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Identification and characterisation of PRXamide peptides in the western flower thrips, *Frankliniella occidentalis*

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Abstract

Insect CAPA-PVK (periviscerokinin) and pyrokinin (PK) neuropeptides belong to the PRX family peptides and are produced from *capa* and *pyrokinin* genes. We identified and characterised the two genes from the western flower thrips, *Frankliniella occidentalis*. The *capa* gene transcribes three splice variants, *capa-a*, *-b*, and *-c*, encoding two CAPA-PVKs (EVQGLFPFPRVamide; QGLIPFPRVamide) and two PKs (ASWMPSSSPRLamide; DSASFTPRLamide). The *pyrokinin* mRNA encodes three PKs: DLVTQVLQPGQTGMWFGPRLamide, SEGNLVNFTPRLamide, and ESGEQPEDLEGSMGGAATSRQLRTDSEPTWGFSPRLamide, the most extended pheromone biosynthesis activating neuropeptide (PBAN) ortholog in insects. Multiple potential endoproteolytic cleavage sites were presented in the prepropeptides from the *pyrokinin* gene, creating ambiguity to predict mature peptides. To solve this difficulty, we used three G protein-coupled receptors (GPCRs) for CAPA-PVK, tryptophan PK (trpPK), and PK peptides, and evaluated the binding affinities of the peptides. The binding activities revealed each subfamily of peptides exclusively bind to their corresponding receptors, and were significant for determining the CAPA-PVK and PK peptides. Our biological method using specific GPCRs would be a valuable tool for determining mature peptides, particularly with multiple and ambiguous cleavage sites in those prepropeptides. Both *capa* and *pyrokinin* mRNAs were strongly expressed in the head/thorax, but minimally expressed in the abdomen. The two genes also were clearly expressed during most of the life stages. Whole-mounting immunocytochemistry revealed that neurons contained PRXamide peptides throughout the whole-body: four to six neurosecretory cells in the head, and three and seven pairs of immunostained cells in the thorax and abdomen, respectively. Notably, the unusual PRXamide profiles of Thysanoptera are different from the other insect groups.

KEYWORDS
CAPA, *Frankliniella occidentalis*, immunocytochemistry, neuropeptide, pyrokinin

INTRODUCTION

Insect neuropeptides represent neurotransmitters, neuromodulators, or neurohormones that regulate various physiological functions and behaviours during immature and adult stages (Nassel & Zandawala, 2019; Schoofs et al., 2017). The PRXamide family peptides are well-characterised and classified with capability (CAPA) peptides, pyrokinin (PK) peptides - pheromone biosynthesis activating neuropeptides (PBAN), diapause hormone (DH), and melanization and reddish coloration hormone (MRCH), and ecdysis-triggering hormone (ETH). The family of these peptides is characterised with PRXamide (X, a variable amino acid) in the C-terminal end, which is conserved for

diverse functions across Insecta (Jurenka, 2015). The names of various PRXamide peptides were initially determined based on their biological functions or peptide sequence similarities in arthropods. In general, these neuropeptides are produced from three genes, *capa*, *pyrokinin*, and *eth* genes.

The first CAPA peptide isolated from the cockroach, *Periplaneta americana*, was designated as periviscerokinin (PVK) (Predel et al., 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-PVK peptides. Three peptides, including two CAPA-PVKs and one PK (= DH-like), were identified in the *capa* gene of *Drosophila melanogaster* (Kean et al., 2002). Insect *capa* genes usually produce CAPA-PVK peptides (CAPA-PVKs) with PRVamide or PRIamide in the C-termini, and PK peptides (CAPA-PKs) with FXPRLamide or WFGPRLamide in the C-termini. Many CAPA-PVK peptides have been identified from various insects (Predel & Wegener, 2006), and their biological functions are involved in desiccation and cold tolerance in *D. melanogaster* (Terhzaz et al., 2015), and anti-diuresis in Malpighian tubules in *Rhodnius prolixus* and other insects (Paluzzi, 2012; Paluzzi et al., 2010).

The first PK peptide was isolated from another cockroach, *Leucophaea maderae* (Holman et al., 1986). The PK peptides encoded by *pyrokinin* genes are conserved with a pentapeptide (FXPRLamide) or similar peptides at the C-terminal end that is diversified with WFGPRLamide (Tryptophan (W) positioned six at the C-terminal end) in trpPK (= DH-like, PK1), and with FXPRLamide in PK (= PBAN-like, PK2) (Ahn et al., 2020; Ahn & Choi, 2018; Choi et al., 2017; Hull et al., 2021; Jurenka, 2015; Redeker et al., 2017). These peptides are involved in a variety of biological functions: hindgut muscle contraction by PK in the cockroach (Holman et al., 1986), sex pheromone biosynthesis by PBAN in many moths (Raina et al., 1989), and cuticle melanization by MRCH in *Leucunia separata* (Matsumoto et al., 1981), induction of embryonic diapause by DH in *Bombyx mori* (Imai et al., 1991), termination of pupal diapause by DH in *Heliothis virescens* (Xu & Denlinger, 2003), and potential seasonal reproductive polyphenism by DH in *Orgyia thyellina* (Uehara et al., 2011).

Thrips are tiny polyphagous plant feeders that are globally distributed with cryptic habits and are among the stealthiest insects. Thrips are frequently intercepted from insect quarantine services at border areas, but their distribution continue to grow due to the global trade of agricultural products and regional diversity (Reitz et al., 2020). Thrips are found on leaves, blossoms, buds, and leaf sheaths of plants in common environments, such as greenhouses, nurseries, gardens, and fields. Many thrips species are notorious for causing extensive crop damage, and vectoring viral pathogens (Morse & Hoddle, 2006; Mouden et al., 2017).

Western flower thrips (WFT), *Frankliniella occidentalis* (Thysanoptera: Thripidae), is one of the most economically important pests due to its serious damage to agricultural crops worldwide. Thrips not only cause direct damage from feeding and oviposition on leaves, flowers, and fruits, they also transmitted economically important plant viruses, such as the tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV) (He et al., 2020; Mouden et al., 2017; Rotenberg & Whitfield, 2018). Numerous studies of WFT have been documented on the species complex, and biological aspects including feeding and oviposition behaviours, control options, and resistance of chemical insecticides (reviewed by Demirozer et al., 2012; Reitz et al., 2020; Zhang et al., 2019). Recently, the WFT genome has provided insights into a variety of molecular and evolutionary processes, including neuropeptides (Rotenberg et al., 2020).

Identification and characterisation of CAPA-PVK and PK peptides related to specific physiological regulations and behaviours will aid in exploring the biological processes on a molecular level, thus helping to identify biological targets that can be utilised for the management of thrips. In the present study, we characterised *capa* and *pk* genes from *F. occidentalis*, their gene structures, and splice variants, and determined differential gene expressions in various adult tissues and during the life stages. We used three G protein-coupled receptors (GPCRs) of the stinkbug (*Halyomorpha halys*) to measure binding affinities of all the potential peptides predicted from the two genes, and discussed peptides from the prepropeptides that contained multiple and ambiguous cleavage sites. In addition, we characterised the immunoreactivity of the PRXamide peptides in thrips, which reveals different spatial patterns compared to other insect groups.

