AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
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TITLE: Biologically based thrips management for Oregon nursery

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EXECUTIVE SUMMARY: Thrips are a common agricultural pest throughout the world. Due to their extremely small size, quick life cycle, and broad host range, detecting and preventing the spread of thrips is extremely difficult. Current control for thrips primarily relies on conventional chemical insecticides despite causing potential adverse effects to human health and environmental degradation. Therefore, it is essential to develop alternative options. Recently, a variety of '-omics' tools such as genomics, proteomics, and metabolomics can be used to propel the identification of biological targets. The research proposal aims to identify potential target genes from thrips to develop biologically-based thrips controls.

We identified biological target genes, including a neurohormone gene and its receptor. We characterized the pheromone biosynthesis activating neuropeptide (PBAN) gene structure and potential peptides from the gene. The G-protein-coupled receptor (GPCR) was expressed in the insect Sf9 cells and characterized with the peptide ligands. Identification and characterization of these peptides related to specific endocrinal regulations and behaviors will aid in exploring the physiological processes on a molecular level, thus helping to identify biological targets to be utilized for biologically-based thrips management that contributes to the reduction of thrips populations and concomitant reduction.

OBJECTIVES: In this project we, particularly, focus on the PBAN and receptors in the thrips. Because these genes might be expected to play chemical communication (using pheromones) in thrips, and good biological targets. This is the initial step towards our long-term goal that is to develop a novel biological insecticide for thrips.

Objective 1. Identify and characterize the *pban* gene in the thrips.
Objective 2. Cloning and identify GPCR genes in the thrips.
Objective 3. Functional expression of the GPCR in insect cells.

PROCEDURES:
Identify PBAN gene from WFT: WFT adults used in this study were maintained under L/D 16:8, RH 60±5% and 25±5 °C at USDA-ARS (Corvallis, OR).

RNA isolation and cDNA synthesis: Total RNA was isolated using the PureLink® total RNA mini Kit from either fresh or frozen samples. Pooled samples were homogenized in cell lysis buffer using a microcentrifuge pestle and tube, proceeded following the manufacturer's protocol and included a DNase digestion step to remove contaminating DNA. The isolated total RNA samples were analyzed on a Nanodrop 2000 to determine RNA concentration and immediately used for cDNA synthesis. Five micrograms (µg) of total RNA from each sample was converted into cDNA using SuperScript III First-Strand Synthesis System using oligo-dT as a primer according to the manufacturer's protocol, and then frozen at -20 °C. The first-strand cDNA synthesized from the different tissues was used for cloning sequences and PCR amplification. Multiple primer sets were designed based on sequences of known insect PBANs, and used for the PCR amplification.

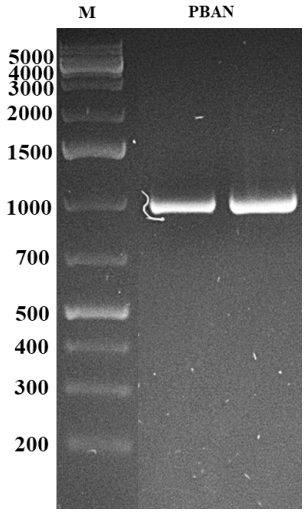
Cloning and expressing receptors in Sf9 cells: Cloning and functional expressions for the G-Protein-coupled receptors (GPCR) was followed by the method described previously (Ahn et al., 2020). Transfected cell lines were selected in the presence of 20 mg/mL blasticidin for ~3 weeks until a blasticidin-resistant cell lines were established and stored in a liquid nitrogen until use. The frozen cell lines were thawed and transferred immediately into a T-25 cell culture flask containing 5ml insectagro Sf9 medium for overnight culture. After a week the cells survived were transferred to a 125 mL glass flask containing 50 mL Sf9 medium for spin culture and prepared for functional assay.

