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Selective Disruption of GWSS Maturation and Reproduction by RNAi

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Introduction

In California, the control of GWSS relies primarily on the use of neonicotinoid insecticides such as imidacloprid and to a lesser extent on biological control using parasitic wasps and on other classes of chemical insecticides. Both metabolic and target site resistance to neonicotinoids are found in hemipterans and other insects (Casida and Durkin, 2013). The effectiveness of imidacloprid treatment against GWSS also appears to be on the decline in California (Porter, 2013). Furthermore, neonicotinoids have been linked to negative off-target effects such as colony collapse disorder in honeybee resulting in restrictions in their use in the European Union. The registration of several neonicotinoids is also under re-review by the US EPA. Because of the potential loss of imidacloprid both in terms of its efficacy and availability (due to regulatory restrictions), alternative technologies to control GWSS should be considered.

RNA interference (RNAi)-based technologies (reviewed in Burand and Hunter, 2013; Gu and Knipple, 2013) that selectively target the GWSS endocrine system is a potential alternative tactic for controlling GWSS and the diseases that it transmits. RNAi is a natural process that is found in a wide range of organisms that regulates gene function and protects against viruses. The natural RNAi process can be artificially induced in insects by the introduction of RNAi effectors, i.e., double-stranded RNA (dsRNA) or small interfering RNA (siRNA) that target a specific messenger RNA. This technology has been shown to work in insects that feed on artificial diet infused with dsRNA or siRNA as well as on transgenic plants that express dsRNAs. Two critical keys for developing an RNAi-based control tactic are (1) the selection of an effective gene target, and (2) the availability of a system to produce and deliver the RNAi effectors in whole insects. In this project, genes that are found in the GWSS endocrine system are being developed as targets for RNAi. Genes in the insect endocrine are ideal targets for knockdown because they are part of an essential and highly sensitive developmental pathway that is only found in arthropods. In the second year of the project, a production and delivery system for RNAi effectors that is based on GWSS pathogenic viruses will be developed.

List of Objectives

I. Develop *jheh* as a model target for RNAi-based control of GWSS maturation
II. Mine the GWSS transcriptome for other RNAi targets
III. Develop virus-based dsRNA production and delivery systems for controlling GWSS

Description of Activities and Summary of Accomplishments

I. Develop *jheh* as a model target for RNAi-based control of GWSS maturation

Juvenile hormones (JHs) and molting hormones (ecdysones) are key components of the insect endocrine system that help to regulate insect development. JHs also regulate other important biological actions such reproduction, mating behavior, feeding induction, and diapause (reviewed in Riddiford, 2008). The level of JH within an insect is determined by a combination of its biosynthesis and degradation. JH acid methyl transferase (JHAMT) is the enzyme that catalyzes the final step of JH biosynthesis. On the other hand, JH degradation occurs through the action two hydrolytic enzymes called JH epoxide hydrolase (JHEH) and JH esterase (JHE). JHEH and JHE metabolize the epoxide and ester moieties that are found on all JH molecules resulting in the formation of JH diol and JH acid, respectively (Figure 1). Minor changes in normal JH levels can result in dramatic alterations in insect development or death. The sensitivity of the insect endocrine system to minor changes is a critical factor in the success of JH analog insecticides such as pyriproxyfen and methoprene. As a class, these insecticides mimic the chemical structure and/or biological action of JH. If insects are exposed to JH analogs at a time during development when JH titer is normally undetectable, these compounds will induce abnormal nymphal development and/or death. The expectation is that the inactivation of JHE and/or JHEH activity will also result in abnormal GWSS nymphal development and/or death.

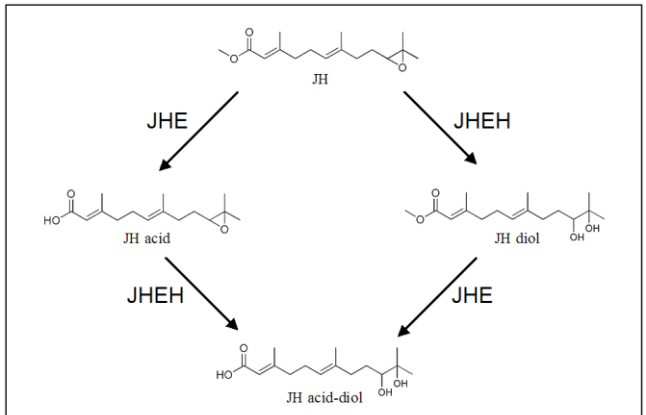

Fig. 1. Structure of juvenile hormone III (JH) and metabolism of JH III by JH esterase (JHE) and JH epoxide hydrolase (JHEH).