MATERIALS AND METHODS

Insect and materials

Frankliniella occidentalis nymphs and adults were collected from soybean or horticultural plants in Corvallis, Oregon, USA, and maintained in a controlled climate chamber at 24 ± 2°C, 16 L: 8 D with cotyledons of soybeans or kidney beans (Price et al., 2022). Eggs, nymphs, and adults were collected from the rearing container for the dissection or gene expression analysis. All the peptides (>95% purity) tested in this study were synthesised from Peptides 2.0 (Chantilly, VA, USA).

Total RNA isolation and cDNA synthesis

More than hundreds of one to two-day-old adults were pooled in lysis buffer provided by PureLink RNA Mini Kit (ThermoFisher Scientific, Waltham, MA, USA) in 1.5 mL RNase-free tube, homogenised using a TissueLyser LT (Qiagen, Germantown, MD, USA), and then sonicated using a probe sonicator (Misonix, Farmingdale, NY, USA) for 5–6 s at level 4–5 for 2 cycles on ice. Total RNA was isolated 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 synthesised to cDNA using a SuperScript III First-Strand Synthesis system (ThermoFisher Scientific) using oligo dT/random hexamer primers according to the manufacturer's instruction. The first-strand

cDNA synthesised from *F. occidentalis* adults was used as a template for PCR amplification and molecular cloning of *capa* and *pyrokinin* genes.

Molecular cloning and sequencing for *capa* and *pyrokinin* genes

BLAST searches for putative *F. occidentalis* orthologs of *capa* and *pyrokinin* genes were performed against its transcriptome and genome databases in the i5k Workspace@NAL (https://i5k.nal.usda.gov/) and NCBI GenBank (https://www.ncbi.nlm.nih.gov/). Primers were designed using Geneious Prime software (Biomatters, Auckland, New Zealand). Sequences of the *capa* and *pyrokinin* genes were amplified using the cDNA of the *F. occidentalis* adult as a template and gene-specific primer sets designed to include coding sequences (Table S1). PCR reactions were performed with Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific) under 98°C for 30 s, 35 cycles of 98°C for 10 s and 60°C for 20 s, and 72°C for 60 s, followed by a final 10 min at 72°C using Veriti 96 Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were run in a 2% agarose gel and visualised with SYBR Safe DNA Gel stain (ThermoFisher Scientific) under UV light. PCR products were then purified using GeneJET Gel Extraction Kit (ThermoFisher Scientific), cloned using CloneJET PCR Cloning Kit (ThermoFisher Scientific) with *E. coli* competent cells, and Sanger sequenced by Eurofins (Louisville, KY, USA). Sequencing results were analysed using Geneious prime software and resulting sequences were used to design primer sets for Rapid Amplification cDNA End (RACE) PCR. RACE PCR was performed using 3′ and 5′ RACE system (ThermoFisher Scientific) according to the manufacture's protocols. The RACE products were separated on 1.2% agarose gels, cloned, and sequenced using the same procedures above.

Reverse-transcriptase PCR (RT-PCR) and qRT-PCR for *capa* and *pyrokinin* expression

Developmental stages of *F. occidentalis* were collected separately from: eggs, 1st nymphs, 2nd nymphs, female prepupae, male prepupae, female pupae, male pupae, female adults, and male adults. The different tissues were collected from head, thorax, and abdomen of female adults. Total RNA was isolated using PureLink RNA Mini Kit (ThermoFisher Scientific). Total RNAs were quantified by a NanoDrop 2000 and adjusted to 250 ng/μl for the developmental stage test and 50 ng/μl for the tissue test and used to synthesise cDNA as described in the section 2.2. The primer sets used for the target and reference genes are listed in Table S1. PCR reaction was performed with DreamTaq poylmerase (ThermoFisher Scientific) under 95°C for 3 min, 35 cycles of 95°C for 30 s and 60°C for 30 s, and 72°C for 30 s, followed by a final 10 min extension at 72°C using the thermal cycler above. Amplified PCR products were visualised in a 1.2% agarose gel electrophorese.

The relative mRNA expression level was assessed by the comparative Ct method after running quantitative real-time PCR (qRT-PCR) according to to Pfaffl's method (Pfaffl, 2001). The qRT-PCR reaction was set up by mixing 0.2 μL of the first-strand cDNA (3.2 ng), 2 μL of 2 μM primer mix, 10 μL of PowerUP SYBR Green master mix (Applied Biosystems), and 7.8 μL of nuclease-free water. The primer sequences used for qRT-PCR are listed in Table S1. The reaction was incubated for 10 min at 95°C followed by 40 cycles of two-step incubation: 95°C for 15 s and 60°C for 1 min. The melt curve was analysed over the temperature range of 60–95°C with a 0.15°C/s increment immediately after PCR amplification. Three *F. occidentalis* housekeeping genes (*Ef1-alpha*, *Rpt6*, *Rpn2*) were initially evaluated for efficiency and variability, then *Ef1-alpha* was selected and used as a control. The *capa* and *pyrokinin* specific signals were normalised by the *elongation factor 1 alpha* (*ef1-α*) control. The statistical significance of the difference was analysed by One-way ANOVA with Bonferroni's multiple comparison test ($p < 0.05$).

Immunohistochemistry

Immunoreactivity of PRXamide peptides in *F. occidentalis* was analysed using a modified whole-mount immunocytochemistry method described previously (Choi et al., 2001; Hull et al., 2021). Polyclonal antiserum was generated from 16 amino acids (DPEQIDSRTKY FSPRLamide) from *Helicoverpa zea* PBAN (HelzePBAN) (Life Technologies, Carlsbad CA, USA). We used 20 female nymphs across three different trials. The whole body was fixed in phosphate-buffered saline (PBS)/10% formalin for 1 h, and then incubated in PBS containing 2% Triton X-100 (PBS-T) overnight. Samples were incubated for 12 h with the polyclonal PBAN antiserum (1:2000), followed by a secondary antibody (goat anti-rabbit IgG-peroxidase 1:2000; Sigma A9169; Mendota Heights, MN, USA), and a rabbit peroxidase anti-peroxidase soluble complex antibody (1:400; Sigma P1291) in PBS-T. Samples were rinsed three times after each incubation with PBS-T. After the last incubation, the samples were washed with PBS and then incubated in 50 mM Tris–HCl buffer (pH 7.6) for 10 min. Immunoreactivity visualisation was achieved with a solution of 3,3′-diaminobenzidine and urea-H_2O_2 (Sigma D4168; tablets dissolved in 1 mL of deionised water). After satisfactory colour development for ~5 min, the samples were transferred to PBS and dehydrated by serial incubation in solutions of 40–100% glycerol. The resulting samples were examined under an ECHO revolve microscope equipped with a digital camera (ECHO, San Diego, CA, USA). No staining was observed in the control tissue prepared using the same procedure but without the polyclonal antiserum.

Functional testing of thrips peptides to activate CAPA and PK receptors

Three GPCRs of *Halyomorpha halys*, HalhaCAPA-R as CAPA receptor (CAPA-R), HalhaPK-R3b as trpPK receptor (trpPK-R, PK1-R), and

HalhaPK-R2 as PK receptor (PK2-R) (Ahn et al., 2020) were used for the binding activity of the thrips PRXamide peptides predicted from the *capa* and *pyrokinin* genes. We hypothesized that the thrips peptides would cross-activate the same family of receptors from a hemipteran species, considering Hemiptera is the closest Order to Thysanoptera (Ishiwata et al., 2011).