Functional testing of receptors: Two days before the binding assay, ~50,000 Sf9 cells expressing the GPCR from suspension cultures were dispensed into each well of a black 96-well plate 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 containing 2.5 mM probenecid at room temperature in the dark for 1 h. The Calcium 6 reagent-loaded cells were transferred to the Flexstation 3 multi-mode microplate reader to measure fluorescence intensity (Δfluorescence) before and after challenging 5 µl of water as a negative control or peptide ligands (20x, 0.00 nM – 1500 nM final concentration), setup emission wavelength at 535 nm and excitation wavelength at 485 nm. Fluorescence measurements from each well on the column were taken every 5 s for 2.5 min. The peptide ligand was added by automatic pipettor once at 30 s from the beginning, and 5 µl of 5 µM ionomycin was added at 2 min to confirm calcium activity as a positive control. Baseline fluorescence was determined by averaging 5 time points for 25 s from each well prior to peptide treatment and the resulting response was expressed as a percent increase in fluorescence relative to the baseline value. For the initial screening from various peptide ligands, each cell line was exposed to a 500 nM single concentration of each peptide or water control. For the dosage-response assay, three-fold serial dilutions of selected ligands from the initial screening were used, giving final ligand concentrations from 0.08nM to 1,500 nM. Data were transformed into a log scale and fit into the non-linear regression curve. EC_{50} values were obtained by statistical analysis using the sum of squares F-test function using a GraphPad Prism.

SIGNIFICANT ACCOMPLISHMENTS

- Identified a full sequence of *Frankliniella occidentalis pban* gene: ~1,000-bp (gel photo).

- Identified three neuropeptides, including diapause hormone and PBAN-likes highlighted with green below.
- The gene structure is unusual compared to other insect PBNAs reported to date.

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TCC AGT ACC TGG ACG CGC ACG TCA TGG GGA CCA TGG CCT CCT ACC AGT TGA GCC GAG CCG   60
GCC TCC TGC TGG CCC TAT GCG CCG TCT GCT TCG GTC TGC ACG GCA CTC GCG GCT GGC CCG  120
CCC CCC ATG CTG CTC GAG GCG GAG CAG GCC CCC GAC GCG GAG GAC GCG GCG CTA CAA ATG  180
        M   L   L   E   A   E   Q   A   P   D   A   E   D   A   A   L   Q   M
GAG CTG CAG ACG CTG ATG AAC CTG TTC CAG GCC AAA GGG AAG CGC GGC CAG GAT CTC GCC  240
 E   L   Q   T   L   M   N   L   F   Q   A   K   G   K   R   G   Q   D   L   A
GAG AAG GGG CTG ACG CGG GAC TTG GTC ACG CAG GTC TTG CAG CCC GGC CAG ACG GGG GTG  300
 E   K   G   L   T   R   D   L   V   T   Q   V   L   Q   P   G   Q   T   G   V
TGG TTC GGG CCG AGA CTA GGG CGG CGC CGC CGC CGC GAC GTC GCC GCT GCC ATG CCC TCC  360
 W   F   G   P   R   L   G   R   R   R   R   R   D   V   A   A   A   M   P   S
GCT ACC TCG GCC ACG GCG TAC AGC GGA CGC CGC AAG CGT TCG GTG CAG ATG TCC CAG GAC  420
 A   T   S   A   T   A   Y   S   G   R   R   K   R   S   V   Q   M   S   Q   D
GGC GAC GTG GAC AAC GAC ATC CCC TGG AAG CTC GCC GAG TTG CTG CGG GAG CTG CAA GGG  480
 G   D   V   D   N   D   I   P   W   K   L   A   E   L   L   R   E   L   Q   G
GCG AAG CGC TCG CCG ACA CCA TGC GAC TCC TCC GAC ATC AAC TGC CTC CTG TCC AAC ATT  540
 A   K   R   S   P   T   P   C   D   S   S   D   I   N   C   L   L   S   N   I
GCT AAC GGC GGC TCG AAC TAC GCT CCG TCA GAG CAG GAG CAG CGC TCT CGC TCT GAG GGC  600
 A   N   G   G   S   N   Y   A   P   S   E   Q   E   Q   R   S   R   S   E   G
AAC CTC GTG AAC TTC ACC CCT CGC CTG GGC CGG GAG AGC GGC GAG CAG CCC GAG GAC CTC  660
 N   L   V   N   F   T   P   R   L   G   R   E   S   G   E   Q   P   E   D   L
GAG GGC TCG ATG GGA GGC GCG GCC ACA TCG CGA CAG CTG CGC ACG GAC AGC GAG CCT ACC  720
 E   G   S   M   G   G   A   A   T   S   R   Q   L   R   T   D   S   E   P   T
TGG GGG TTC TCG CCA CGT TTG GGC CGT CGC CTG CTG CCA CCG GGC ACC GTG GAC GTG CAC  780
 W   G   F   S   P   R   L   G   R   R   L   L   P   P   G   T   V   D   V   H
GCC CCG GCT GGA GTC CCA CCC TCC CCG GCC AGC CTA CAA CCC TTG TTG GCC CTC CAG CGG  840
 A   P   A   G   V   P   P   S   P   A   S   L   Q   P   L   L   A   L   Q   R
TCC AGG GGT GGT CGC TCC GCG CGG TCC GCG CCG ACG GAC AAG GGA ACA GGG AGC CAA GGA  900
 S   R   G   G   R   S   A   R   S   A   P   T   D   K   G   T   G   S   Q   G
CAA GCT CAG GTG TAG CCG ATA AAC TGC ATT AAG AAA TGA AGG TGG AAA AAT GAG AAA GAA  960
 Q   A   Q   V   *
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Figure 1. Wester flower thrips (Frankliniella occidentalis) *pban* transcript (gel photo, left) and prepropeptides. Three neuropeptides are predicted by putative endoproteolytic cleavages indicated with bold and italic letters (right). The first ATG was used as a initiation codon, TAG as a termination codon, as highlighted.

