A THESIS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

Transcriptomic Identification and Characterization of Genes Responding to Sublethal Concentrations of Different Insecticides in Three Insect Species

아치사량 살충제에 반응하는 3 종 해충의 유전자 동정 및 특성 분석

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UNDER THE DIRECTION OF ADVISER SI HYEOCK LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Since one of the major insecticide resistance mechanisms is the enhanced xenobiotic detoxification, characterization of these detoxification factors would facilitate the understanding how insects develop metabolic resistance to insecticides. As the expression of many detoxification gene is inducible by sublethal treatment of insecticides, analysis of the transcriptome profiles of insects treated with a sublethal dose of insecticide has been employed as a general method for identifying the major metabolic factors associated with insecticide tolerance and resistance. In this study, *Plutella xylostella* (diamondback moth, DBM), *Frankliniella occidentalis* (western flower thrips, WFT)

and *Drosophila melanogaster* (common fruit fly, CFF) were selected as model insect species. These insects were treated with sublethal amounts of various insecticides, and their transcriptomes were analyzed and compared within and between species to common metabolic factors possibly associated with insecticide tolerance and resistance.

In chapter I, third instar larvae of the *P. xylostella* were pretreated with sublethal concentrations (LC_{10}) and then subsequently exposed to medium lethal concentrations (LC_{50}) of chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad via leaf dipping, their tolerance to insecticides was significantly enhanced. Transcriptome data determined that 125, 143, 182, 215 and 149 transcripts were overexpressed whereas 67, 45, 60, 60 and 38 transcripts were underexpressed following treatments with chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad, respectively. When further characterized the differentially expressed genes (DEGs), the most notable over-transcribed genes were two cytochrome P450 genes (*Cyp301a1* and *Cyp9e2*) and nine cuticular protein genes. On the contrary, several genes composing the mitochondrial energy generation system were under-transcribed in all treated larvae. These results showed at least in the case of *P. xylostella*, the common DEGs appeared to be involved in general chemical defense, regardless of the structures and

modes of actions of these insecticides, at the initial stage of intoxication.

In chapter II, pretreatment with sublethal concentrations (LC_{10}) of chlorfenapyr, dinotefuran and spinosad, then subsequently treated with medium lethal concentrations (LC_{50}) of the respective insecticide via residual contact vial plus water (RCVpW) method and the pretreatments enhanced the tolerance in *F. occidentalis* female adult significantly. Transcriptome analysis showed that 404, 386 and 756 genes were up-regulated, meanwhile 124, 107 and 169 genes were down-regulated following the treatment of chlorfenapyr, dinotefuran and spinosad, respectively. Among these, 199 transcripts were commonly up-regulated, whereas 31 transcripts were commonly down-regulated. Most up-regulated transcripts were categorized as basic biological processes, including proteolysis and lipid metabolism. Detoxification genes, such as one glutathione S transferase, three UDP-glucuronosyltransferases, four CYP450s, and one ABC transporter, were commonly up-regulated in all three insecticide-treated groups. RNA interference of five commonly overexpressed genes increased mortalities to all three insecticides, since these three tested insecticides have distinct structures and modes of action, the roles of commonly expressed genes in tolerance were supported and further discussed.

In chapter III, sublethal concentrations (LC_{10}) of chlorantraniliprole,

cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad were introduced to *D. melanogaster* female adults, and subsequently treated these insecticides with medium lethal concentrations (LC_{50}) via topical treatment. Similar with the previous cases, the tolerance to insecticides was enhanced significantly. Transcriptome analysis identified 123, 173, 75, 245, 368 and 145 over-transcribed genes, as well as 137, 108, 202, 83, 59 and 126 under-transcribed genes in chlorantraniliprole-, cypermethrin-, dinotefuran-, indoxacarb-, ivermectin- and spinosad-treatment, respectively. Among these DEGs, 26 and 30 genes were found commonly up- and down-regulated in all insecticide treated groups. The major part of commonly up-regulated genes are immune induced antibacterial peptides, such as attacin-A/C, diptericin A/B, drosocin and immune induced molecule 18, etc. On the other hand, many components of mitochondrial respiratory chain were commonly down-regulated in all treatments. Their roles in general chemical tolerance were discussed.

