AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
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TITLE: Screening of RNAi targets to develop a novel RNAi-based control method for spotted wing drosophila

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SUMMARY: Spotted wing drosophila (SWD), *Drosophila suzukii*, is a serious invasive pest from Asia that is now in the United States, Canada, and Europe. The severe damage caused by this destructive pest affects ripening all small fruits, and the infestation area is rapidly spreading through North America as well as Europe. Growers are facing economic losses by increased spending on management costs, the loss of production and market values, and rejection of exports if unacceptable levels of insecticide residues and damage are found. Major control of SWD relies on the spray of chemical insecticides which have negative impacts on agricultural ecosystems affecting non-target insects and human health. In addition, there is an inevitable risk that SWD populations in the field will develop insecticide resistance from the continuous use of chemical controls. Therefore, the heavy reliance on chemical insecticides should be replaced or at least complemented with biologically-based environmentally friendly alternatives.

RNA interference (RNAi) technology is a new direction of the insect pest management, a biologically-based and target specific strategy. During the past decade the availability of insect genomics and computational biology has further enabled the implementation of RNAi technology to target economically important insect pests. One of the key advantages of RNAi technology is its high degree of species-specificity for the target pests; this is a unique point compared other conventional insecticides. Recently it has shown striking results in various insect groups, suggesting that it will be a promising tool for the next generation of pest management. To date, a variety of RNAi targets are being screened and evaluated for specific impacts applicable to pest management of agricultural crops or insect vector-borne diseases. However, virtually nothing is known for RNAi targets for SWD although a lot of RNAi tests has been done to identify biological functions in *D. melanogaster*.

To successfully develop RNAi applications, a critical initial step is screening for appropriate RNAi target genes because degrees of gene silencing impacts vary from RNAi target genes and insects. The challenge with gene selection is to select suitable insect-specific target genes that provide fast-acting mortality or suppression and long-term population suppression without affecting other non-target organisms. Therefore, it is important to screen multiple and key RNAi candidates to improve the chance for identifying an effective RNAi target. This is the initial step towards our long-term-goal that is to develop a novel RNAi-based control for SWD. Successful achievement of our *goal* will add a new environmentally-friendly SWD control method to the IPM tool box and contribute to the reduction of SWD populations and concomitant reduction in environmental impacts and losses in agriculture.

Our 2016-18 **OBJECTIVES** are as follows:

Objective 1. RNA sequencing SWD midgut, and analyze the RNA expression profile.
Objective 2. Select and identify RNAi targets – ten (10) genes from the midgut.
Objective 3. Design and synthesis dsRNAs for RNAi materials.
Objective 4. Bioassay to measure RNAi impacts on SWD.

PROCEDURES

RNA sequencing and analyze RNA expression profile on SWD midgut (2016): A SWD colony was maintained with an artificial diet in the laboratory. Adult midguts were dissected, and collected in an RNase/DNase free tube on dry ice and stored at -80 °C to isolate total RNA. Total RNAs were isolated from SWD whole body, midgut tissue and whole body. The isolated total RNA samples were analyzed to determine RNA concentration and immediately used for cDNA synthesis. Purified total RNA samples (whole body and midgut) was prepared for the RNA sequencing and differentially expressed gene (DEG) analysis.

Select and identify RNAi targets (2016): With RNA sequencing data and DEG analysis from the midgut and the whole body specific genes for RNAi targets were selected and identified from the midgut. In this initial project, we decided ten candidate genes for SWD RNAi targets. The selection of targets was based on the previous studies for insect RNAi targets and biological functions. These target genes were classified into essential housekeeping genes that are required for the maintenance of basic cellular functions, neuropeptide hormones and G-protein-coupled receptors.

RESULTS AND DISCUSSION

1. SWD midgut transcriptome analysis

The midgut and whole body transcriptomes were analyzed by next-generation sequencing using Illumina HiSeq2000 platform. The sequencing data were produced and trimmed to have over 5.6 G bases, resulting in 56 million reads. Based on the SWD official gene set (OGS) provided in the SpottedWingFlyBase (http://spottedwingflybase.org), over 73% and78% of reads from the midgut and whole body transcriptomes, respectively, were mapped to the reference and assembled into 13,583 contigs. By expression analysis, contigs with at least one zero FPKM were excluded, leaving 8,871 contigs to be further analyzed. The distributions of the contig expression levels were similar between the midgut and whole body (Fig. 1A). DEG analysis between the two transcriptomes showed that 873 contigs were up-regulated and 1,117 contigs were down-regulated in the midgut compared to the whole body (Fig. 1B), whereas the rest (6,881 contigs) were similarly expressed between two samples. Scatter plot of expression levels between two groups showed that the up-regulated contigs in gut are less than in the whole body and their up-regulation levels are also smaller (Fig. 1C). Similar result was obtained from the volume plot analysis, where expression levels were also counted when the two groups' expression levels were compared (Fig. 1D).