- Identified a GPCR (unpublished data): The GPCR gene was identified with 560 amino acids that contain 7-transmembrane domains forming an extra binding pocket in the receptor.

MDVAANGTLDLAGLGLSLGGLVDAEPWNASADPSAAGPWPPPNMTLEHYLERMRGPKHLPLTVVWPLTV
IYVVIFVTGVIGNVAVCVVIVRNASMHTATNYYLFSLAVSDLALLLLGLPYELSVYWQQYPWTYGEVLCKFRAL
VSEMTSYTSVLTIVAFSLERYLAICHPLQSYRMSGLRRAVRIIAVLWIVSFVAALPFSVLSTVHYLEWPIGSGLLA
PESAFCAMLDSYMVYETSSLLFFLLPMLVMVVIYGRIGSKIRSRGRHSLGKRVEGTMHGETKQTQTRKAIIRM
LSAVVIAFFLCWAPFHSQRLVYLYGQDLPHFAEINAWLYYVTGVLYFFGSTVNPILYNLMSVKYRMAFRETLC
GSLSPSAAAARGGFREQSSFRDTSVHQVGESGNGVKWHHRGGNGTAAGGSTRRFQRHGVHSLGPAVVVA
PPASPQAAQLLENGSVLEPHWKDPADPARVAADVLLDVDVVDGDADGVGADLDGCQSVDDGDAGSELVV
MISPAHRKPRVYAVGKVSRKWPKVLMLKVARKQTPAAEAVPAQHPAAATAQQGETCI

Figure 2. Amino acid sequences translated from the GPCR mRNA

- Functional assay of the GPCR with peptides: We synthesized five peptides from the thrips PBAN gene. Thrips PK2-1 and PK2-2 peptides are possibly processed with short (S) and long (L) forms, from the potential processing sites indicated the bold and italic letters in Table 1. We found it looks like the PK2-1S and PK2-2L forms have more binding affinity to the GPCR (Fig. 3). This finding is important, particularly PK2-2L was not expected before the binding test. So, the thrips PBAN mRNA could translates peptides PK1, PK2-1S, and PK2-2L.

