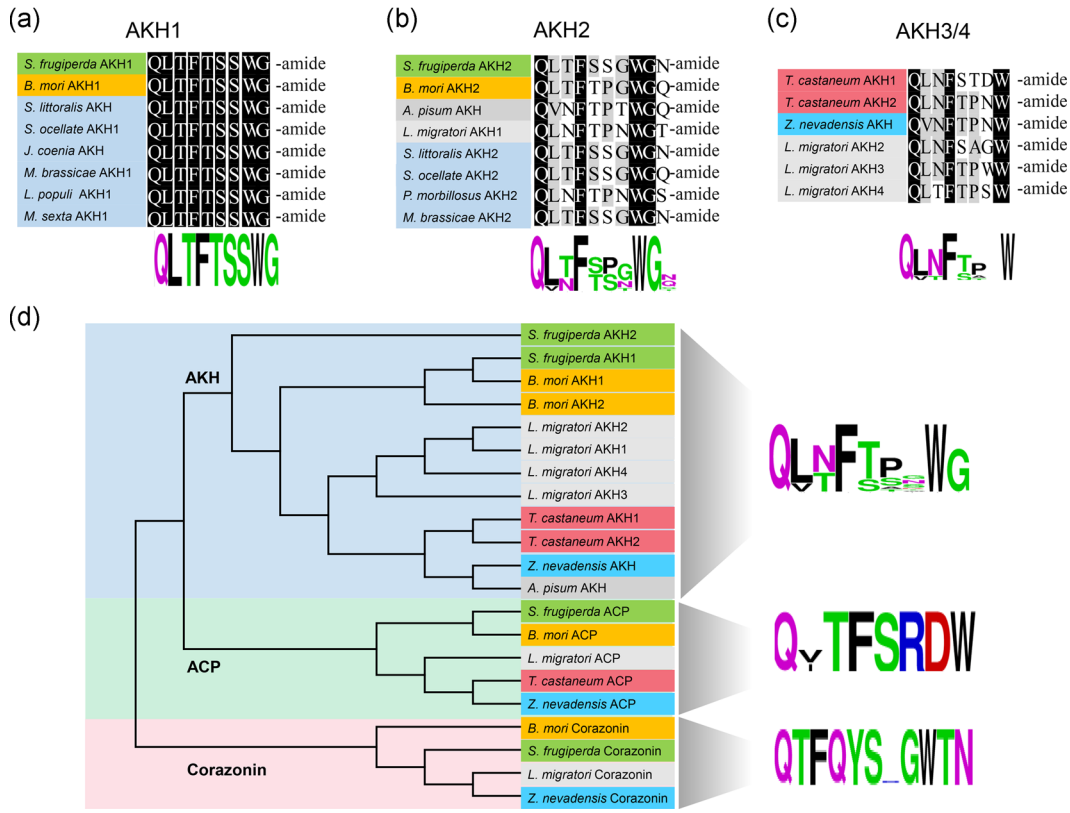

FIGURE 4 (a) Multiple sequence alignments of AKH1 mature peptides; (b) multiple sequence alignments of AKH2 mature peptides; (c) multiple sequence alignments of AKH3/4 mature peptides; (d) the hypothetical evolutionary tree and the C-terminal motifs of ACP, AKHs, and Corazonin. The C-terminal region in ACP, AKHs, and Corazonin are shown for the frequencies of specific amino acids

We identified two AKHs (AKH1 and 2) in *S. frugiperda*. The mature peptide of AKH1 (qQLTFTSSWG) is more conservative than AKH2 and AKH3/4 by sequence alignment (Figure 4a–c). Phylogenetic analysis of AKHs, ACP, and Corazonin revealed that the three neuropeptides have independent branches (Figure 4d). Nevertheless, the mature peptides of ACP and AKH3/4 have certain structural similarities and are composed of eight amino acids. The ACP peptide, lost in many species, is synthesized and released in the brain. It does not produce an adipokinetic effect (Siegert, 1999). The *S. frugiperda* ACP precursor encodes the core peptide pQITFSRDWTG-amide, which was predicted simultaneously in the genome, transcriptome, and peptidome (Figure 4d and Table S2). The mature peptide sequences of ACP and AKH are very similar (Figure 4d), but unlike classic AKHs, which are produced in corpora cardiaca and mobilize lipids from the fat body, ACP is synthesized in the brain and does not produce an adipokinetic effect (Patel et al., 2014; Siegert, 1999). The mature peptide of Corazonin is similar in structure to the AKHs of insects and are composed of 11 amino acids, and they have widely been considered to have a common evolutionary origin (Hansen et al., 2010). Our sequence alignment results showed that the Corazonin peptide (qQTFQYSxGWTN) is conserved among insects, and all Corazonin peptides have an N-terminal pyroglutamate and a C-terminal amide. Although this neuropeptide sequence is conserved among various species, this gene is missing in *T. castaneum*.