The study results opened an avenue to understand gene expressions of SWD midgut in molecular level, which is the first battle field of plant defense compounds, insecticides, Bt toxins, and oral RNAi agents. The midgut-specific and/or -abundant genes will be further explored for screening biological targets for RNAi approach to control SWD.

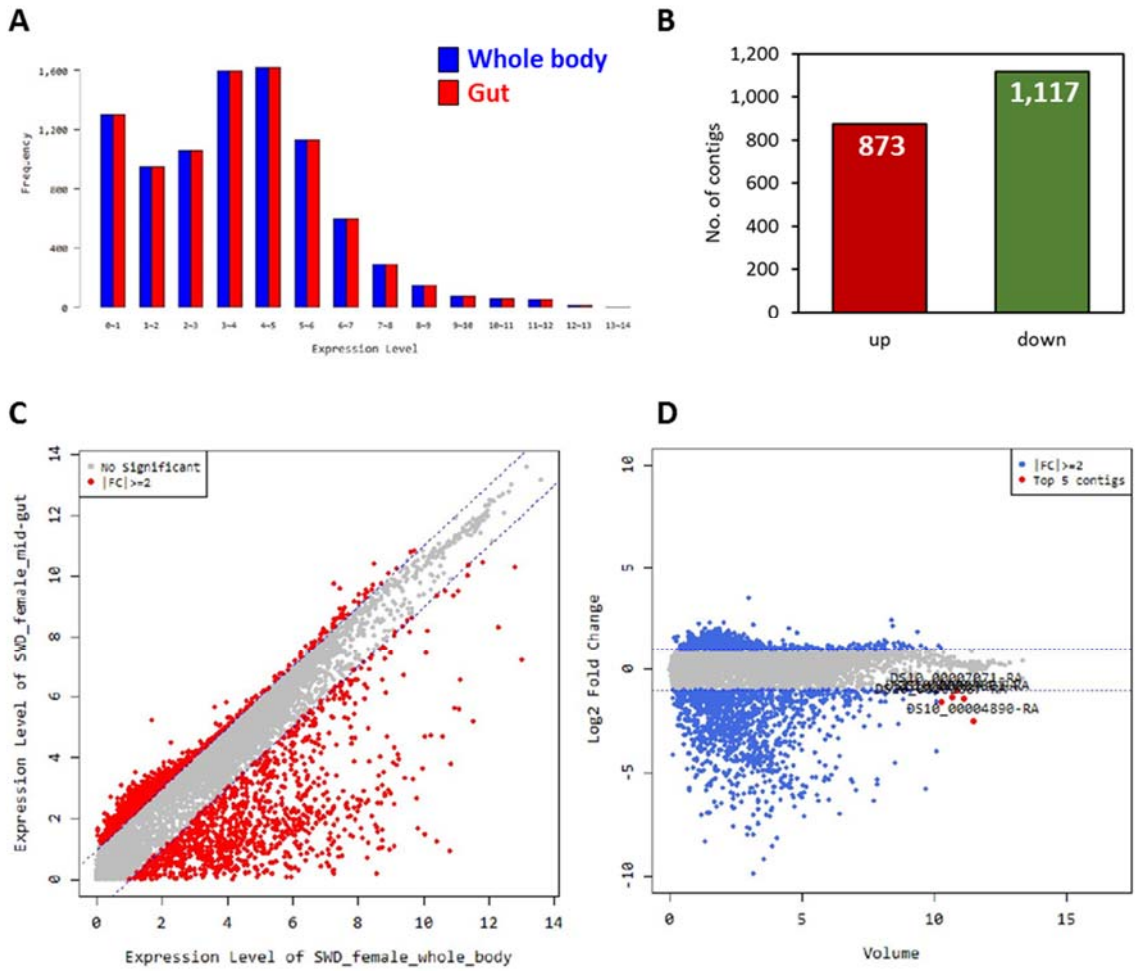

Figure 1. Deferentially-expressed gene (DEG) analysis of the SWD midgut transcriptome compare to the whole body transcriptome. (A) Distribution of expression level between the midgut and whole body. The expression level is shown as normalized Log2(FPKM+1) of each groups. Genes with fold change over 2 (lFCl ≥ 2) are indicated in red dots. **(D)** Volume plot showing DEGs as a function of geometric mean of two group's expression levels. Red dots indicate top five transcripts by volume which satisfy FCl ≥ 2.

