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Identification and characterization of *capa* and *pyrokinin* genes in the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera): Gene structure, immunocytochemistry, and differential expression

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Abstract

CAPA and pyrokinin (PK) neuropeptides are produced from two different genes, *capa* and *pyrokinin*, respectively. In this study, we identified and characterized the *capa* and *pyrokinin* genes from the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera). The *capa* gene encodes two CAPA-PVK (periviscerokinin) peptides (DAGLFPFPRVamide and EQ-LIPFPRVamide) and one CAPA-DH (diapause hormone; NGASGNGGLWFGPRLamide). The *pyrokinin* gene encodes three PK2 peptides (QLVSFRPRLamide, SPPFAPRLamide, and FYAPFSPRLamide). The whole-mounting immunocytochemistry revealed the neurons contained PRXamide-like peptides throughout the cerebral ganglia (CRG), gnathal ganglia (GNG), thoracic ganglia (TG), and abdominal ganglia (AG). A pair of neurosecretory cells in the CRG and three cell clusters in the GNG were found with the axonal projections extended through the lateral side. A pair of immunostained cells were found in the TG, while three pairs of cells were present in the fused AG. Different expression patterns of *capa* and *pyrokinin* genes were observed in the CRG–GNG, TG, and AG. The *capa* gene was highly expressed in the AG tissue, whereas the *pyrokinin* gene was strongly expressed in the CRG–GNG. Interestingly, different developmental stages showed similar expressions of both genes, with the highest from the first nymph, gradually decreasing to the female adult. Comparison of peptide

sequences encoded from *pyrokinin* genes showed the PK1 peptide is lost in Heteroptera suborders including *H. halys*, but retained in other suborders. The missing PK1 from the *pyrokinin* gene might be compensated by CAPA-DH (=PK1-like) produced by the *capa gene*.

1 | INTRODUCTION

Insect neuropeptides represent neurotransmitters, neuromodulators, or necrohormones to regulate a variety of physiological functions and behaviors during developmental and adult stages in insects (Nässel & Winther, 2010; Schoofs, De Loof, & Van Hiel, 2017). The PRXamide family is a well-characterized neuropeptide group classified into three subfamilies: CAPA (peptide produced by *capa* gene), pyrokinin (PK) including pheromone biosynthesis activating neuropeptides (PBAN)/diapause hormone (DH), and ecdysis-triggering hormone (ETH). The peptides in this family have a common amino acid sequence, PRXamide (X, a variable amino acid), at the C-terminal end, which is conserved for diverse functions across Insecta (Jurenka, 2015).

The first CAPA peptide, isolated from the cockroach *Periplaneta americana*, was designated as periviscerokinin (PVK; Predel, Linde, Rapus, Vettermann, & Penzlin, 1995). At about the same time, the cardioacceleratory peptide 2b (CAP_{2b}) was identified from *Manduca sexta* (Huesmann et al., 1995), which was later determined to be one of the CAPA peptides. It was then discovered the capability (*capa*) gene in *Drosophila melanogaster* producing three CAPA peptides (Kean et al., 2002). The CAPA subfamily peptides encoded from *capa* genes usually include CAPA-PVK with common PRVamide or PRIamide, and CAPA-PK/DH with WFGPRLamide at the C-terminal ends, respectively. CAPA peptides have been reported to be involved in desiccation and cold tolerance in *D. melanogaster* (Terhzaz et al., 2015), and antidiuresis in Malpighian tubules in *Rhodnius prolixus* (Paluzzi & Orchard, 2010).

The first PK peptide was isolated from another cockroach, *Leucophaea maderae* (Holman et al., 1986). The PK subfamily peptides encoded from *pyrokinin* genes are conserved with a pentapeptide (FXPRLamide) at the C-terminal end that is diversified with WFGPRLamide in PK1 or DH, and with FXPRLamide in PK2 or PBAN (Jurenka, 2015). These peptides are involved in a variety of biological functions including the hindgut muscle contraction in the cockroach (Holman, Cook, & Nachman, 1986), sex pheromone biosynthesis in many moths (Raina et al., 1989), cuticle melanization in *Leucuniu separutu* (Matsumoto et al., 1990), induction of embryonic diapause in *Bombyx mori* (Imai et al., 1991), pupal development in *Heliothis virescens* (Xu & Denlinger, 2003), and trail pheromone production in *Solenopsis invicta* (Choi & Vander Meer, 2012).

As described above, various PRXamide peptides were initially determined based on biological functions or amino acid sequence similarities. CAPA, PK1/DH, and PK2/PBAN are sometimes confused due to different names from structural and functional diversities in many insect groups. In general, these neuropeptides are produced from two different genomic sources, *capa* and *pyrokinin* genes, where the former encodes for CAPA-PVK and CAPA-PK1 (=DH-like) peptides, and the latter encodes for PK1 (=DH-like) and/or PK2 (=PBAN-like) peptides.

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Hemiptera: Pentatomidae), an invasive pest native to Asia, was introduced into the United States a decade ago. The spread of BMSB populations is threatening a wide range of economic crops (Leskey & Nielsen, 2018). It has spurred to develop various management options including pheromone-based monitoring and trapping (Khrimian et al., 2014), biological control utilizing entomopathogenic fungi (Gouli et al., 2012), and a recent RNA interference (RNAi)-mediated approach (Ghosh, Hunter, Park, & Gundersen-Rindal, 2017, 2018).