Key words: Transcriptome analysis; Insecticide tolerance; Sublethal concentration; Diamondback moth; Western flower thrips; Common fruit fly; Detoxification gene; Mitochondrial respiratory chain; Common DEGs

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ABBREVIATION USED

ABCT: ATP-binding cassette transporter

DBM: Diamondback moth

CFF: Common fruit fly

CYP: Cytochrome P 450

DEG: Differentially expressed gene

GO: Gene ontology

GST: Glutathione S-transferase

FC: Fold change

LC_{10} : 10% lethal concentration

LC_{50} : 50% lethal concentration

PPM: Part per million

RCVpW: Residual contact vial plus water

TF: Transcription factor

UDP: Uridine 5`-diphosphate

UGT: UDP-glucuronosyltransferase

WT: Wild type

WFT: Western flower thrips

GENERAL INTRODUCTION

One of the major mechanisms for insecticide resistance is the enhanced xenobiotic detoxification, including proteins that can metabolize or efflux xenobiotics (James et al. 2009). Therefore, characterization of these detoxification factors would facilitate the understanding how insects develop metabolic resistance to insecticides. Metabolic resistance to insecticides can be developed when pests acquire heritable traits that result in either constitutive/inducible over-expression or functional alteration of protein products involved in metabolism (Yoon et al. 2011). Since the expression of many detoxification gene is inducible by sublethal treatment of insecticides, analysis of transcript profiles, either in a small scale of using a subset of detoxification genes or in a full scale of using entire transcriptome, has been employed as a general method for identifying the major metabolic factors associated with insecticide resistance (Yoon et al. 2011; Vontas et al. 2005; Willoughby et al. 2006). In the transcriptional profiling of body louse detoxification genes [cytochrome P450 (Cyp450) and ABC transporters (ABCT)], three Cyp450 and one ABCT genes, which are also known to be involved in insecticide metabolism in other organisms, were significantly overexpressed upon induction by a brief, sublethal exposure

to ivermectin that resulted in tolerance (Yoon et al. 2011). Heterologous expression of CYP6CJ or ABCC4 resulted in the oxidative metabolism or ATP-dependent efflux of ivermectin, respectively (Kim et al. 2018). In the case of *P. xylostella* larvae induced by sublethal doses of cypermethrin, eight of 11 Cyp450 genes tested were over-transcribed in a cypermethrin-resistant strain whereas only a single Cyp450 gene was induced in a susceptible strain, suggesting that the selective Cyp450 induction by cypermethrin is also a metabolic resistance mechanism (Baek et al. 2010).

To investigate the involvements of these detoxification genes, non-invasive induction assays have been used in previous studies (Kim et al. 2018; Yoon et al. 2011). Since the lethal concentrations treatments of insecticides not only induce the detoxification related metabolic factors, but also a lot more genes related to general physiological stress. On the other hand, a brief and sublethal concentrations treatment induce the genes which are more closely related with actual metabolism, makes it a better tactic for identifying the metabolic factors in detoxification metabolism. Due to the expression of many detoxification genes are inducible, analysis of transcript profiles using s subset of detoxification genes or entire transcriptome has been used as a general method to identify the differentially expressed detoxification genes (Vontas et al.

2005; Willoughby et al. 2006).

In my study, I used three insect pest species as models to identify insecticide-inducible detoxification factors using the non-invasive induction assay: *Plutella xylostella* (diamondback moth, DBM), *Frankliniella occidentalis* (western flower thrips, WFT) and *Drosophila melanogaster* (common fruit fly, CFF). These insects were treated with sublethal amounts of various insecticides, and their transcriptomes were analyzed and compared within and between species to common metabolic factors possibly associated with insecticide tolerance and resistance.