Two days before the binding assay, ~50,000 Sf9 cells expressing each of the HalhaCAPA-R (CAPA-R), HalhaPK-R3b (PK1-R) and HalhaPK-R2 (PK2-R) from suspension cultures were dispensed into each well of a black 96-well plate (Corning C3603) (Corning, NY, USA) and incubated at 28°C for 48 h. After removing the media from the plate, cells were incubated with 95 µL of 1× FLIPR Calcium 6 reagent (Molecular Devices, San Jose, CA, USA) containing 2.5 mM probenecid at 28°C in the dark for 1 h. The Calcium 6 reagent-loaded cells were transferred to the FlexStation 3 multi-mode microplate reader (Molecular Devices) to measure the fluorescent intensity (Δfluorescence) before and after the addition of 5 µL peptide ligands or water as a negative control (excitation: 485 nm and emission: 525 nm). Fluorescent measurements from each well on the column were taken every 5 s for 2.5 min. The peptide ligand was added by automatic pipettor at 30 s from the beginning, and 5 µL of 20 µM ionomycin was added at 2 min to confirm calcium activity as a positive control. Baseline fluorescence was determined by averaging five time points for 25 s from each well prior to peptide treatment, and the resulting response was expressed as a percentage increase in fluorescence relative to the baseline value. For the initial screening from various peptide ligands, each GPCR was tested with a 500 nM single concentration of each peptide or water control. For the dosage-response assay, three-fold serial dilutions of selected peptide ligands from the initial screening were used, giving final ligand concentrations from 0.08 to 1500 nM. The fluorescent intensity for each concentration was averaged across eight replicates for each peptide treatment. Data were transformed into a log scale and fit to the non-linear regression curve. EC_{50} values were obtained by statistical analysis using the sum of squares F-test function using GraphPad Prism version 7.0 (San Diego, CA, USA).

RESULTS

Characterisation of *capa* and *pyrokinin* genes and peptides

The *capa* gene of *F. occidentalis* was identified to have three splice variants (GenBank Accession No. MW699931, ON210284, MW699932), *capa-a*, *capa-b*, and *capa-c*, that were found three different sizes of PCR products using 5′ and 3′-RACEs with different primer sets (Table S1). They are composed of five or four exons encoding two or one CAPA-PVK peptides (-PRV) and two PK peptides (-PRL). Open reading frames (ORFs) of the three variants are 576 nucleotides (nt) encoding 191 amino acids (aa) for *capa-a*, 558-nt encoding 185-aa for *capa-b*, and 450-nt encoding 149-aa for *capa-c* (Figures 1 and S1). The longest variant *capa-a* is composed of five exons encoding two CAPA peptides (CAPA-PVK1 and CAPA-PVK2), and two PKs (CAPA-PK2-1 and CAPA-PK2-2), predicted by potential endoproteolytic cleavage sites (bold and italic; Figure 1). The variant *capa-b* is also composed of five exons, encoding one CAPA (CAPA-PVK2) and the two PKs. The shortest variant *capa-c* is composed of four exons, like the variant *b*, encoding the same CAPA-PVK2 and the two PKs (Figure 1). The cleaved peptides (bold and italic) are predicted to have

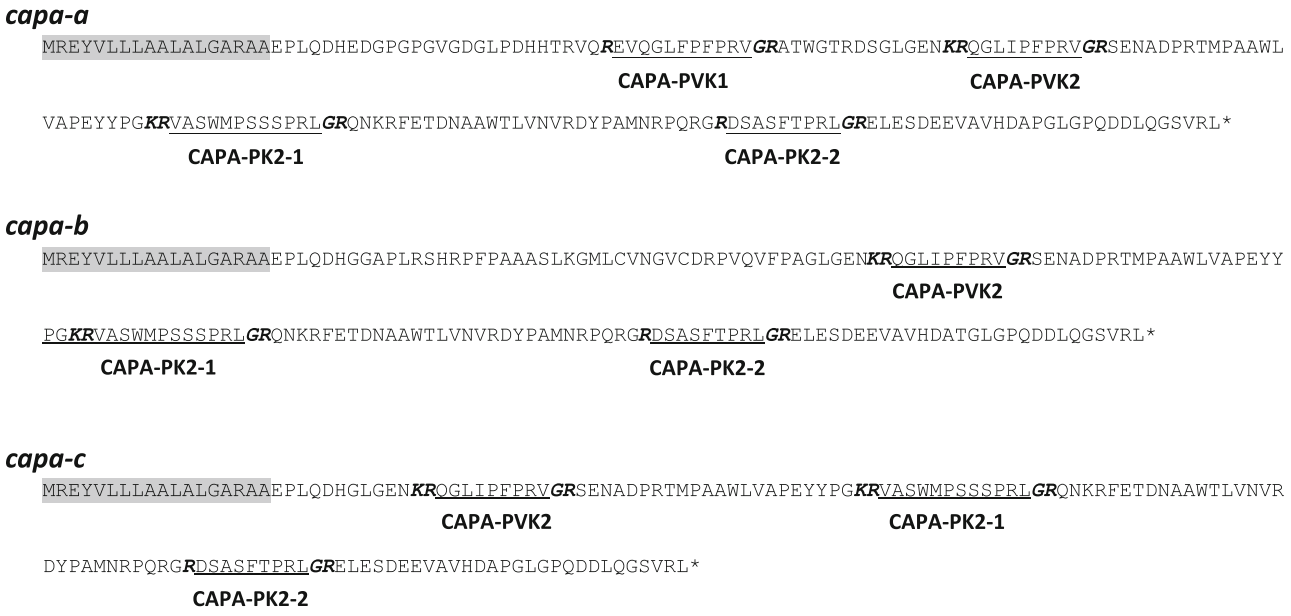

FIGURE 1 Prepropeptide sequence of *Frankliniella occidentalis* capa variants. Signal peptides (grey), prepropeptides (underlined) for CAPA and PK peptides, glycine (G) providing amide group and potential endoproteolytic cleavage sites (bold and italic), and stop codon (*) are indicated. CAPA-PVK, CAPA- periviscerokinin (PVK); PK, pyrokinin.

C-terminal amide group provided by glycine (G) (Southey et al., 2008; Veenstra, 2000). The considered mature peptides are EVQGLFPFPR-Vamide (CAPA-PVK1), QGLIPFPRVamide (CAPA-PVK2), VASWMP SSSPRLamide (CAPA-PK2-1), and DSASFTPRLamide (CAPA-PK2-2) (underlined in Figure 1).

The *pyrokinin* gene of *F. occidentalis* (GenBank Accession No. MW699930) is composed of five exons, translating all three peptides in the second and third exons (Figures 2 and S2). The *pyrokinin* gene contained 888 nt encoding 295-aa with three putative FXPRL peptides predicted by potential endoproteolytic cleavage sites (bold and italics in Figure 2). They are classified by trpPK1 (= DH-like): DLVTQVLQPGQTGVWFGPRLamide, and two PKs, PK2-1(SEGNLV NFTPRLamide) and PK2-2 (ESGEQPEDLEGSMGGAATSRQLRTDSEPT WGFSPRLamide). The PK2-2 corresponds to the insect PBAN orthologs, that is the longest PBAN ortholog in insects so far.