Identification and characterization of CAPA and PK peptides related to specific endocrinal regulations and behaviors will aid to explore the physiological processes on a molecular level, thus helping to identify biological targets to be utilized for BMSB control. In the current study, we identified and characterized the *capa* and *pyrokinin* genes of *H. halys*, and determined differential gene expressions in nerve tissues and developmental stages. We also elucidated the immunoreactivity of FXPRLamide peptides in the central nervous system (CNS). The deduced amino acid sequences from over 30 hemipteran species were compared, providing the evolutionary feature of CAPA and PK precursors in Hemiptera.

2 | MATERIALS AND METHODS

2.1 | Insect

H. halys nymphs and adults were collected from ornamental plants in the Willamette Valley of Oregon and maintained in a controlled climate chamber at 21°C, 16 L: 8 D with peanut, carrot, candy ball, and water. Eggs, nymphs, and adults were collected from the rearing cage for the dissection and gene expression analysis.

2.2 | Total RNA extraction and complementary DNA (cDNA) synthesis

CNS was obtained from 5 to 10 days old adults in cold phosphate-buffered saline (PBS; 100 mM of NaH_2PO_4, 150 mM of NaCl, 3 mM of KCl, pH 7.4), pooled in lysis buffer provided by PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA) in 1.5 ml of RNase-free tube, and homogenized using TissueLyser LT (Qiagen, Germantown, MD) for 2 min at 50/s frequency with two sterilized metal beads in the tube. Total RNA was extracted from the samples using the PureLink RNA Mini Kit under the manufacturer's instruction, with DNase I treatment to remove remaining genomic DNA. The total RNA was quantified by NanoDrop 2000 (Thermo Fisher Scientific) and immediately used for cDNA synthesis. One microgram (μg) of total RNA was used to synthesize cDNA using a SuperScript IV First-Strand Synthesis system (Thermo Fisher Scientific) using oligo dT/random hexamer primers according to the manufacturer's instruction. The first-strand cDNA synthesized from the CNS tissue was used as a template for PCR amplification and molecular cloning of *capa* and *pyrokinin* genes.

2.3 | Molecular cloning and sequencing

Blast searches for the putative orthologs of *capa* and *pyrokinin* genes were performed against the *H. halys* transcriptome and genome databases in i5k Workspace@NAL (https://i5k.nal.usda.gov/). Sequences of the *capa* and *pyrokinin* genes were amplified using the cDNA of the adult CNS as a template and gene-specific primer sets designed to include coding sequences (Table 1). PCR reaction was performed with an Advantage 2 Polymerase Mix (Takara Bio USA, Mountain View, CA) under 95°C for 5 min, 35 cycles of 95°C for 30 s, and 68°C for 60 s, then 68°C for 5 min using Veriti 96 Fast Thermal Cycler (Applied Biosystems, Foster City, CA). PCR products were run in 1.2% agarose gel and visualized with GelRed (Biotium, Fremont, CA) under UV light. The PCR products were then purified using QIAquick Gel Extraction Kit (Qiagen), cloned into pSC-A-amp/kan vector (Agilent, Santa Clara, CA), and sequenced by Center for Genome Research and Biocomputing in Oregon State University. The sequencing results were analyzed using a Geneious 8.1 software (Biomatters, Newark, NJ) to the genomic scaffolds from the database to elucidate sequence fidelity and genomic architecture including intron and exon parts.

2.4 | Quantitative real-time polymerase chain reaction (qRT-PCR)

For the gene expression analysis of the CNS tissue segments, the CNS was dissected and divided into three segments: the cerebral–gnathal ganglia complex (CRG–GNG), thoracic ganglia (TG), and abdominal ganglia (AG).

TABLE 1 List of primers used in this study

Target gene	Primer	Sequence (5′–3′)	Length (bp)	Product size (bp)	Usage
capa	HhCapa-F1	TTTACCATGTCCAACGTCTTCTG	23	554	Full length PCR and qPCR
	HhCapa-R1	AAGAAAAGCGTTAAACACATAGACATAAC	29		
	HhCapa-F2	TTGGGAAATCCGGTCCGAAG	20	105	qRT-PCR
	HhCapa-R2	CGACAGGGATGAGCTCCATC	20		
pyrokinin	HhPK-F1	AAGCACACCATGGCACCG	18	380	Full length PCR and qPCR
	HhPK-R1	TTTATTGGTTGGGACGATGTTG	22		
	HhPK-F2	GGTAGCCGCCAACTAGTGAG	20	116	qRT-PCR
	HhPK-R2	AAAGGAGCGTAGAACCTGCC	20		
rpn2	HhRpn2-F1	ACAGCAACCGTGATAGCCAA	20	266	qPCR
	HhRpn2-R1	GCACCGTGGTTTGCATGAAT	20		
	HhRpn2-F2	GCCCAGCTTATCTCCGACAA	20	110	qRT-PCR
	HhRpn2-R2	TGTAGCAGCTTCCTGATGGC	20		

Note. rpn2 (regulatory particle non-ATPase 2) was used as a reference gene.
qRT-PCR: quantitative real-time polymerase chain reaction.