Diamondback moth (DBM), *Plutella xylostella* (L.), which originated from the Mediterranean region, is one of the most devastating agricultural pests. It feeds on foliar tissue of cruciferous crops, including cabbage, broccoli, cauliflower, etc. The economic loss caused by *P. xylostella* is estimated about US $ 4–5 billion worldwide annually (Talekar et al. 1993; Tang et al. 2014). The western flower thrips, *Frankliniella occidentalis* Pergande, is a serious polyphagous pest that causes severe damage to horticultural crops (Reitz et al. 2011; Woo 1988).

F. occidentalis causes direct damages such as silvery scar, and also indirect damages by transmitting plant viruses such as tomato spotted

wilt virus (TSWV) and impatiens necrotic spot virus (INSV), leading to substantial economic loss (Webster et al. 2011; Zhao et al. 1995). This species has been widely distributed throughout the world since the 1970s, as the international trade in horticultural products expanded from Europe to Asia (Kirk et al. 2003).

The common fruit fly (CFF), *Drosophila melanogaster*, originates from Africa and now distributes to all continents and islands (Baudry et al. 2004; Markow 2015). *D. melanogaster* is one of the most intensively studied organisms in biology and serves as a model system for the investigation of many developmental and cellular process (Adams et al. 2000). *D. melanogaster* has also been used as a model insect for insecticide resistance studies (Wilson 1988). The well-studied genomics of *D. melanogaster* facilitate discovering the underlying mechanisms related with insecticide resistance such as overexpressed CYPs, target site mutations in acetylcholinesterase and voltage sensitive sodium channel (vssc), etc (Scott et al. 2019; Daborn et al. 2007).

Many insecticides have been used to control *P. xylostella* in the past decades. As a result of the overuse of insecticides and its high adaptability, *P. xylostella* has developed resistance to most commercial insecticides, including organophosphates, carbamates and pyrethroids (Shelton et al. 1993). The use of insecticides with different modes of

action is an important strategy for controlling *F. occidentalis* populations, but it has also resulted in the development of insecticide resistance because of the short life cycle and high fecundity of thrips, which are traits conducive to the evolution of resistance (Bielza et al. 2007; Brodsgaard 1994). *D. melanogaster* also has developed resistance to many different insecticides including DDT (organochlorine), cypermethrin (pyrethroid), and malathion (organophosphate), etc (Catania et al. 2004; Sun et al. 2019). To overcome the resistance problem, various novel groups of insecticides, including neonicotinoids, macrocyclic lactones, diamides, etc., have been introduced to control these pests (Ninsin 2004; Jinfeng et al. 2008; Wang et al. 2013; Gao et al. 2012; Sun et al. 2019). Although some cases of resistance to these newly introduced insecticides have been reported, resistance is not yet widespread and underscores the critical need for proactive resistance management strategies for them.

To identify the genes commonly involved in insecticide tolerance, various insecticides with different modes of action and structures were used in my studies, including chlorantraniliprole, chlorfenapyr, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad. Chlorantraniliprole (IRAC class: 13) is an anthranilic diamide, it targets the muscle calcium channel ryanodine receptor, caused the uncontrolled

calcium ion release, rapid paralysis and death of insects (https://pubchem.ncbi.nlm.nih.gov/compound/Chlorantraniliprole).

Chlorfenapyr (IRAC class: 13) belongs to a new class of chemicals - the pyrroles. This compound is a pro-insecticide and the activity depends on the oxidative removal of its *N*-ethoxymethyl group by mixed function oxidases, it is an uncoupler of oxidative phosphorylation via disruption of H^+ gradient, thus resulting in disruption of ATP production, cellular death and ultimately organism mortality (https://pubchem.ncbi.nlm.nih.gov/compound/Chlorfenapyr).