The coding sequence of the *jheh* gene of GWSS has been identified and confirmed to encode a biologically active JHEH in a previous project (Kamita et al., 2013). This gene is now being developed as a target for RNAi in GWSS. Plasmid constructs for the expression of full-length dsRNAs corresponding to *jheh* of GWSS have been designed and are in the construction process. The baseline levels of JHEH and JHE activities in control 5th instar GWSS has been quantified (Figure 2). Detailed information of these activities is critical for quantification of the efficacy of RNAi against the genes that encode these enzymes. During the first four days of the 5^{th} instar, JHE activity was relatively low (1.5 to 4.4 pmol of JH acid formed min^{-1} ml^{-1} of hemolymph) and found at relatively constant levels. JHE activity dramatically increased (by about 7-fold) on the 5^{th} day of the 5^{th} instar. JHE activity remained high (9- to11-fold higher than

that found on the 4th day of the 5th instar) on the 6th, 7th, and 8th days of the 5th instar, then started to decline on the 9th day of the 5th instar. The dramatic increase in JHE activity during the second half of the 5th instar is predicted to remove residual JH from the hemolymph so that (in conjunction with small spikes of ecdysteroids) the juvenile insect undergoes a nymph-to-adult molt instead of a nymph-to-nymph molt. JHEH activity was lower than JHE activity during all of the time points tested. JHEH activity increased by about 4-fold on the 6th day of the 5th instar, a delay of about 1 day in comparison to the spike in JHE activity. These findings suggested that JHE may play a more predominant role than JHEH in JH metabolism in GWSS.

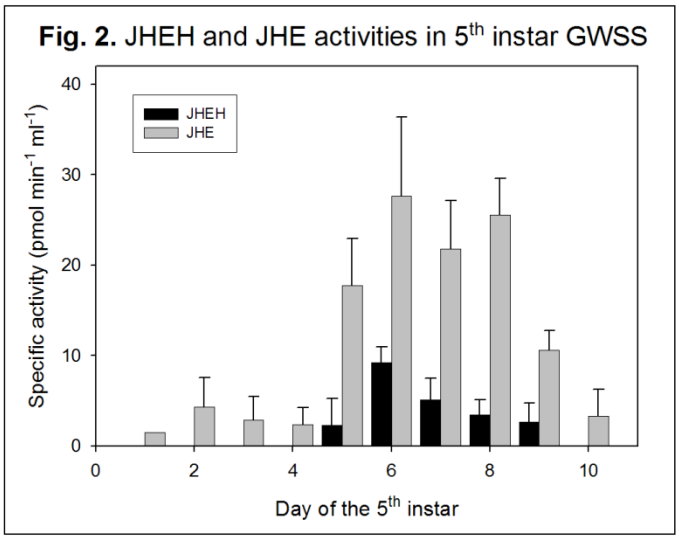

Fig. 2. JHEH and JHE activities in 5th instar GWSS

II. Mine the GWSS transcriptome for other RNAi targets

A transcriptome is defined a set of all of the RNA molecules that are found in a specific set of cells at particular moment in time. The cooperator Prof. Bryce Falk's laboratory has recently determined the sequence of the transcriptome of 5th instar GWSS (Nandety et al., 2013). By computer software-based screening of the GWSS transcriptome, multiple *jhe*-like coding sequences were identified. These potential JHE encoding sequences were manually analyzed (24 deduced amino acid sequences during the initial screening) for the presence of conserved motifs that are found in biologically active JHEs (see Kamita and Hammock, 2010). A rank order of the potential of these sequences to encode a biologically active JHE was determined and primer sequences were designed for the amplification of the full-length sequences of the top three candidates. In order to mine the full-length JHE sequence from GWSS, double-stranded cloned DNA (ds cDNA) libraries were generated from a developmentally mixed population of 5th instar GWSS (30 individuals) as well as individual GWSS at day 7, 8, and 9 of the 5th instar. The ds cDNAs were used as template sequences for 3'- and 5'-random amplification of cDNA ends (RACE) procedures to generate full-length gene coding sequences.