Each part dissected was pooled in the lysis buffer in 1.5 ml of nuclease-free tube, frozen in liquid nitrogen and kept at −80°C until RNA extraction. Each of three biological replicates was pooled with five individuals for each segment. Homogenization and total RNA isolation steps were performed as described above. For the gene expression analysis of the developmental stages, whole bodies of egg, first, third, and fifth nymphs, and adults were separately frozen in the liquid nitrogen and kept at −80°C until RNA extraction. The samples with three biological replicates were homogenized in the lysis buffer using PYREX® glass pestle tissue grinder (Corning, Corning, NY) by hand in ice. The homogenates were centrifuged at 13,000*g* for 10 min at 4°C, and the supernatant was used for total RNA isolation.

Total RNAs were quantified by the NanoDrop 2000 and adjusted to 50 ng/μl for the CNS tissues and 200 ng/μl for adult and developmental stages samples. cDNAs were synthesized from 250 and 1,000 ng of total RNA, respectively, using a Verso cDNA Synthesis Kit with oligo dT/random hexamer primers (Thermo Fisher Scientific) according to the manufacturer's instruction. Same cDNA preparation without reverse transcriptase was also performed for each RNA sample as a control. Synthesized cDNAs were stored at −20°C until use. The qRT-PCR was conducted with different cDNA templates using a StepOnePlus Real-Time PCR System (Applied Biosystems) and a FAST SYBR Green Master Mix (Applied Biosystems). The primer sets used for target and reference genes are listed in Table 1. The qRT-PCR reaction mixture was prepared in optical 96-well plate with 20 μl-volume contained 10 μl of FAST SYBR Green Master Mix, 1 μl of cDNA template, 1 μl of primer pair (5 μM each), and 8 μl of nuclease-free water. Reaction condition was: 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis over the range of 60–95°C with 0.3°C/min increment. Six concentrations of cDNA were used to construct a standard curve for each primer set to determine primer efficiency. At least three repetitions were performed using different preparations of *H. halys* cDNA and the average calculation of the repetitions gave a final efficiency. Four candidate reference genes (*rpn2*, *rps4*, *rpl19*, and *rpt6*) had been evaluated for their primer efficiency and variability through pilot tests, then the regulatory particle non-ATPase 2 (*rpn2*) gene was selected as a reference gene due to less variability compared to other reference genes tested (Supporting Information Table S1).

2.5 | PCR amplification

Using the same cDNA samples above the PCR amplification was performed with primer sets of *capa* and *pyrokinin* genes to visualize the amplification intensity. A fragment of 266-base pair (bp) of *rpn2* gene was amplified as a reference. The PCR reaction was performed under the follow condition: 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 50 s, then 72°C for 5 min using a Green *Taq* DNA polymerase (GenScript, Piscataway, NJ). PCR products were run in 1.2% agarose gel and visualized using the GelRed (Biotium) under UV light to verify expression levels.

2.6 | Immunocytochemistry

The distribution of PRXamide-like immunoreactivity in the *H. halys* CNS was observed using the whole-mount immunocytochemistry method described previously (Choi, Rafaeli, & Jurenka, 2001; Choi, Raina, & Vander Meer, 2009). A synthetic truncated Hez-PBAN, 18 amino acids (Cys-Nle-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-NH$_2$), was used to generate the polyclonal antisera (Ma & Roelofs, 1995). Adult CNS including CRG–GNG, TG, and AG was dissected in cold PBS, fixed in 10% formalin in PBS for 1 hr, and then incubated in PBS containing 2% Triton X-100 (PBS-T) overnight. The tissues were sequentially incubated for 6 hr in the PBAN antiserum (diluted 1:2,000), anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat (Sigma A9169; diluted 1:2,000, Mendota Heights, MN), and peroxidase anti-peroxidase soluble complex antibody produced in rabbit (Sigma P1291; diluted 1:400) in PBS-T with three times washing with PBS-T after each incubation. After the last incubation, the tissues were washed with PBS and then incubated in 50 mM of Tris-HCl buffer (pH 7.6) for 10 min. Visualization of the immunoreactivity was performed in the solution of 3,3′-diaminobenzidine and urea–H$_2$O$_2$ (Sigma D4168; tablets dissolved in 1 ml of deionized water). After satisfactory color development was achieved, the tissue was transferred to PBS and dehydrated by incubations in a series of glycerol solutions (40–100%). Tissues were examined under a Leica DMRB microscope equipped with digital camera (Leica Microsystems, Wetzlar, Germany). No staining was observed in the control tissue prepared under the same procedure without the PBAN antiserum treatment.

2.7 | Comparative analysis of hemipteran CAPA and PK peptides

The hemipteran CAPA and PK precursor sequences were obtained from the literatures published or the hemipteran transcriptome databases (TSA and SRA) in the NCBI GenBank and the i5k Workspace@NAL. All candidate genes were examined to find peptide coding regions. The mature peptides were predicted by sequence similarity with other known hemipteran peptide sequences as well as by potential mono- and di-basic cleavage sites (Southey, Sweedler, & Rodriguez-Zas, 2008; Veenstra, 2000). CAPA or PK precursors were compared among the hemipteran species according to taxonomic hierarchy using the Geneious 8.1.5 software (Biomatters).