Expression of the *capa* and *pyrokinin* genes in different tissues and life stages

The three splice variants of *capa* were differentially expressed in the head, thorax, and abdomen (Figure S2). Since there is only an 18-nt difference in the ORFs of *capa a* and *b*, only two PCR amplicons (*capa-a/b* and *capa*-c) were detected in the gel electrophoresis (Figure 3a), revealed the upper band included two variants (*capa-a* and *capa-b*). The lower band represents the *capa-c* variant verified by sequencing (Figure 3). Overall, *capa* was highly expressed in the head, followed by the thorax, and minimally in the abdomen (Figure 3a,b). The quantitative expression level combined with the three variants showed that mRNA expression in the head is approximately 2.5 times greater than in the thorax ($p < 0.05$), but there is no significant difference in expression between the thorax and abdomen (Figure 3b). The *pyrokinin* mRNA was also highly expressed in the head, with lower expression in the thorax, and minimally or no expression in the abdomen (Figure 3c,d). The *pyrokinin* expression was 3 times higher in the head than in the thorax ($p < 0.001$; Figure 3d). Comparing the expression of the two genes, *capa* transcripts were ~2.5 times more abundant than *pyrokinin* in the head and thorax (Figure 3b,d).

When compared across developmental stages of *F. occidentalis*, the expression levels of *capa* and *pyrokinin* were similar in 1st and 2nd nymphs and pupal stages (Figure 3e). However, *capa* was only minimally detected in the eggs, while *pyrokinin* was clearly expressed (Figure 3e). Interestingly, both genes were expressed higher in male adults than in female adults, which was further confirmed by qRT-PCR (Figure S3).

Functional testing of thrips peptides to activate PK and CAPA receptors

Ten CAPA and PK peptides from *F. occidentalis* and three CAPA and PK peptides from *H. halys* were tested to activate three GPCRs: CAPA-R, PK1-R (trpPK-R, DH-R), and PK-R2 (= PBAN-R) (Table 1 and Figure S4). These GPCRs have been characterised with PRXamide peptides of *H. halys* in our previous study (Ahn et al., 2020). For initial screening, 500 nM of each peptide was challenged to the three GPCRs to determine ligand-induced receptor activation (Figure S4). The CAPA-R responded only to CAPA-PVK peptides, CAPA-PVK1, CAPA-PVK2, and HalhaCAPA-PVK2, and not to the other PK peptides. PK1-R strongly responded to HalhaPK1 (= trpK) and PK2-1, followed by trpPK1, HalhaPK2-3, and trpPK1L. The other peptides slightly activated the receptor but was not significant compared to the water control. As expected, PK2-R was strongly responded by PK peptides (= PK2, PBAN-like): PK2-1, CAPA-PK2, and HalhaPK2-3, followed PK2-1 L, PK2-2, and trpPK peptides: HalhaPK1 and trpPK1.

To determine activities of each peptide to their receptors, serial concentrations of the *F. occidentalis* CAPA-PVK, trpPK and PK peptides were tested with the corresponding GPCRs of *H. halys*, measured EC_{50} values of the peptides, and compared with *H. halys* CAPA-PVK, trpPK and PK peptides, as true ligands (Figure 4 and Table 1). CAPA-PVK2 (QGLIPFPRVamide, $EC_{50} = 337$ nM) actively bound to CAPA-R, compared to CAPA-PVK1 (EVQGLFPFPRVamide, $EC_{50} > 1$ μM). However, *F. occidentalis* CAPA-PVK2 was less active than as the true ligand of *H. halys*, HalhaCAPA-PVK2 (EQLIPFPRVamide, EC_{50} 47 nM) (Figure 4a). The two trpPKs peptides, trpPK1 (DLVTQVLQPGQTGVWFGPRLamide) and HalhaPK1 (NGASGNGGLWFGPRLamide), both activated PK1-R at similar EC_{50} levels, 560 nM and 480 nM, respectively (Figure 4b). Another trpPK candidate, trpPK1L (GQDLAEKGLTRDLVTQVLQ PGQTGMWFGPRLamide), can be processed by the potential cleavage site, but was minimally or non-specific active ($EC_{50} > 1$ μM) to the

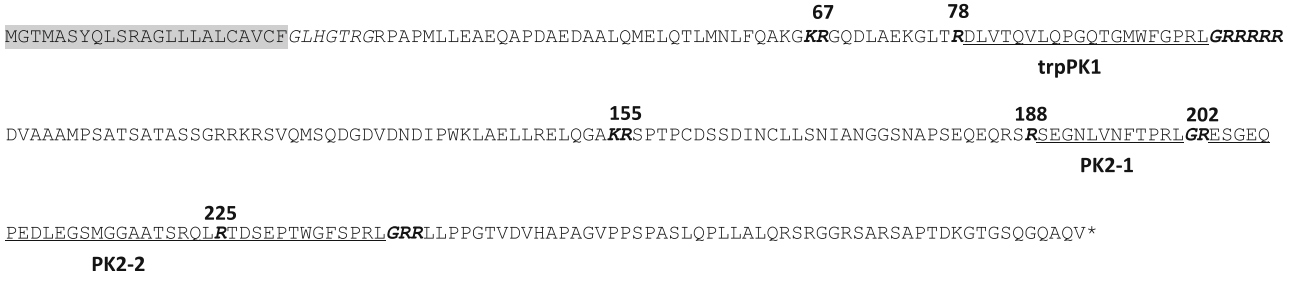

FIGURE 2 Prepropeptide sequence of *Frankliniella occidentalis* pyrokinin. Signal peptides (grey), prepropeptides (underlined) for PK peptides, glycine (G) providing amide group and potential and alternative (superscript number) endoproteolytic cleavage sites (bold and italic), and stop codon (*) are indicated. PK, pyrokinin; TrpPK, tryptophan pyrokinin.