Cypermethrin (IRAC class: 3A) is a synthetic pyrethroid and mainly prolongs the opening of voltage-gated sodium channel and cause the hypo-polarization and hyperexcitation of the cell, and finally insect death (https://pubchem.ncbi.nlm.nih.gov/compound/2912). Dinotefuran (IRAC class: 4A) is a member of neonicotinoids, acts as an agonist of the insect nicotinic acetylcholine receptors and thus disrupts the acetylcholine-mediated neurotransmission (C. Abstracts 2004). Dinotefuran has been widely used to control many agricultural and veterinary pests. Indoxacarb (IRAC class: 22A) is an oxadiazine pesticide, and blocks the ion conductance of the neuronal sodium channel and certain subtypes of nicotinic receptors (https://pubchem.ncbi.nlm.nih.gov/compound/1077-20). Indoxacarb is widely used for control lepidopteran larvae. Ivermectin (IRAC class: 6)

is a macrocyclic lactone derived from *Streptomyces avermitilis*, it binds and activates glutamate-gated chloride channels (GluCls) on neurons and pharyngeal muscle cells (https://pubchem.ncbi.nlm.nih.gov/compound/6321424). Oftentimes, ivermectin was used to treat ectoparasites, therefore, the resistance of ivermectin was reported in *Rhipicephalus microplus* and *Anoplura* species (Klafke et al. 2012; Amanzougaghene et al. 2018). Spinosad (IRAC class: 5) is a macrocyclic lactone which derived from *Saccharopolyspora spinose*. The spinosyns mainly target binding sites on nAChRs and therefore disrupt acetylcholine neurotransmission. Spinosyns also have secondary effects as a γ-aminobutyric acid (GABA) neurotransmitter agonist (Sparks et al. 2001). Spinosad is highly effective via both contact and ingestion in many insect species, it has been use to control various insect pests, including lepidoptera, diptera, thrysanoptera, coleopteran, orthopteran and many others.

Resistance to chlorantraniliprole has been reported in several lepidopteran insect species, such as *P. xylostella*, *Spodoptera exigua*, and *Chilo suppressalis*, etc (Wang et al. 2013; Lai et al. 2011; Xu et al. 2019). Several species have been reported to generate resistance to chlorfenapyr, include *Tetranychus urticae*, *Anopheles gambiae* and *P. xylostella,* etc (Nicastro et al. 2013; N'Guessan et al. 2007; Wang et al. 2019). Many

arthropod species have developed resistance to cypermethrin since it is used widely as an agricultural insecticide as well as a public health insecticide to control medical pests (Wang et al. 2018; Valles et al. 2000; Zhang et al. 2007). Resistance to dinotefuran has been reported in *Bemisia tabaci*, *Leptinotarsa decemlineata* and *Nilaparvata lugens*, etc (Meng et al. 2016; Mota-Sanchez et al. 2006; Sun et al. 2018). Resistance to indoxacarb has been mainly reported in lepidopteran species as well as in other insect orders, for example, *P. xylostella*, *S. litura*, *Helicoverpa assulta* Guenee and *Sitophilus zeamais* all developed resistance to indoxacarb (Nehare et al. 2010; Shi et al. 2019; Pang et al. 2012; Haddi et al. 2015). Oftentimes, ivermectin was used to treat ectoparasites, therefore, the resistance of ivermectin was reported in *Rhipicephalus microplus* and *Anoplura* species (Klafke et al. 2012; Amanzougaghene et al. 2018). Spinosad resistance has been widely reported in various arthropod species including *P. xylostella, F. occidentalis and Drosophila Suzukii* (Zhao et al. 2002; Herron et al. 2005; Gress et al. 2019).

Among the insecticides mentioned above, I used chlorantraniliprole, cypermethrin, indoxacarb and spinosad for sublethal treatment to *P. xylostella* as they are primarily used for *P. xylostella* control (Riley et al. 2017). In addition, dinotefuran was also included as one of common insecticides that are supposed to treat other two species (i.e., *F.*

occidentalis and *D. melanogaster*). Chlorfenapyr, dinotefuran and spinosad were selected for treating *F. occidentalis* as they are widely used for thrips control (Mouden et al. 2017). As for the treatment to *D. melanogaster*, the same set of insecticides (chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad) as used for *P. xylostella* were used for an easy comparison between the two species. In addition, ivermectin was included as a chloride channel agonist because it causes the hyperpolarization of neuronal cells unlike above mentioned insecticides casing depolarization and thus serving as a reference to compare the altered energy metabolism under the influence of insecticide. The overall information on test insects, insecticides and treatment methods listed in the Table 1. By treating dinotefuran and spinosad to all the three insect species, common responses to the same insecticides were investigated across different insect species. Transcriptomes of insecticide-treated samples were compared with untreated controls to identify the genes commonly responding to sublethal insecticide stress in each insect species. Characterization of these general differentially expressed genes (DEGs) may help us to determine the potential generalist defense factors associated with tolerance. Furthermore, comparing these commonly responded detoxification genes among three species of insects may improve our knowledge of the general insecticide defense mechanisms in these three insect species and the different

strategies used by different insect when they experience a similar xenobiotic stress.