3 | RESULTS

3.1 | Identification of *capa* and *pyrokinin* genes

H. halys capa gene (GenBank Accession No. MG735192) was composed of four exons in its genomic locus, translating CAPA-PVK1 in the second exon, and CAPA-PVK2 and CAPA-DH in the third exon without intron disruption (Figure 1a). The *capa* gene contained 465 nucleotides encoding 154 amino acids with two PRV and one FXPRL homologs predicted by potential endoproteolytic cleavage sites (italicized in Figure 1a). The cleaved peptides were predicted to have C-terminal amide group provided by glycine (G; Southey et al., 2008; Veenstra, 2000). The considered homologs were
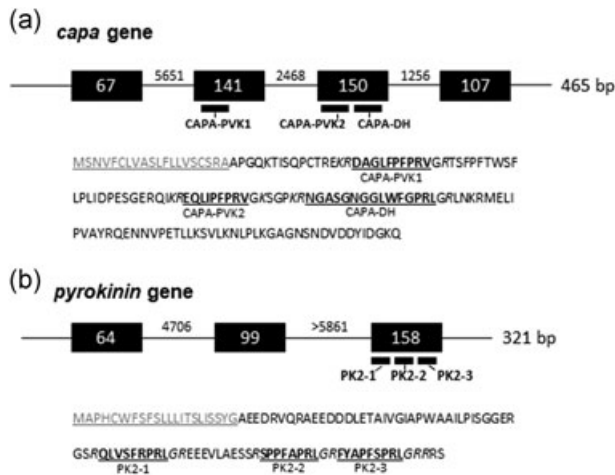
FIGURE 1 Genomic architecture of *capa* (a) and *pyrokinin* (b) genes, and their deduced peptide precursor sequences in *Halyomorpha halys*. Rectangular boxes indicate exon regions on the line of genomic scaffold. Numbers refer to the nucleotide lengths. The deduced amino acid sequences of the putative precursors indicate the N-terminal signal peptide underlined with gray, the putative peptides underlined with bold, cleavage sites italicized, and designated peptide names
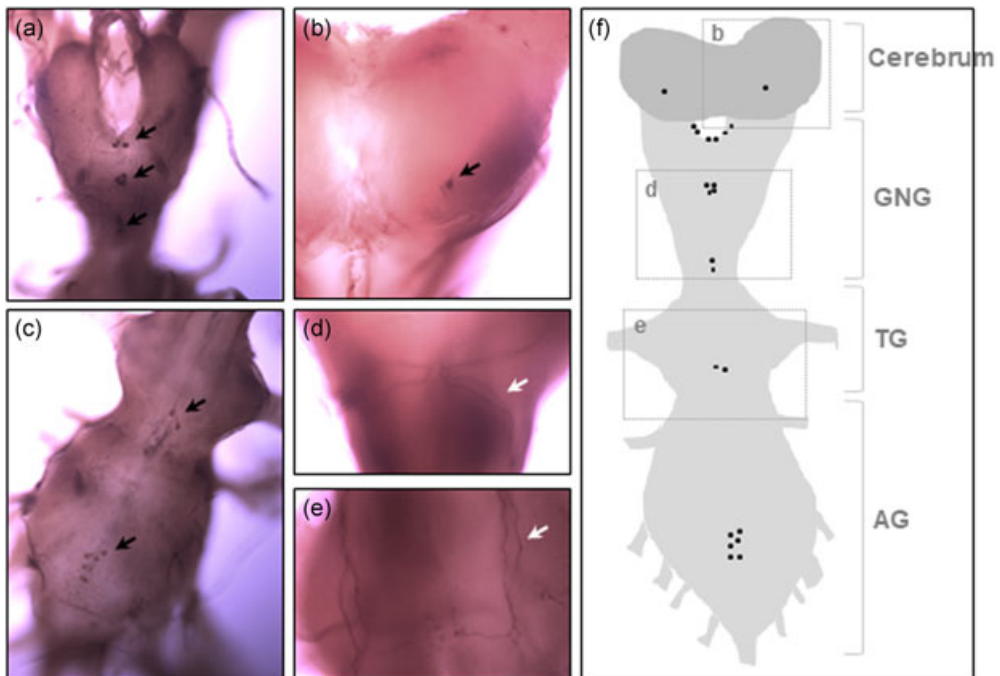

FIGURE 2 Localization of PRXamide-like immunoreactivity in the central nervous system (CNS) of *Halyomorpha halys*. Photomicrographs of PRXamide-like immunoreactivity in ventral view of gnathal ganglia (GNG) (a), dorsal view of cerebrum (b), ventral view of the fused thoracic ganglia (TG) and abdominal ganglia (AG) (c), dorsal view of the anterior part of GNG (d), and dorsal view of TG (e). Neurosecretory cells and the projections are indicated by black and white arrows, respectively. Schematic diagram of the CNS (f) include cerebrum, GNG, TG, and AG with corresponded neurosecretory cells marked by black dots

DAGLFPFPRVamide (CAPA-PVK1), EQLIPFPRVamide (CAPA-PVK2), and NGASGNGGLWFGPRLamide (CAPA-DH; underlined with bold in Figure 1a). The amino acid sequence at the N-terminus was predicted as a signal peptide (underlined with gray in Figure 1a).