Among *S. frugiperda*, TK and NTL were identified in the genome and peptidome, and both contain 3 and 15 mature peptides, respectively (Figure 5a). The two neuropeptides differ greatly in the number of mature peptides, and it seems that NTL has more mature peptides than TK in Lepidoptera, but this is different from *L. migratoria*,

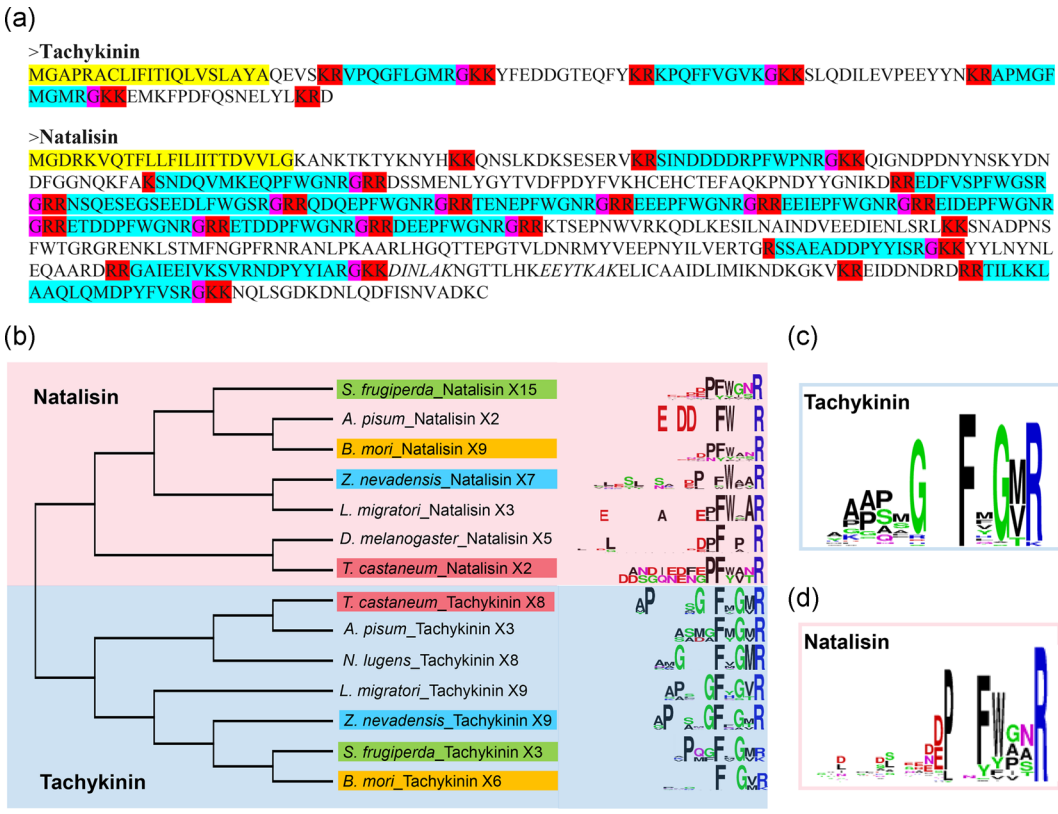

FIGURE 5 The C-terminal consensus sequences of NTL and TK and the phylogenetic tree. (a) The *Spodoptera frugiperda* NTL and TK precursor sequences, the color notes as above. (b) The hypothetical evolutionary tree and the C-terminal motifs of Natalisin and Tachykinin. The C-terminal region in NTL and TK are shown for the frequencies of specific amino acids. (c) Sequence alignment C-terminal motif of NTL/TK sequences

Z. nevadensis, and *A. pisum*. Moreover, the ligand-receptor interactions studied by functional assays found that DmNTL4 activates DmTRPR and BmNTL1 activates BmTRPR at high concentrations, indicating that they have a moderate to low degree of cross-activation specificity (Jiang et al., 2013). The two neuropeptides still have a certain interaction relationship in the process of evolving in different directions. Considering the difference in the number of mature peptides, we speculate that their functional specificity is different between Lepidoptera and other species. TK of *S. frugiperda* has a conserved motif PxGFxGMR-amide and clusters with the *B. mori* (Figure 5b). The mature peptide conserved motif of *S. frugiperda* NTL is xxxPFWxxR-amide. Comparison of mature peptides from multiple species showed that NTL and TK are closely related neuropeptides with the common sequence motif FxxxRamide (C-terminally amidated; Figure 5c,d).

5 | CONCLUSION

In this study, we analyzed the newly discovered CNS transcriptome, proteome, and genomic data to comprehensively identify the neuropeptide precursors of the fall armyworm *S. frugiperda*, one of the most destructive agricultural pests in the world. The identification of 57 neuropeptide precursors in *S. frugiperda* has provided important new insights into the evolution and diversity of neuropeptide signaling systems. By integrative methods of multi-omics data, the unique characteristics of the neuropeptides can be identified. Our results indicated that multi-omics comprehensive analysis is necessary for the identification of neuropeptides. These peptidome data also provide a reliable reference for their identification in other species and the finding of potential candidates as selective for pests versus beneficial insects.

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AUTHOR CONTRIBUTIONS

Yan Shi: Project administration (equal); Writing-review & editing (equal). **JiangJie Li:** Formal analysis (equal); Writing-original draft (equal). **LinYu Li:** Data curation (equal); Formal analysis (equal). **GanLin Lin:** Investigation (equal); Methodology (equal). **Amir M. Bilal:** Investigation (equal); **Guy Smagghe:** Validation (lead); Writing-review & editing (equal). **Tong-Xian Liu:** Project administration (equal); Supervision (lead); Writing-review & editing (lead).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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