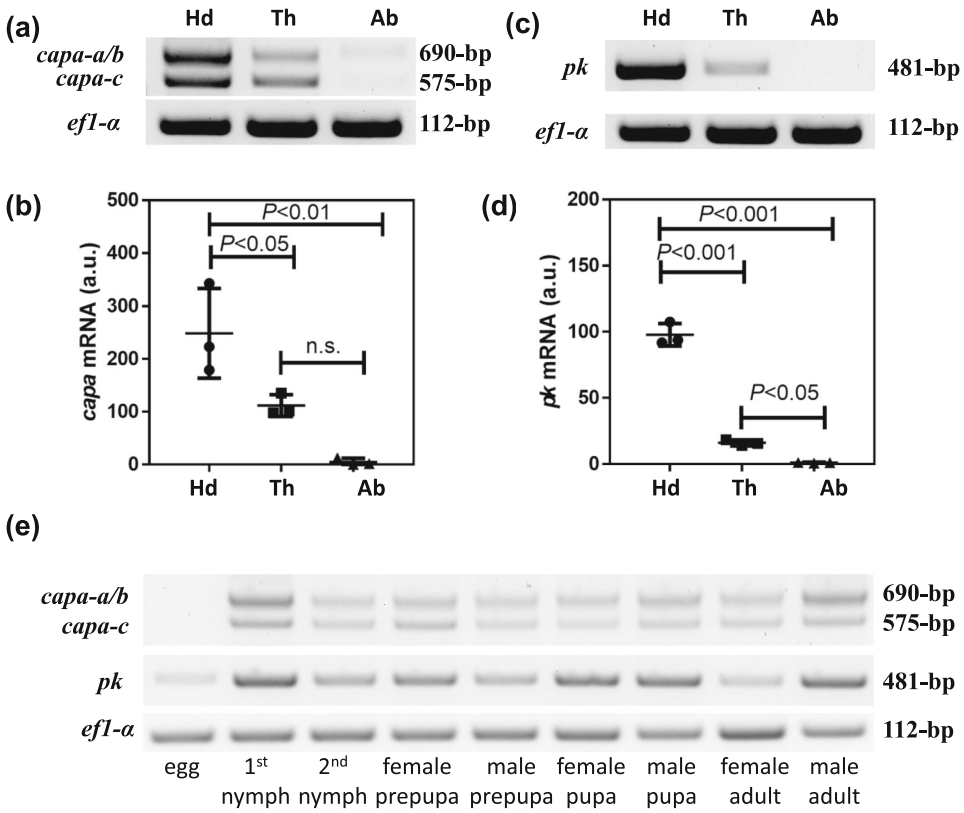

FIGURE 3 The expressions of *capa* (a,b) and *pyrokinin* (*pk*) (c,d) genes was in head, thorax, and abdomen of *Frankliniella occidentalis*. Expression profiles were visualised in 1.2% agarose gel electrophoresis and the gel photo of the reference gene (*ef1-α*) amplified from the tissues was used in both panels (a,c), and measured by a real-time quantitative PCR (b,d). Relative expressions of *capa* and *pyrokinin* genes were visualised in developmental stages (e). The same cDNA samples above were used. Primer information is in Table S1. The specific signal was normalised by *ef1*-alpha control. The statistical significance of difference was analysed by One-way ANOVA with Bonferroni's multiple comparison test. Ab, abdomen; Hd, head; n.s., not significant; PK, pyrokinin; Th, thorax.

TABLE 1 Half-maximum effective concentrations (EC_{50}) of the peptides from *Frankliniella occidentalis* and *Halyomorpha halys* tested on heterologously-expressed CAPA and PK GPCRs of *Halyomorpha halys*.

PRXamide	Peptide	Amino acid sequence	CAPA-R	PK1-R	PK2-R
CAPA-PVK	FraocCAPA-PVK1	EVQGLFPFPRVa	>1 μM	>1 μM	>1 μM
	FraocCAPA-PVK2	QGLIPFPRVa	337 nM	>1 μM	>1 μM
	HalhaCAPA-PVK2	EQLIPFPRVa	47 nM	>1 μM	>1 μM
trpPK (= DH-like, PK1)	FraoctrpPK1	DLVTQVLQPGQTGVWFGPRLa	>1 μM	480 nM	>1 μM
	FraoctrpPK1L	GQDLAEKGLTRDLVTQVLQPGQTGMWFGPRLa	>1 μM	>1 μM	>1 μM
	HalhaPK1	NGASGNGGLWFGPRLa	>1 μM	560 nM	>1 μM
Pyrokinin (= PBAN-like, PK2)	FraocPK2-1	SEGNLVNFTPRLa	>1 μM	>1 μM	19 nM
	FraocPK2-1 L	SPTPCDSSDINCLLSNIANGGSNYA PSEQEQRSRSEGNLVNFTPRLa	>1 μM	>1 μM	211 nM
	FraocPK2-2	ESGEQPEDLEGSMGGAATSRQLRTDSEPTWGFSPRLa	>1 μM	>1 μM	122 nM
	FraocPK2-2S	TDSEPTWGFSPRLa	>1 μM	>1 μM	295 nM
	FraocCAPA-PK2-1	VASWMPSSSPRLa	>1 μM	>1 μM	>1 μM
	FraocCAPA-PK2-2	DSASFTPRLa	>1 μM	>1 μM	15 nM
	HalhaPK2-3	FYAPFSPRLa	>1 μM	>1 μM	45 nM

Abbreviations: a, amide in C-termini; CAPA-PVK, CAPA-periviscerokinin; Fraoc, *Frankliniella occidentalis*; Halha, *Halyomorpha halys*; PK, pyrokinin; trpPK, tryptophan pyrokinin.

PK1-R (Table 1). Up to six *F. occidentalis* PK peptides (= PK2) are possibly translated from the *capa* and *pyrokinin* genes (Figures 1 and 2). The peptides were challenged to PK2-R and their binding activities were compared to HalhaPK2-3 as a true ligand of the receptor (Figure 4c–e and Table 1). The binding activities of PK2-1 (SEGNLVNFTPRLamide) and the long-form FraocPK2-1 L (SPTPCDSSDINCLLSNIANGGSNYAPSEQEQRSRSEGNLVNFTPRLamide) revealed that PK2-1 is almost 10 times more active than PK2-1 L at their EC_{50} values of 19 and 211 nM, respectively (Figure 4c and Table 1). Another two possible PK peptides, PK2-2S (TDSEPTWGFSPRLamide) and FraocPK2-2 (ESGEQPEDLEGSMGGAATSRQLRTDSEPTWGFSPRLamide), were tested with the same PK2-R. Interestingly, the short PK2-2S was ~2.4 times less active than the long PK2-2 at their EC_{50} values of 295 and 122 nM, respectively (Figure 4d and Table 1), indicating PK2-2 might be processed from the *pyrokinin* gene of *F. occidentalis*. The other two short PK peptides, CAPA-PK2-2 (DSASFTPRLamide) and HalhaPK2-3 (FYAPFSPRLamide), strongly activated the same receptor at low concentrations, as expected (EC_{50}, 15 and 45 nM, respectively) (Figure 4e and Table 1).

Localization of PRXamide-like immunoreactivity in the whole body

To determine the localization of *F. occidentalis* PRLamide peptides, immunohistochemical imaging was performed using a custom antiseruma that recognizes the characteristic PRXamide C-terminus. Immunostaining revealed localization to the neurosecretory cells of the central nervous system within head, thorax, and abdomen (Figure 5). In the head, PRXamide peptides localised in four to six immunoreactive cells (Figure 5a–c). In the thorax, a total of three pairs of immunoreactive cells were identified, with each pair of the cells distributed in the pro-, meso- and meta-thorax regions, respectively (Figure 5a,d). In the abdomen, seven pairs of the immunoreactivity cells were found and distributed in each segment (Figure 5a,b). However, the immunostaining results could not identify specific cell bodies, ventral nerve cords, or neurohemal organs due to the cuticle interfering with the tissue transparency in this study. However, further studies are needed. No staining was observed in the control sample prepared using the same procedure except the polyclonal antiserum.