H. halys pyrokinin gene (GenBank Accession No. MG735193) was composed of three exons, translating all three neuropeptides in the third exon (Figure 1b). The *pyrokinin* gene contained 321 nucleotides encoding 106 amino acids with three putative FXPRL homologs predicted by potential endoproteolytic cleavage sites (underlined with italics in Figure 1b). All the three peptides were classified as PK2 type peptides based on C-terminal sequences. The three homologs were QLVSFRPRLamide (PK2-1), SPPFAPRLamide (PK2-2), and FYAPFSPRLamide (PK2-3; underlined with bold in Figure 1b). The amino acid sequence at the N-terminus was predicted as a signal peptide (underlined with gray in Figure 1b).

3.2 | Localization of FXPRLamide-like immunoreactivity in the CNS

The morphological structure and names of the BMSB CNS including cerebrum, GNG, TG, and AG shown in Figure 2 followed a systematic nomenclature suggested by Insect Brain Name Working Group (Ito et al., 2014). The TG and AG were fused into the ganglionic mass of TG and AG. In the ventral part of GNG, three immunoreactive cell clusters were found: three pairs in the anterior, two pairs in the medial, and one pair in the most posterior abdominal neuromeres (Figure 2a,f). The dorsal protocerebrum part contained a pair of neurosecretory cells immunostained by the FXPRLamide (Figure 2b,f). Axonal projections in the cerebrum looked extended through the ipsilateral side to the
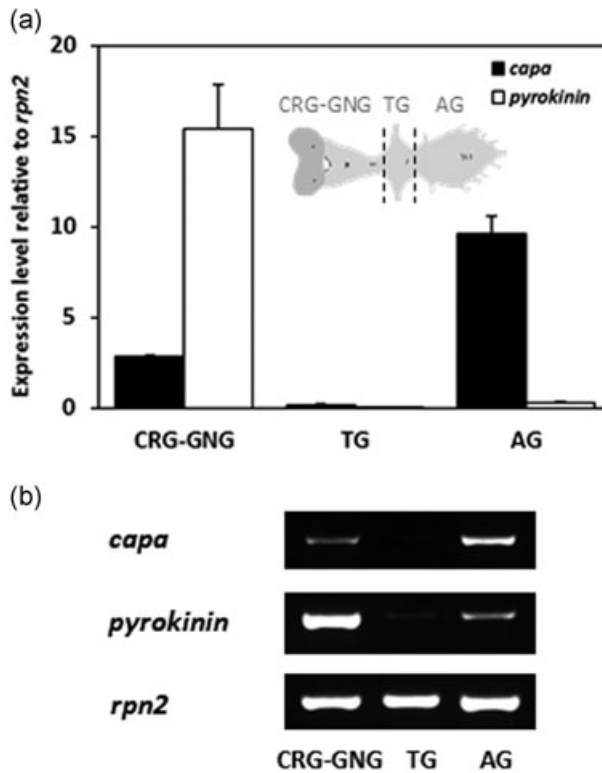
FIGURE 3 Relative expressions of *capa* and *pyrokinin* genes in the cerebral ganglia–gnathal ganglia complex (CRG–GNG), thoracic ganglia (TG), and abdominal ganglia (AG) of *Halyomorpha halys*. (a) Quantitative real-time PCR (qRT-PCR) analysis: cDNA was synthesized from 250 ng of total RNA from each segment tissue. (b) Expression profiles were visualized in 1.2% agarose gel electrophoresis using the same cDNA samples above. Primer information is in Table1

median bundle of the tritocerebrum where they form a densely stained region of varicosities (Figure 2b). The somata in the GNG and in the TG appeared to project axons to the ventral nerve cord (Figure 2d).

Only a pair of cells was immunostained in the posterior part of the TG, which looked projecting toward the dorsal part (Figure 2c,f). The fused AG contained three pairs of cell bodies showing the FXPRLamide-like immunoreactivity (Figure 2c,f). The cell bodies were located near the ventral midline, and their axons projected to the dorsal surface of the ganglia (Figure 2e).

3.3 | Expression of the *capa* and *pyrokinin* genes in the CNS

The *capa* gene was highly expressed in the AG, but relatively low in the CRG–GNG complex. In contrast, the expression of the *pyrokinin* gene was very strong in the CRG–GNG, but it was barely detectable in the AG (Figure 3). The expressions of two genes were consistent from both qRT-PCR and PCR results, but neither of the genes were virtually expressed in the TG (Figure 3).