Table 1. Overall information on test insects, insecticides and treatment methods

Target Insect Species	Insecticide	Insecticide Treatment Method
Plutella xylostella (Diamondback moth)	Chlorantraniliprole, Cypermethrin, Dinotefuran, Indoxacarb, Spinosad	Leaf dipping
Frankliniella occidentalis (Western flower thrips)	Chlorfenapyr, Dinotefuran, Spinosad	Residual contact vial plus water (RCVpW)
Drosophila melanogaster (Common fruit fly)	Chlorantraniliprole, Cypermethrin, Dinotefuran, Indoxacarb, Ivermectin Spinosad	Topical application

CHAPTER I Transcriptomic identification and characterization of genes responding to sublethal concentrations of five different insecticides in the diamondback moth, *Plutella xylostella*

ABSTRACT

When the 3rd instar larvae of the diamondback moth (DBM), *Plutella xylostella*, were pretreated with sublethal concentrations (LC_{10}) and then subsequently exposed to medium lethal concentrations (LC_{50}) of chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad via leaf dipping, their tolerance to insecticides was significantly enhanced. To identify genes that commonly respond to the treatment of different insecticides and are responsible for the tolerance enhancement, transcriptomic profiles of larvae treated with sublethal concentrations of the five insecticides were compared with that of untreated control. A total of 117,181 transcripts with a mean length of 662 bp were generated by de novo assembly, of which 35,329 transcripts were annotated. Among them, 125, 143, 182, 215 and 149 transcripts were determined to be up-regulated whereas 67, 45, 60, 60 and 38 genes were down-regulated following treatments with chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad, respectively. Gene ontology (GO) analysis of differentially expressed genes (DEGs) revealed little differences in their GO profiles between treatments with different insecticides except for

spinosad. Finally, the DEGs commonly responding to all insecticides were selected for further characterization, and some of their over-transcription levels were confirmed by quantitative PCR. The most notable examples of commonly responding over-transcribed genes were two cytochrome P450 genes (*Cyp301a1* and *Cyp9e2*) and nine cuticular protein genes. In contrast, several genes composing the mitochondrial energy generation system were significantly down-regulated in all treated larvae. Considering the distinct structure and mode of action of the five insecticides tested, the differentially expressed genes identified in this study appear to be involved in general chemical defense at the initial stage of intoxication. Their possible roles in the tolerance/resistance development were discussed.

Keywords: Diamondback moth; Insecticide; Sublethal concentration; Tolerance; Cuticle protein; Cytochrome P450

1. INTRODUCTION

Diamondback moth (DBM), *Plutella xylostella* (L.), which originated from the Mediterranean region, is one of the most devastating agricultural pests. It feeds on foliar tissue of cruciferous crops, including cabbage, broccoli, cauliflower, etc. The economic loss caused by *P. xylostella* is estimated about US $ 4–5 billion worldwide annually (Talekar et al. 1993; Tang et al. 2014). Many insecticides have been used to control *P. xylostella* in the past decades. As a result of the overuse of insecticides and its high adaptability, *P. xylostella* has developed resistance to most commercial insecticides, including organophosphates, carbamates and pyrethroids (Shelton et al. 1993). To overcome the resistance problem, various other groups of insecticides, including neonicotinoids, macrocyclic lactones, diamides, etc., have been introduced to control *P. xylostella* (Ninsin 2004; Jinfeng et al. 2008; Wang et al. 2013). Although some cases of *P. xylostella* resistance to these newly introduced insecticides have been reported, resistance is not yet widespread and underscores the critical need for proactive resistance management strategies for them. Since one of the major mechanisms for insecticide resistance is enhanced xenobiotic detoxification, including proteins that can metabolize or efflux xenobiotics (James et al.2009),

characterization of these detoxification factors would facilitate the understanding how insects develop metabolic resistance to insecticides.