DISCUSSION

PRXamide family peptides including CAPA, and PK peptides are found across Arthropoda, and they are well characterised with conserved C-terminal motifs, and specifically, PRVamide or similar peptides for the CAPA-PVKs and PRLamide or similar peptides for the PKs (Jurenka, 2015; Predel & Wegener, 2006). Receptors for the PRXamide peptides were also characterised and classified using binding affinity of each ligands, CAPA-PVK, trpPK/PK1/DH, and PK/PK2/PBAN, in various insects (Ahn et al., 2020; Cazzamali et al., 2005;

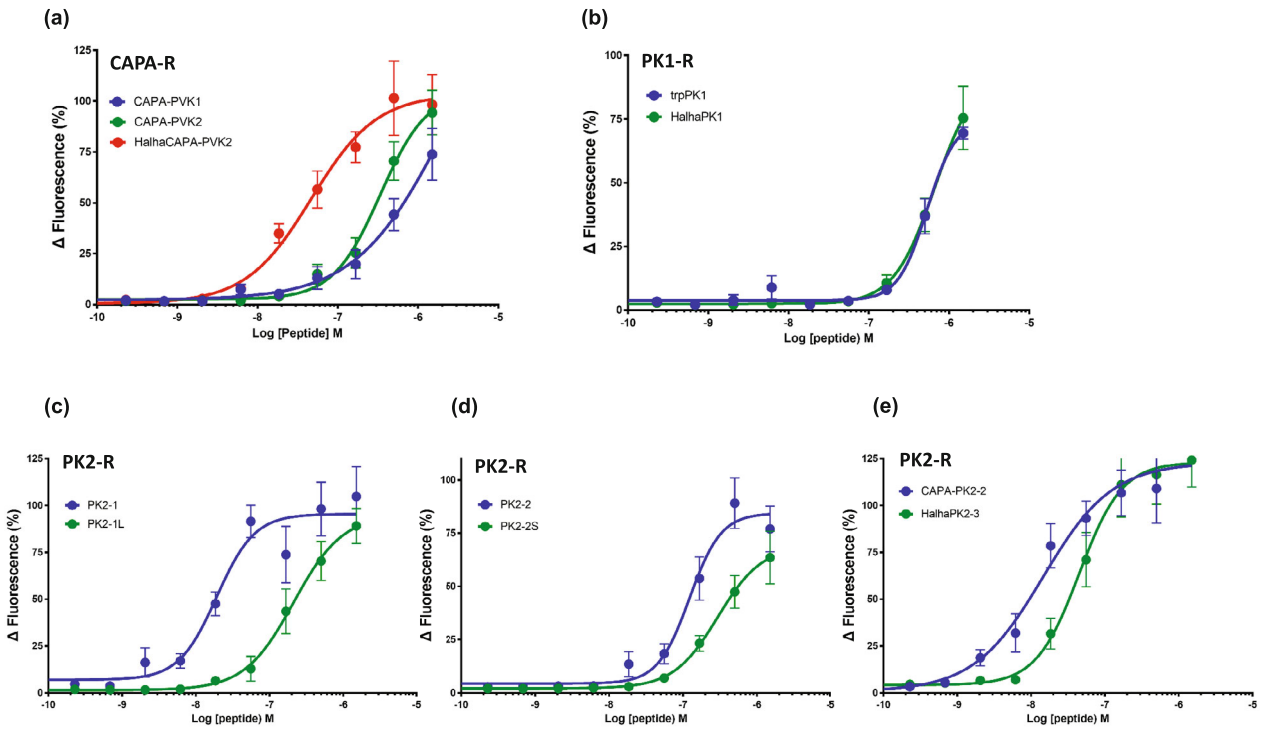

FIGURE 4 Dose–response profiles of CAPA-PVK and PK peptides of *Frankliniella occidentalis* and *Halyomorpha halys* to activate the three GPCRs from *H. halys*. Data represent the mean ± s.e.m response of cells. EC_{50} values and amino acid sequences for all the peptides to the GPCRs are detailed in Table 1. CAPA-PVK, CAPA- periviscerokinin (PVK); PK, pyrokinin.

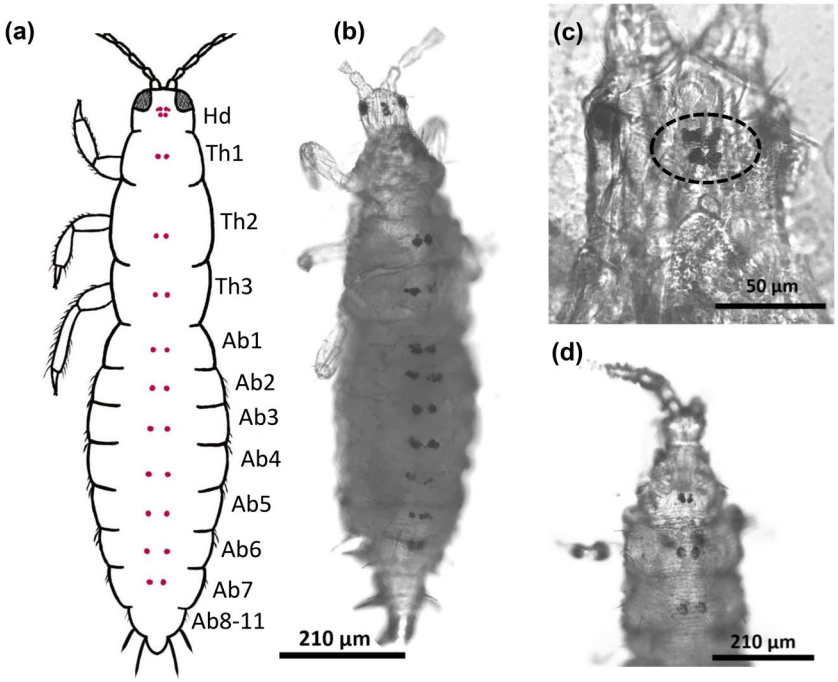

FIGURE 5 Localization of PRXamide immunoreactivity in *Frankliniella occidentalis*. Schematic diagram of the whole-body which includes the central nervous system (CNS) with the corresponding PRXamide immunoreactive neurosecretory cells indicated by red dots (a). Location of somata clusters in the whole-body (b), magnified image of immunoreactive cells (circle) in the head (c), and three pairs of immunoreactive cells in the thorax (d). Ab, abdomen; Hd, head; Th, thorax.

Jiang et al., 2014; Nusawardani et al., 2013; Olsen et al., 2007; Paluzzi & O'Donnell, 2012). The family peptides are known to involve various biological functions, such as feeding and diuresis in different life stages. In this study, we characterised two PRX peptide groups, including CAPA-PVK and PK peptides from *F. occidentalis*, their gene structures, mRNA expressions, and binding affinities of these peptides to corresponding receptors. We also identified PRXamide neurosecretory cell clusters using a whole-mount immunostaining.

The cleavage sites of the prepropeptides are usually predicted based on basic site cleavages within their sequences (Southey et al., 2008; Veenstra, 2000). Although bioinformatics algorithms and tools have been improved and provide insight into the patterns and locations of amino acids in the cleavage of prepropeptides, there are still uncertainties in predicting mature forms of bioactive peptides (natural peptide ligands). Various peptidomic methods employed with mass spectrometry (MS) and protein analytical tools are useful when using concentrated nerve tissue samples to confirm the sequences of mature peptides. Previously, we isolated crude proteins from a hundred whole bodies of the thrips and over 500 heads and analyzed small peptides to identify the target CAPA-PVK and PK peptides using a peptidomic analysis. Our own attempts to identify these peptides by MS were not successful (unpublished data).