Expression levels of *capa* and *pyrokinin* genes in different developmental stages revealed that the *capa* gene expression was highest in the first nymph, then decreased with development to the adult stage, with lower levels observed in the adult female than the adult male (Figure 4). The *pyrokinin* gene also showed a similar expression pattern in the same developmental stages, thus highest in the first nymph. But, the *pyrokinin* gene consistently
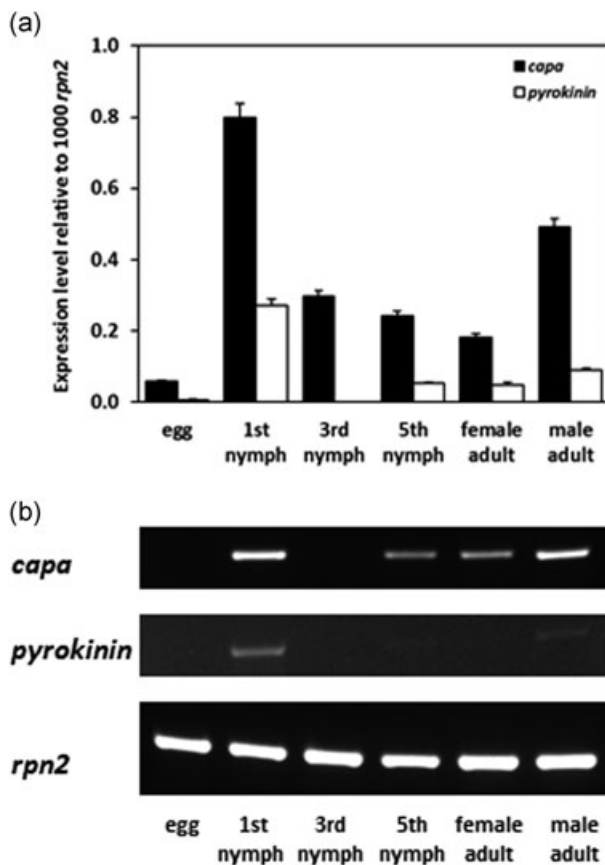
FIGURE 4 Relative expressions of *capa* and *pyrokinin* genes in developmental stages of *Halyomorpha halys*. (a) Quantitative real-time PCR (qRT-PCR) analysis: complementary DNA (cDNA) was synthesized from 1,000 ng of total RNA from each stage. (b) Expression profiles were visualized in 1.2% agarose gel electrophoresis. The same cDNA samples above were used. Primer information is in Table1

showed lower expression levels than the *capa* gene for all life stages. Although some copies were measured by PCR (Figure 4b), the *pyrokinin* gene was hardly visible by gel electrophoresis in the fifth nymph on to the adult stage. Both *capa* and *pyrokinin* expressions were lower in adult females than in the males.

3.4 | Comparative analysis of CAPA and PK amino acid sequences

In general, CAPA precursors deduced from Hemiptera *capa* genes showed two CAPA-PVKs and one CAPA-DH. But, additional CAPA-PK2 type peptides were found in some suborders including Cicadomorpha (ex: cicadas) and Fulgoromorpha (ex: leafhoppers), as well as two species in Sternorrhyncha (ex: aphids) (Figure 5).

PK precursors deduced from Hemiptera *pyrokinin* genes were mainly divided into two groups based on the presence or absence of PK1 (=DH-like). Pentatomorpha (ex: stink bugs) and Cimicomorpha suborders, together belonging to Heteroptera group, were shown to produce only PK2 (=PBAN-like) peptides (PK2-1, PK2-2, and PK2-3), but not PK1 (=DH-like; Figure 6). The other suborders, Cicadomorpha, Fulgoromorpha, and Sternorrhyncha, appear to produce PK1 plus two or three PK2 peptides (Figure 6).