Metabolic resistance to insecticides can be developed when pests acquire heritable traits that result in either constitutive/inducible over-expression or functional alteration of protein products involved in metabolism (Yoon et al. 2011). Since the expression of many detoxification gene is inducible by sublethal treatment of insecticides, analysis of transcript profiles, either in a small scale of using a subset of detoxification genes or in a full scale of using entire transcriptome, has been employed as a general method for identifying the major metabolic factors associated with insecticide resistance (Yoon et al. 2011; Vontas et al. 2005; Willoughby et al. 2006). In the transcriptional profiling of body louse detoxification genes [cytochrome P450 (Cyp450) and ABC transporters (ABCT)], three Cyp450 and one ABCT genes, which are also known to be involved in insecticide metabolism in other organisms, were significantly overexpressed upon induction by a brief, sublethal exposure to ivermectin that resulted in tolerance (Yoon et al. 2011). Heterologous expression of CYP6CJ or ABCC4 resulted in the oxidative metabolism or ATP-dependent efflux of ivermectin, respectively (Kim et al. 2018). In the case of *P. xylostella* larvae induced by sublethal doses of cypermethrin, eight of 11 Cyp450 genes tested were over-transcribed

in a cypermethrin-resistant strain whereas only a single Cyp450 gene was induced in a susceptible strain, suggesting that the selective Cyp450 induction by cypermethrin is also a metabolic resistance mechanism (Baek et al. 2010).

In this study, significant enhancements of tolerance to insecticides were verified in the *P. xylostella* larvae individually pretreated with sublethal doses of five different insecticides (chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad). To identify genes that commonly respond to different insecticides, thereby being putatively responsible for tolerance, the transcriptomes of the treated larvae were compared with that of untreated larvae, and a systematic analysis of metabolic factors that are induced by different groups of insecticides was carried out. In addition, by comparing deferentially expressed genes between insecticides, gene groups that commonly responded to different groups of insecticide were identified in order to determine generalist detoxification (defense) factors associated with tolerance. Identification of these insecticide-induced genes would contribute to our understanding on the xenobiotic detoxification factors in *P. xylostella* and their role in developing tolerance and perhaps eventually resistance.

2. MATERIAL AND METHODS

2.1 Diamondback moth stocks and rearing conditions

The insecticide-susceptible strain of *P. xylostella* was obtained from Rural Development Administration, South Korea. They were reared in plastic cages (40 cm × 35 cm × 30 cm) using Chinese cabbage plants (*Brassica napus*) at 24 °C, 60% humidity and a 16-h light:8-h dark cycle. This strain has not been exposed to any insecticide to more than ten years, however, since it was originally collected in the field, a few resistant traits could be still maintained in this strain.

2.2 Determination of insecticide sublethal concentrations and tolerance bioassay

Technical grade insecticides (> 93.9% purity) Insecticides were dissolved in acetone to obtain 100X test stock solutions. The insecticide stock solutions in acetone were then diluted 100-fold in water containing 0.5% Triton X-100 to their final test concentrations and used in the leaf dipping bioassays. Third instar larvae were used for determining the sublethal concentration (LC_{10}) values for each insecticide. In order to simulate the natural exposure route for insecticides and minimize any possible solvent stress, a leaf dipping bioassay was employed as the insecticide delivery method instead of topical application (Yoon et al.

2011). Cabbage leaf discs (d = 5 cm) were dipped into different concentrations of the test insecticide for 1 min. Control leaf discs were treated with distilled water containing only 1% acetone and 0.5% Triton X-100. After air drying for 1 h, the leaf discs were placed onto fitted filter paper in plastic petri dishes (d = 5.5 cm). A total of 10 3rd instar larvae (body weight: 0.6–0.7 mg/larva) were placed onto each leaf disc and each treatment replicated three times. The larvae were allowed to feed for 24 h at 24 °C and their mortality recorded at 3, 6, 10, 16, 21 and 24 h post-exposure. The LC_{10} concentrations were calculated by Probit analysis at 10 or 20 h (for dinotefuran) post-treatment (Polo Plus 2.0, LeOra Software, Northampton, UK).