2. Identification of RNAi candidate genes from SWD: We identified DNA sequences for 11 candidate genes, designed and synthesized 11 dsRNAs (Table 1).
We completed Obj. 1 &2 in 2016. RNA-sequences for SWD midgut tissue, female ovary and whole body were competed and analyzed up and down regulated transcripts, mRNA copies. The methodology we outlined in the proposal has been successful; we have been able to dissect and collect target tissues, then total RNAs have been done for RNA sequencing 2016.

We found 873 genes are up-regulating in the midgut compare to the whole body whereas almost two times of genes (1,921 genes) are up-regulating in the ovary compare to the whole body. We completed to classify these all genes for further consideration and characterization for RNAi targets. This is very important data because selection of RNAi targets can be narrowed

down. Based on our RNAi experience, knowledge and previous RNAi reports, we selected 11 potential candidates (Table 1) including neuropeptide hormones, receptors and housekeeping genes for SWD RNAi target(s).

Table 1. SWD RNAi candidates from three different groups and green fluorescence protein (GFP), and nucleotide lengths of dsRNAs.

RNAi candidates	DNA template for RNAi synthesis	Gene group
SWD ID1	296 nucleotides	Neurohormone
SWD ID2	195 nucleotides	Neurohormone
SWD ID3	399 nucleotides	Hormone receptor
SWD ID4	244 nucleotides	Housekeeping
SWD ID5	253 nucleotides	Housekeeping
SWD ID6	255 nucleotides	Housekeeping
SWD ID7	253 nucleotides	Housekeeping
SWD ID8	250 nucleotides	Housekeeping
SWD ID9	251 nucleotides	Housekeeping
SWD ID10	254 nucleotides	Housekeeping
SWD ID11	254 nucleotides	Housekeeping
(GFP)	350 nucleotides	control gene – unrelated in SWD

We found some genes identified in this study were very different from those sequences published on the SWD genome data, indicating a wrong annotated or uncompleted the SWD genome that should need to be confirmed actual sequences for each target genes. The length of dsRNA for SWD ID2 was designed a short because the size corresponds roughly to full sequence (~200 base pairs). SWDID3 is a receptor for a neuropeptide hormone functioning to egg development in the female SWD.

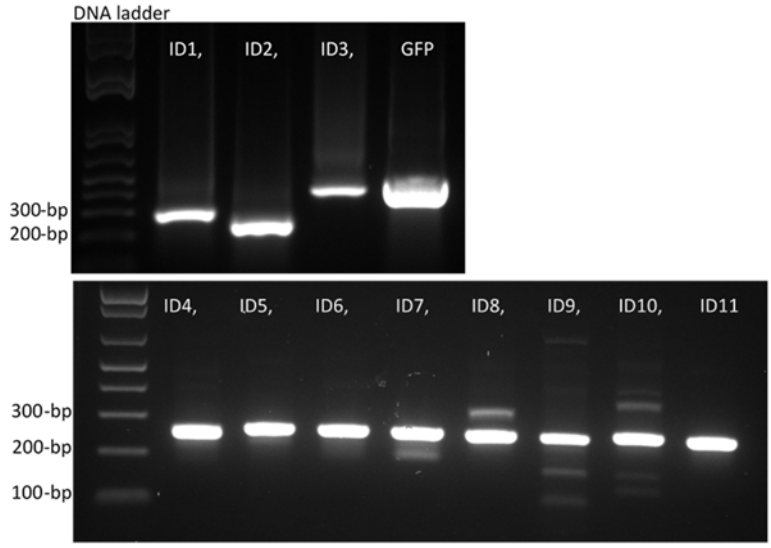

Figure 2. Photos of synthesized dsRNAs of SWD ID1-3 & GFP (upper) and ID4-11 (lower) corresponding to DNA templates from Table 1.