- A part of research results has been submitted or prepared for peer-reviewed journals.

1) B. E. Price, C. Raffin, S.-H. Yun, K. Velasco-Graham, M-Y. Choi. 2022. A sustainable mass rearing method for western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae). Florida Entomologist (under revision).
2) S. H. Yun, S. J. Ahn, H. S. Jang, B. E. Price, M.-Y. Choi. 2022. Identification and characterization of PRXamide peptides in the western flower thrips, *Frankliniella occidentalis* (Thysanoptera). Insects (in preparation).

Table 1. Synthetic peptides tested with the GPCR expressed in Sf9 cells

Thrips Peptides	
Thrips PK1	DLVTQVLQPGQTGVWFGPRLamide
Thrips PK2-1S	SEGNLVNFTPRLaminde
Thrips PK2-1L	SPTPCDSSDINCLLSNIANGGSNYAPSEQEQRS***R***SEGNLVNFTPRLamide
Thrips PK2-2S	TDSEPTWGFSPRLamide
Thrips PK2-2L	ESGEQPEDLEGSMGGAATSRQL***R***TDSEPTWGFSPRLaminde

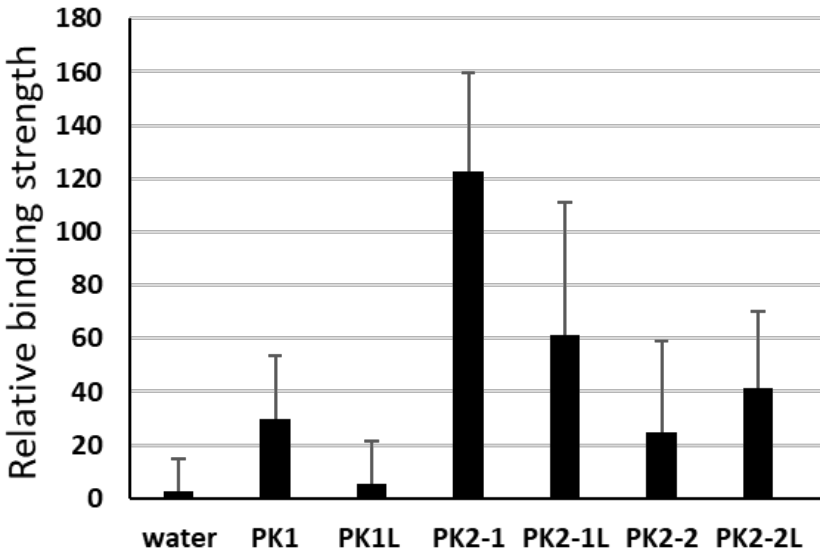

Figure 3. Responses of candidate the GPCR to thrips peptides at 500 nM concentration. Data represent the mean ± SEM response of cells from three independent experiments.

BENEFITS & IMPACT: Oregon has a variety of nursery crops from greenhouse to field. Management of thrips under biological controls is one of the top priorities of nursery growers. Target genes identified from the western flower thrips will be great potential to approach molecular and biological tools such as RNA interference (RNAi), receptor interference (Receptor-i), or pheromone. Insect PBAN peptides are known to involve various biological functions, including pheromone biosynthesis. So, the peptides identified in this project can be introduced into thrips to find if thrips pheromone is produced. We are currently developing a micro-injection technique for this study.

The impact of developing a biologically-based thrips control method, which is a chemical pesticide alternative, would be significant for thousands of growers and stakeholders in the nursery and horticulture industry. Thus, the outcomes of this research are expected to address fundamental requirements for the application of biological tools for controlling other thrips species.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM: Dr. Choi received additional funds from the Oregon Association of Nursery for thrips research.

FUTURE FUNDING POSSIBILITIES: We Will continue to apply for the thrips research proposal to Oregon Nursery and AFRI grants, and currently, the project collaborates with scientists from South Korea.