The RACE procedures identified a full-length JHE coding sequence (Figure 3A) from the ds cDNA library generated from a mixed population of 5th instar GWSS. This sequence encoded an open reading frame of 550 amino acid residues. Seven amino acid sequence motifs that are found in known biologically active JHEs were highly conserved in the putative GWSS JHE sequence. Surprisingly, phylogenetic analysis (Figure 3B) did not place GWSS JHE in a clade with NlJHE from the hemipteran *N. lugens*. In order to confirm that this sequence encodes a biologically active JHE, the coding sequence was cloned into a baculovirus transfer vector, and

the resulting construct was used to generate a recombinant baculovirus expression vector for the expression of the putative GWSS JHE protein. Experiments to purify and characterize the putative GWSS JHE are ongoing.

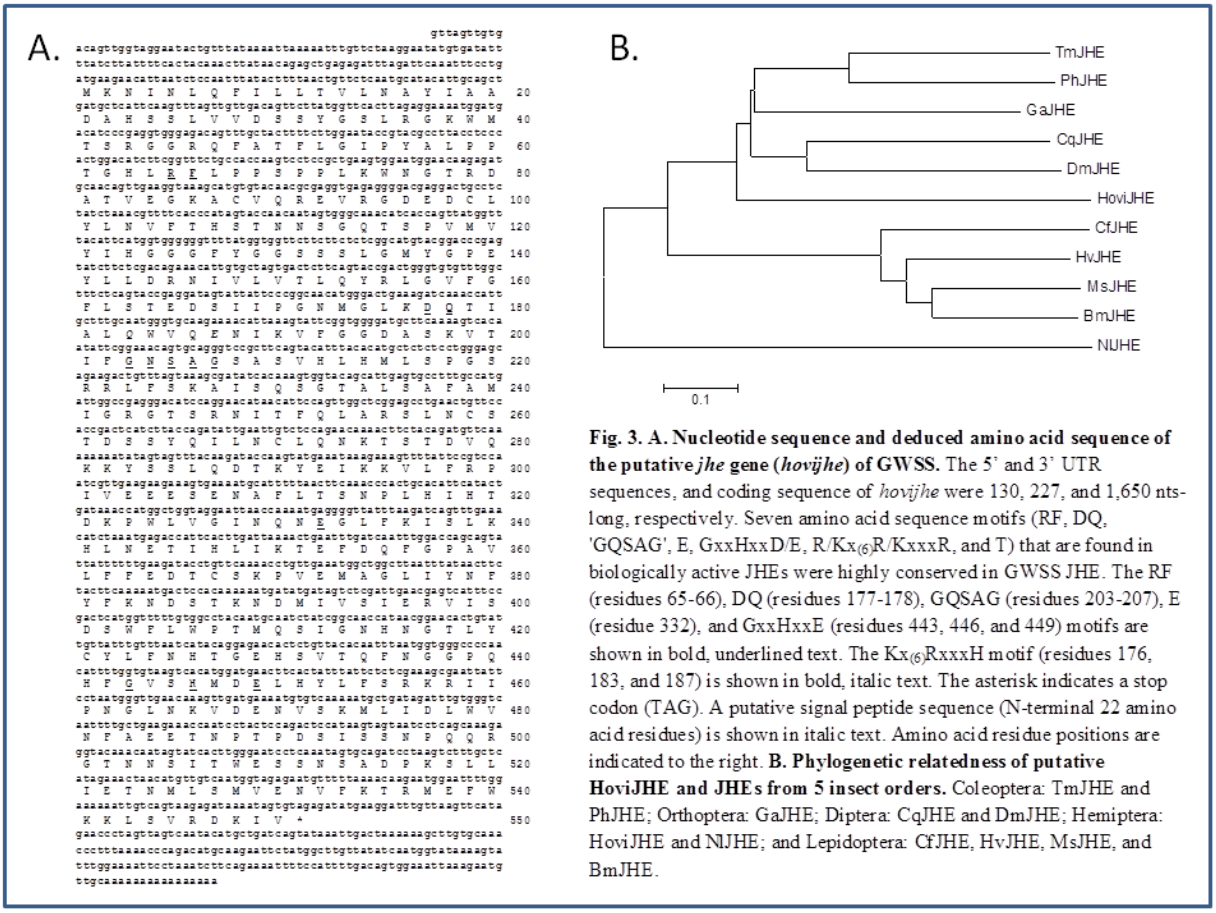

Fig. 3. A. Nucleotide sequence and deduced amino acid sequence of the putative *jhe* gene (*hovijhe*) of GWSS. The 5' and 3' UTR sequences, and coding sequence of *hovijhe* were 130, 227, and 1,650 nts-long, respectively. Seven amino acid sequence motifs (RF, DQ, 'GQSAG', E, GxxHxxD/E, R/Kx$_{(6)}$R/KxxxR, and T) that are found in biologically active JHEs were highly conserved in GWSS JHE. The RF (residues 65-66), DQ (residues 177-178), GQSAG (residues 203-207), E (residue 332), and GxxHxxE (residues 443, 446, and 449) motifs are shown in bold, underlined text. The Kx$_{(6)}$RxxxH motif (residues 176, 183, and 187) is shown in bold, italic text. The asterisk indicates a stop codon (TAG). A putative signal peptide sequence (N-terminal 22 amino acid residues) is shown in italic text. Amino acid residue positions are indicated to the right. **B. Phylogenetic relatedness of putative HoviJHE and JHEs from 5 insect orders.** Coleoptera: TmJHE and PhJHE; Orthoptera: GaJHE; Diptera: CqJHE and DmJHE; Hemiptera: HoviJHE and NlJHE; and Lepidoptera: CfJHE, HvJHE, MsJHE, and BmJHE.

III. Develop virus-based dsRNA production and delivery systems for controlling GWSS

Insect viruses are used as highly effective biological insecticides to protect against pest insect of forests and agricultural planting such as soybean. Genetic modifications have been used to further improve the efficacy of insect viruses for crop protection. For example, tomato leaf damage caused by tobacco budworm *Heliothis virescens* can be reduced by up to 45% when they are infected with a genetically modified virus that expresses a *jhe* gene (El-Sheik et al., 2011). Unfortunately, only a few viruses such as *Homalodisca coagulata* virus-1 (HoCV-1) (Hunnicutt et al., 2006) and *Ho. vitripennis* reovirus (HoVRV) (Stenger et al., 2009) are well-characterized from GWSS. HoCV-1 or HoVRV are naturally found in GWSS populations in the field but they are not severely pathogenic against GWSS. Additionally, an in vitro system (e.g., a continuous cell line) that can be used to produce and to help make genetic modifications in these viruses is unavailable. Thus, the genetic modification of HoCV-1 or HoVRV is difficult. The primary goal of Objective III is to identify new GWSS pathogenic virus(es) that are supported by a robust in