To overcome the limitation of the nerve tissue collection of the thrips and isolation of a sufficient amount of protein samples, we employed a biological method instead to evaluate binding affinities of all the predicted CAPA-PVK and PK peptides from *F. occidentalis* to corresponding receptors that have been identified from the stinkbug *H. halys* in our previous study (Ahn et al., 2020). Thrips (Thysanoptera) and stinkbug (Hemiptera) are phylogenetically very close and belong to the superorder Paraneoptera (Ishiwata et al., 2011). The same families of neuropeptides and their receptors are usually characterised at the same or very similar binding patterns and strengths (personal communication). PRXamide peptides and their receptors have been well deorphanized and classified across Arthropoda (Jurenka, 2015), indicating these receptors were selectively activated by CAPA-PVK, trpPK (= DH-like or PK1) or PK (= PBAN-like or PK2) peptides, and characterised with the three GPCR groups, CAPA-R, PK1-R, and PK2-R, across different insect orders (Ahn et al., 2020; Choi et al., 2017; Nusawardani et al., 2013) and this study. Therefore, biological data demonstrating binding activities would be informative for determining the mature forms of bioactive peptides from the *capa* and *pyrokinin* transcripts. Particularly, if there are multiple potential endoproteolytic cleavage sites present in prepropeptides, the measurement of the peptide binding affinities to specific receptors can help to predict mature peptides.

While the cleavage sites for the two CAPA peptides of *F. occidentalis* were clear, there were multiple ambiguous cleavage sites in the prepropeptides in the *pyrokinin* transcripts for trpPK (= DH-like, PK1) and PK (= PBAN-like, PK2) peptides. Mono- and dibasic amino acids such as lysine (K) and arginine (R) are common sites for proteolytic processing in prepropeptides, however, is not always true (Southey et al., 2008; Veenstra, 2000). With the basic residue ($K\text{-}R^{67}$), the trpPK (31-aa: GQDLAEKGLTRDLVTQVLQPGQTGM WFGPRLamide is predicted), which was also described in the genome

annotation of *F. occidentalis* (Rotenberg et al., 2020). Although there is a low or no possibility, the arginine residue (R^{78}) might be processed for the short trpPK (20-aa, DLVTQVLQPGQTGMWFGPRLamide) (Southey et al., 2008; Veenstra, 2000). We synthesised both peptides and evaluated the binding affinities to the trpPK receptor (PK1-R). The short 20-aa trpPK1 was to activate the PK1-R, which is similar to the binding strength of the true ligand, *H. halys* PK1 (HalhaPK1) (Table 1 and Figure 4b). However, activation of the 31-aa long PK1L to the same receptor was very poor (EC_{50} > 1 μM). The results indicate *F. occidentalis* trpPK might be the 20-aa trpPK1, not the 31-aa trpPK1L. In other insects, trpPK (= DH-like) peptides are relatively short, including those in moths, which are about 24-aa in length, and are much shorter in non-lepidopteran species (Choi, 2022). Therefore, the 31-aa trpPK in *F. occidentalis* would be unusual, but further peptidomic analysis should be performed to confirm its actual sequence. The position of CAPA-PK2-1 is typically for CAPA trpPK precursor, but the sequence of the thrips (VASWMPSSSPRLamide) is not like the insect trpPK. None of the three GPCRs was activated by this peptide, indicating that they might not be processed. Deletion and expression of trpPK peptides from *capa* and *pyrokinin* genes might be associated with the biological function(s) through the evolution of Hexapoda (Diesner et al., 2021).

The two PK prepropeptides from the *pyrokinin* transcript, PK2-1 and PK2-2, also contain multiple cleavage sites. Prepropeptide for PK2-1 can be processed at the dibasic (*K-R*^{155}) or monobasic (R^{188}) sites, and will produce a 46-aa PK2-1 L (SPTPCDSSDINCLLSNIA NGGSNYAPSEQEQRSRSEGNLVNFTPRLamide) or a 12-aa PK2-1 (SEGNLVNFTPRLamide). When the two synthetic peptides were challenged to the PK2-R, the 12-aa PK2-1 was 10 times more active (EC_{50} = 19 nM) to the PK2-R than the long-form FraocPK2-1 L (EC_{50} = 211 nM) (Figure 4c and Table 1). We expect the 12-aa prepropeptide would be processed for a mature FraocPK2-1, and it was also predicted in the *F. occidentalis* genome (Rotenberg et al., 2020). It was interesting that even the long 46-aa peptide fairly activated the PK2-R.

Another PK2 peptide, PK2-2, which corresponds to the insect PBAN orthologs, was predicted to have two different lengths of prepropeptides by two potential monobasic cleavage sites, R^{202} or R^{225} (Southey et al., 2008; Veenstra, 2000). If the peptide is cleaved by the R^{202}, it would result in PK2-2, a 36-aa peptide, and would be one of the most extended peptides discovered among the insect PBANs (Choi, 2022). If the prepropeptide is cleaved by R^{225}, the 13-aa peptide, PK2-2S (short form), would be a potential precursor, which is similar in length to many non-lepidopteran species, between 8-aa and 20-aa long peptides (Choi, 2022). We evaluated and compared the binding affinities of PK2-2 and PK2-2S to the PK2 receptor. Unexpectedly, the longer form, the 36-aa PK2-2, was almost two times more active (EC_{50} = 122 nM) than the shorter form, 13-aa PK2-2S (EC_{50} = 295 nM) (Figure 4d and Table 1), indicating PK2-2 might be a true ligand, that is one of the longest PBAN orthologs reported in insects. The same peptide was also predicted in the genome of *F. occidentalis* (Rotenberg et al., 2020). Another 36-aa PBAN was recently predicted in the moth *Spoladea recurvalis*, but has not been characterized yet (Senthilkumar & Srinivasan, 2019). Interestingly, the *pyrokinin* gene of *F. occidentalis* does not produce another short FSPRL motif that is always found in insect *pyrokinin* genes (Choi, 2022).

In this study, we found three *capa* variants in *F. occidentalis*. Two of them are found in the *F. occidentalis* genome (Rotenberg et al., 2020) and correspond to the *capa-a* and *capa-c* variants, however, the *capa-b* variant was found in this study. The gene structure of the *b* variant is unusual with the second exon that is not found from the *capa-a* and *-c* variants (Figure S1). Another case study in the fire ant showed that two *capa* variants were found with different exon numbers encoding two CAPA-PVKs and two PKs from both variants (Choi et al., 2014). The *capa* gene of *F. occidentalis* is expressed in all the life stages except for the embryo. Interestingly, the expression levels of the three *capa* variants showed different patterns: variant *a* is dominant in the head and thorax, while variant *c* is dominant in the abdomen. The differential expressions of insect *capa* variants in different life stages and tissues are not unusual, as described in the fire ant (Choi et al., 2014).

For two CAPA-PVKs, CAPA-PVK2 (QGLIPFPRVamide) are translated from all three variants, and CAPA-PVK1 (EVQGLFPFPRVamide) is only translated from *capa-a*; therefore, CAPA-PVK2 would present more abundant in *F. occidentalis*. The binding affinity of CAPA-PVK2 (EC_{50} = 337 nM) was much more active than CAPA-PVK1 (EC_{50} > 1 μM) to the CAPA receptor. As compared to the gene expression of the *capa* variants and binding affinities of the two CAPA-PVKs, CAPA-PVK2 would be the major CAPA-PVK ligand in *F. occidentalis*. The same peptide was also confirm in mantids (Koehler & Predel, 2010), but the thrips own peptides need to be confirmed in the future. The CAPA receptor is exclusively activated by CAPA peptides even if they are from different species, with little or no binding to other PRXamide peptides (Figure S4). Although the two CAPA-PVKs of *F. occidentalis* were characterised with the *H. halys* CAPA receptor in this study, they might have different binding activities to GPCR(s) of *F. occidentalis* that need to be identified and evaluated in the future study.