Eight housekeeping genes as constitutive genes are expressed in all cell types at a level that does not fluctuate with the cell cycle. Functional examples of housekeeping genes for RNAi targets are related in the muscle physiology, detoxification, ATP metabolism, protein sorting and transporting, and cell membrane structure in cells. These genes have been selected for RNAi candidates to develop RNAi-based control for insect pests.

3. Initial screening of 11 potential RNAi targets

During two years the 1st screening with over 11 RNAi candidates was completed with over 2,000 nano-injections to flies. We found some effective phenotypic impacts, mainly mortality, from some of the RNAi injection into SWD flies (Figs. 3 and 4).

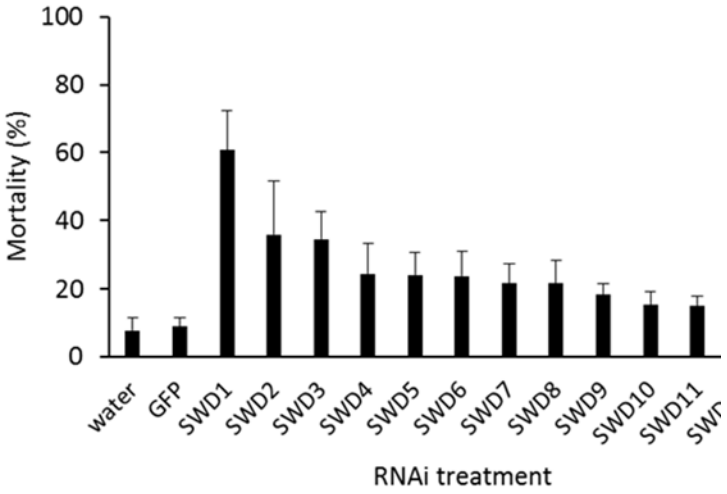

Figure 3. Mortality of SWD adult flies after injected dsRNA (1µg/fly) within 48h. Twenty flies were used with 3 replications at least for each RNAi target.

Figure 4. Photos of dead SWD adults after RNAi treatment within 48h.

4. Genotypic impact of the potential RNAi targets

Three most effective RNAi candidates were selected for further genotypic test that is quantitative PCR (qPCR) analysis for the gene expression after RNAi injection. We investigated the gene expression levels to find whether those genes are being suppressed or not after target RNAi (dsRNA) injected into SWD. Using the quantitative gene analysis we found all three RNAi target genes have been knock downed by dsRNA introduction to SWD (Fig. 5).

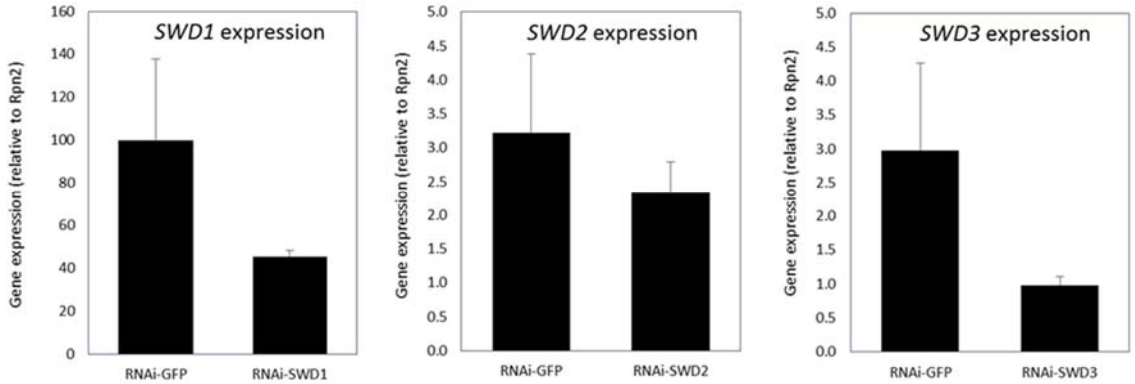

Figure 5. Knock-down of target gene expression by RNAi. Messenger RNA expression levels of SWD1, SWD2, and SWD3 were compared between RNAi-GFP and RNAi-target in *D. suzukii* 12h after dsRNA injection of SWD1, SWD2, SWD3, and GFP. Gene expression estimates

The research results have been presented at the meetings of International Entomology Congress, Entomological Society of America, and Pacific Northwest Insect Management Conference, and preparing a manuscript to be published.

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