vitro system that can be used in the genetic modification of these viruses. The experiments to accomplish these goals are planned for the second year of the project.

Publications Produced and Pending, and Presentations Made

No publications (produced or pending) have been generated from this project to date. One oral and one poster presentation have been prepared and presented at the 2014 Pierce's Disease Research Symposium, Sacramento, California, December 15-17, 2014. The titles of both presentations were the same as the title of the grant (i.e., Selective Disruption of GWSS Maturation and Reproduction by RNAi).

Research Relevance Statement

The overall goal of this project is to study and exploit targets within the endocrine system of GWSS that can be used to control GWSS or reduce its ability to spread Pierce's Disease. The approach involves the identification and characterization of genes that are unique to the GWSS endocrine system that metabolize a key insect hormone called JH. Once characterized the genes will be targeted for gene knockdown using a process called RNAi. A potential outcome of this project is the development of an alternative control strategy for GWSS.

Layperson Summary of Project Accomplishments

A natural process called RNAi is used by a wide range of organisms to regulate normal gene function and defend against viruses. RNAi can be artificially manipulated to knock down the activity of a targeted gene. Two critical keys for developing an RNAi-based control tactic are (1) the selection of an effective gene target, and (2) the availability of a system to produce and deliver the RNAi effectors in whole insects. In this project, genes that are found in the GWSS endocrine system have been identified and are being developed as targets for RNAi. A field-applicable delivery system for inducing RNAi will be developed during the second year of the project.

Status of Funds

As of the end of January 2015, approximately $35,000 ($6,000 in Supplies/Expenses/Travel as well as $29,000 in Salaries/Benefits) of the grant's first year budget is remaining.

Summary and Status of Intellectual Property

At least one peer-reviewed publication is expected from the research results that are generated from this project. Any reagent, technology, nucleotide sequence or other material that is generated from this project will be made freely available to other researchers.

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