Neurosecretory cells producing PRXamide peptides using immunocytochemistry methods have been characterised and localised in the central nervous system (CNS) in a variety of insect groups (Jurenka, 2015; Predel & Wegener, 2006). All the immunoreactive neurons were consistently observed with several cell clusters in the brain and the gnathal ganglia (GNG) (also known as suboesophageal ganglia). However, a variable number of the immunoreactive neurons containing PRXamide peptides were found in thorax and abdominal ganglia of different insect groups. In *D. melanogaster*, no PRXamide immunoreactive neuron was observed in the thoracic ganglia (Choi et al., 2001), and a single pair was found in the mosquitoes *Aedes sub-albatus* and *A. aegypti* (Hellmich et al., 2014). In thoracic ganglia of other insects, there are a single pair in the stinkbug *H. halys* (Hemiptera) (Ahn & Choi, 2018), and two pairs in *Lygus hesperus* (Hemiptera) (Hull et al., 2021) and the fire ant, *Solenopsis invicta* (Hymenoptera) (Choi et al., 2009). Three to five pairs of PRXamide immunoreactive neurons have been found in moths (Lepidoptera)

(Chen et al., 2019; Choi et al., 2004; Ma et al., 1998; Ma & Roelofs, 1995), while three pairs of the neurosecretory cells (a pair per thoracic ganglion) were observed in *F. occidentalis* (Thysanoptera). In abdominal ganglia, PRXamide immunoreactive neurons are usually found with three to four pairs in most insects, but six pairs in mosquitoes. In this study, seven pairs of PRXamide immunoreactive neurons were detected in the abdominal ganglia of *F. occidentalis*, but it is unknown the number of the abdominal ganglia and whether they are confused or not. The abdomens of thrips is 10-segmented and 9 segments are visible (Gibb, 2015). Recently, nine pairs of immunostained cell bodies with their locations and projections were described in the silverfish (*Lepisma saccharina*), a primitive insect (Diesner et al., 2021). The patterns of PRXamide immunoreactive neurons appear to be variable depending on insect groups and not related among the holometabolous and hemimetabolous insects.

Although strong immunoreactive detections exist in the head, thorax, and abdomen, the *capa* and *pyrokinin* mRNAs in the head were strongly expressed compared to the mRNAs of the thorax and abdomen. One possible reason that the *capa/pyrokinin* mRNAs could be more concentrated from the tiny head than thorax and abdomen tissues. Using the two PCR methods, the *capa* transcripts were expressed in the abdominal tissues, while the *pyrokinin* was only minimally or not detected (Figure 3c,d). Therefore, the *capa* transcripts are more dominantly expressed in the abdomen to produce the CAPA-PVK and PK peptides. The PRLamide antiserum used in this study possibly recognise all the CAPA and PK peptides. Because similar results for *capa* mRNA expressions and PRXamide-like immunoreactivities have been demonstrated in *Rhodnius prolixus* (Paluzzi & Orchard, 2006), *S. invicta* (Choi et al., 2014), *H. halys* (Ahn & Choi, 2018), *L. hesperus* (Hull et al., 2021), and *L. saccharina* (Diesner et al., 2021).

Many CAPA-PVK and PK peptides are involved in various physiological functions across Arthropoda, such as feeding regulation, nutritional digestion in the midgut, and diuretic or anti-diuretic processes in the hindgut and Malpighian tubules (Jurenka, 2015; Paluzzi, 2012; Predel & Wegener, 2006). Identifying agonists or antagonists to disrupt water regulation and osmotic balance could be a strategy to manage piercing-sucking type pests. (Pietrantonio et al., 2018; Scherkenbeck & Zdobinsky, 2009; Van Hiel et al., 2010). Like other piercing-sucking insects, mouthparts of thrips enable them to penetrate the leaf and feed on plant cells and sap. The feeding manner of thrips is not only causing physical damage on plants, but potentially transmitting various plant viruses, as a vector (Mouden et al., 2017; Reitz et al., 2020; Rotenberg & Whitfield, 2018). While the specific receptors for CAPA and PK peptides remain to be identified in *F. occidentalis*, the molecular and physiological data in this study provide new insight to discover potential biological functions of the neuropeptides, and to assist in development of control methods for the western flower thrips.

AUTHOR CONTRIBUTIONS

Seung-Hwan Yun: Funding acquisition; writing – original draft; investigation; data curation; methodology; validation; resources. **Hyo Sang Jang:** Writing – original draft; methodology; investigation; data curation. **Seung-Joon Ahn:** Investigation; writing – original draft; conceptualization; methodology; writing – review and editing; data curation. **Briana E. Price:** Investigation; writing – original draft; methodology; data curation. **Daniel K. Hasegawa:** Writing – original draft; writing – review and editing; funding acquisition. **Man-Yeon Choi:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; methodology; supervision; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Schematic diagram of the *capa* and *pyrokinin* gene structures of *Frankliniella occidentalis*. The nucleotide numbers of each exon (in boxes) between introns (solid lines) and prepropeptides. Signal peptides (highlighted with yellow), prepropeptides (underlined) for CAPA and PK peptides, potential endoproteolytic cleavage sites (bold italic), and start (ATG) and stop codon (*) are indicated. CAPA-PVK, capability (CAPA) periviscerokinin (PVK); PK, Pyrokinin.

Figure S2. Relative mRNA expressions of *capa* variants (a, b, c) in adult body sections of *Frankliniella occidentalis*. The gel photo (left) of *F. occidentalis*'s *capa* variants amplified with 5 ng cDNA from whole-body (Wb), head (Hd), thorax (Th), or abdomen (Ab) using 2x Dream-Taq master mix (Thermo Fisher Scientific) under 98°C for 30 sec, 35 cycles of 98°C for 5 s and 55°C for 30 s, and 72°C for 30 s, followed by a final 5 min extension at 72°C. PCR products were run and visualised in 2% E-gel with SyBR safe agarose gel (Thermo Fisher Scientific) under UV light. Relative mRNA expressions of *capa* variants were measured by the qRT-PCR method as described in Materials and Methods.

Figure S3. The expression of thrips *capa* and *pk* mRNA in developmental stages. The relative expression levels of *capa* and *pk* were assessed by real-time quantitative PCR. The specific signal was normalized by *ef1-alpha* control. The statistical significance of the difference was analyzed by One-way ANOVA with Bonferroni's multiple comparison test. n.s., not significant; *pk*, *pyrokinin;* N, nymph; F, female; M, male.

Figure S4. Responses of *Halyomorpha halys* receptors to CAPA-PVK and Pyrokinin peptides at 500 nM concentration. Data represent the mean ± SEM response of cells from three independent experiments. Halha, *Halyomorpha halys*.

Table S1. List of primers used for in this study.

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