4 | DISCUSSION

The pierce-sucking type mouthparts of hemipteran insects enable feeding on plant sap or blood meal from hosts, potentially taking an excessive amount of water into the body. In these insects, therefore, it is important to control water balance after feeding. Neuropeptide hormones including CAPA and PK peptides are involved in antidiuretic processes by controlling urine production via water absorption between Malpighian tubules and hindgut
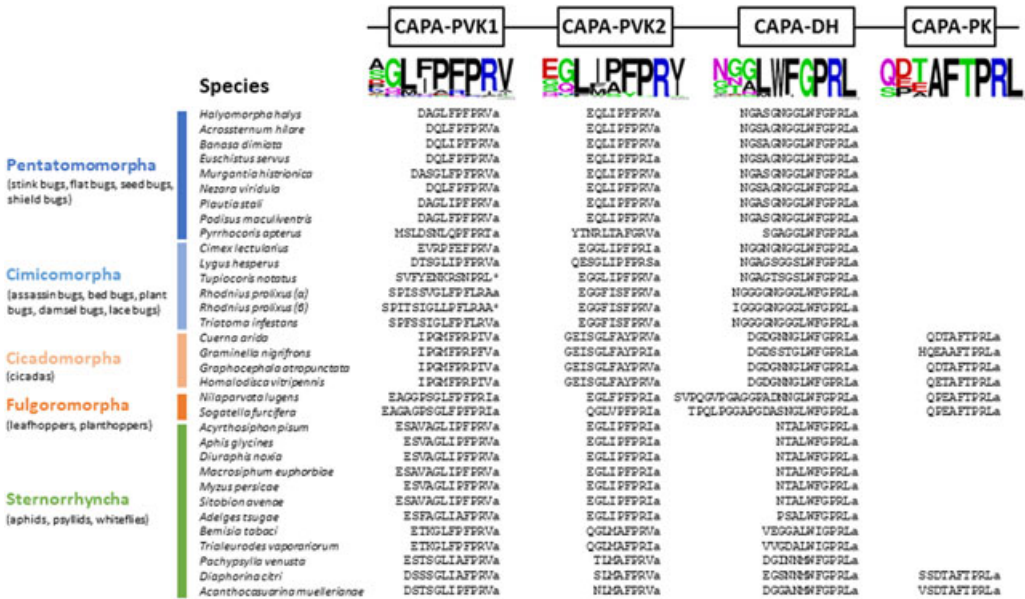
FIGURE 5 Comparison of peptide sequences encoded from *capa* genes in Hemiptera. The peptides were predicted from each precursor sequence based on the conventional mono- and/or di-basic cleavage sites. Consensus sequences of the C-terminal amino acids of peptides are presented as the sequence logo above created by WebLogo (Crooks, Hon, Chandonia, & Brenner, 2004). Peptides are indicated with C-terminal end amidated (a) or no-amidated (*)
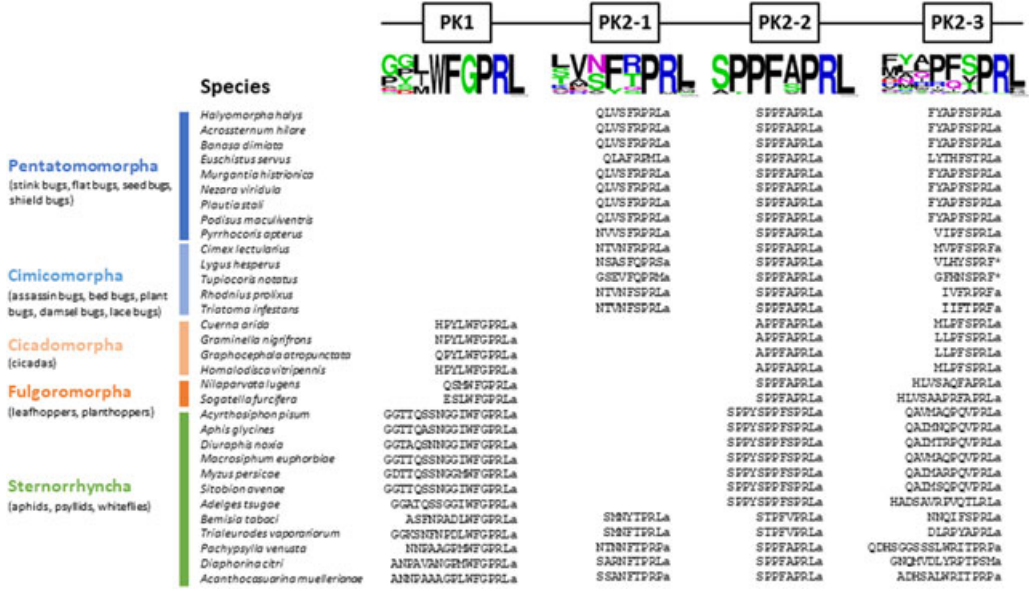
FIGURE 6 Comparison of peptide sequences encoded from *pyrokinin* genes in Hemiptera. The peptides were predicted from each precursor sequence based on the conventional mono- and/or di-basic cleavage sites. Consensus sequences of the C-terminal amino acids of peptides are presented as the sequence logo above created by WebLogo (Crooks, Hon, Chandonia, & Brenner, 2004). Peptides are indicated with C-terminal end amidated (a) or no-amidated (*)

(Jurenka, 2015; Predel & Wegener, 2006). Although various CAPA and PK peptides have been identified from hemipteran species, most of them are focused on *Rhodnius prolixus*, a blood-feeding disease vector, to study physiological functions, gene characterization and expression, but limited in crop pests (Ons, 2017; Paluzzi & Orchard, 2010; Paluzzi, Russell, Nachman, & Orchard, 2008; Predel et al., 2008). In this study, we identified *capa* and *pyrokinin* genes from the brown marmorated stink bug, *H. halys*, and characterized PRXamide peptides, the localization of the peptidergic neurons in the CNS, differential gene expressions, and comparisons of peptide compositions in Hemiptera.

The first CAPA family peptides in the hemipteran species were determined to be two CAPA-PVKs and one CAPA-PK in the southern green stink bug, *Nezara viridula* (Predel et al., 2006). These peptides were confirmed by MALDI-TOF mass spectrometry, and characterized in the neurons producing the peptides in the AG. Hemipteran CAPA peptides have been intensively studied for *R. prolixus*, a major vector of Chagas disease (Ons, 2017), and the bed bug, *Cimex lectularius*, a global human ectoparasite (Predel, Neupert, Derst, Reinhardt, & Wegener, 2018). The *Rhodnius* genome contains two *capa* genes, *RhoprCAPA-α* and *RhoprCAPA-β*, encoding three putative neuropeptides (two CAPA-PVKs and one CAPA-DH) from each gene. The transcripts of both genes are expressed in all developmental stages and predominantly localized in the CNS (Paluzzi & Orchard, 2010; Paluzzi et al., 2008). Recently, the bed bug genome showed one *capa* gene encoding two CAPA-PVKs and one CAPA-DH peptides (Benoit et al., 2016; Predel et al., 2018).

Like many other hemipteran species, *H. halys* has a single *capa* gene producing two PRVamide (CAPA-PVKs) and one WFGPRLamide (CAPA-DH) peptides. Many neurons recognized by the PBAN-like antisera were observed in the cerebrum, GNG, TG, and AG. CAPA peptides are typically synthesized in the median neurosecretory neurons of the AG and released into the hemolymph via the abdominal perisympathetic organ (PSO) as a neurohemal organ (Predel & Wegener, 2006; Predel et al., 2018). As shown in *H. halys*, three paired cells in the fused AG projecting to the ventral nerve cords were observed in many insect groups including stink bugs (Paluzzi & Orchard, 2006; Predel et al., 2006), *Drosophila melanogaster* (Choi et al., 2001), and *C. lectularius* (Predel et al., 2018).

Therefore, we anticipate the *H. halys capa* gene expression in the AG occurs most likely in the six neurosecretory cells, rather than in other nerve tissues. The FXPRLamide antisera used in this study can recognize not only FXPRLamide peptides including three PKs from the *pyrokinin* gene, but can also detect CAPA-DH and possibly CAPA-PVKs encoded from the *capa* gene. A similar study of the antiserum against the MasPETH (pre-ecdysis-triggering hormone) from *Manduca sexta* recognized the PRXamide-like immunoreactivity for *R. prolixus* (Paluzzi & Orchard, 2006). To avoid potential cross-immunoreactivity, it is better to investigate the *capa* gene expression in different CNS tissues. The highest *capa* expression was detected in the AG among the CNS tissues observed in *H. halys*. A similar result for the *capa* mRNA expression has also been demonstrated in the fire ant CNS (Choi, Köhler, Vander Meer, Neupert, & Predel, 2014). While not as much as the AG, the cerebrum also expressed the *capa* gene from a few neurosecretory cells, but not in the TG. Unlike the *capa* gene, the *pyrokinin* gene was predominantly expressed in the CRG–GNG complex, but barely detected in the AG (Figure 3). Normally, the *pyrokinin* gene is highly expressed in the median ventral cells of the GNG (previously named subesophageal ganglia) in insects (Choi, Sanscrainte, Estep, Vander Meer, & Becnel, 2015; Choi, Vander Meer, Shoemaker, & Valles, 2011; Predel & Nachman, 2006).

In *R. prolixus*, blood meal feeding is under control of the antidiuretic hormone through the *capa* gene upregulation. CAPA-PVK peptides are released into the hemolymph via the abdominal neurohemal organs 3–4 hr after blood feeding, when the ceasing of diuresis begins (Paluzzi & Orchard, 2006). In *H. halys*, the immunoreactivity of the abdominal CAPA neurons was not always strongly visualized in different CNS preparations (observed data). The varied immunoreactive signals could be explained by weak penetration of the antiserum into the CNS tissues, and/or from short incubation periods with the primary and secondary antibodies. More evidence remains to elucidate whether phloem-feeding controls the gene expression and/or peptide release in *H. halys*.

Insect *capa* and *pyrokinin* genes are considered to have a common evolutionary origin (Derst et al., 2016). From over 30 hemipteran species across five suborders, overall peptide sequences produced by two genes reveals that CAPA peptides have relatively simple and conserved structures. They consist of similar amino acid sequences, and the number of peptides with two CAPA-PVKs and one CAPA-DH, except for eight species. In fact, CAPA genes are ubiquitously found across Invertebrate phyla (Predel & Wegener, 2006). Across developmental stages of *H. halys*, the *capa* gene was also more abundant than the *pyrokinin* gene.

In contrast, *pyrokinin* genes are more diversified in terms of the amino acid sequences and the number of peptides, even within same suborders. The *pyrokinin* gene of *H. halys* encodes three PK2 (=PBAN-like) peptides characterized with the FXPRLamide at the C-terminal end, but no PK1 (=DH-like) defined by the WFGPRLamide to activate the corresponding receptors (Jurenka & Nusawardani, 2011). The lack of PK1 in *pyrokinin* genes appear to be common in Heteroptera group including Pentatomomorpha and Cimicomorpha suborders, although so far two *pyrokinin* genes without PK1 have been characterized in *D. melanogaster* (Meng et al., 2002) and the sand fly, *Phlebotomus papatasi* (Choi et al., 2015). The *D. melanogaster* PK1 (=CAPA-3) is produced by the *capa* gene instead of the *pyrokinin* gene (Kean et al., 2002). The other suborders, Cicadomorpha, Fulgoromorpha, and Sternorrhyncha, produce PK1, and they are phylogenetically distant from Heteroptera species including *H. halys*. The phylogenetic distance implies either that PK1 had probably been lost in a common ancestor of the Pentatomomorpha and Cimicomorpha suborders before they split, or that PK1 was incorporated into a common ancestor of the other three suborders. The missing PK1 might be compensated by CAPA-DH (=PK1-like) produced by the *capa* gene targeting the corresponding receptor to keep its biological function (Paluzzi & O'Donnell, 2012). Although biochemical verifications of the active forms of the CAPA and PK peptides in *H. halys* remain, the current results provide a new insight to discover potential biological function(s) of the neuropeptides, and to assist in development of control methods for the brown marmorated stink bug.

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

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