To determine whether DBM larvae exposed to sublethal doses of insecticide can develop tolerance, the 3rd instar larvae were pretreated with sublethal concentrations (LC_{10}) of insecticides via leaf dipping as described above. For the control larvae, leaf discs were treated with distilled water containing only 1% acetone and 0.5% Triton X-100. After 24 h, the pretreated and control larvae were treated again with medium lethal concentrations (LC_{50}) of the same insecticides by leaf dipping, and mortalities were evaluated at 24 h post-treatment. The tolerance bioassay was conducted with three replicates, each with 15 larvae in the LC_{10} pretreatment and 10 larvae in the second LC_{50} treatment. Significant

differences between the mortality responses of the insecticide-pretreated and control larvae were determined by Student's *t*-test.

2.3 Insecticide treatment and total RNA extraction

Thirty 3rd instar larvae were treated with LC_{10} concentrations of each insecticide by the leaf dipping method as described above. After treating LC_{10} concentrations of chlorantraniliprole, cypermethrin, indoxacarb and spinosad, treated DBM larvae were collected for RNA extraction at 10 h post-treatment as these insecticides caused rapid intoxication. In the case of dinotefuran, which showed a relatively slower intoxication response, treated larvae were collected for RNA extraction at 20 h post-treatment. Both treated and control larvae were directly collected into a 1.5-ml tube at 10 or 20 h post-treatment. Whole bodies of 26–27 larvae (any dead larvae were removed) were homogenized in TRIzol (MRC, Cincinnati, OH, USA) reagent and total RNA was extracted according to the manufacturer's instruction.

2.4 Library construction and sequencing

RNase-free DNase I was added to the prepared total RNA for removing genomic DNA. Concentration and integrity of the RNA samples were measured by NanoDrop 8000 spectrophotometer (Thermo, Waltham, MA, USA) and Agilent 2100 bioanalyzer (Agilent

Technologies, Santa Clara, CA, USA), respectively. Only RNA samples that achieved an OD260/280 value of ≥ 1.8 and an integrity number ≥ 7.0 were selected for subsequent steps. The qualified total RNA (2.5 μg) samples were used for mRNA preparation and cDNA library construction by Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instruction. The amplified libraries yielded 400 ng of cDNA with an average fragment length of 350 bp. The final cDNA libraries were then paired-end sequenced (2 × 100 bp) using an Illumina Hiseq 2500 (Illumina, San Diego, CA, USA).

2.5 Sequence processing, de novo assembly and annotation

The obtained paired-end sequence files (Fastq) were subjected to Cutadapt with default parameters for adapter removal, quality trimming and length trimming (Martin 2011). For the process of quality trimming, the qualified reads with Phred quality > 30 were obtained and then the reads with length of < 100 bp were trimmed. Whole processed sequences were de novo assembled with Trinity assembler v.2.0.6 under default values that can recover more full-length transcripts compared with other de novo assemblers (Grabherr et al. 2011). The transcripts with > 200 bp were selected and then included in the reference transcriptome. The assembled unigenes were mapped by BLASTX (e-value cut-off $1e^{-5}$)

against the database of non-redundant (Nr) protein on NCBI and Swiss-Prot. The Gene Ontology (GO) terms annotated were assigned to each unigene that were annotated as homologs to GOseq (Young et al. 2012).

2.6 Differentially expressed gene (DEG) analysis

To overview the expression pattern of unigenes in all the samples, reads from individual samples were aligned to the reference transcriptome using bowtie2 with custom parameters (Langmead et al. 2012). Transcript abundances in reads per kilobase per million reads mapped (RPKM) were estimated using RSEM (RNA-Seq by Expectation Maximization) (Li et al. 2011) through the Trinity plug-in, run_RSEM.pl (Grabherr et al. 2011). In order to identify the differential expression patterns of transcripts, the TMM-normalized RPKM matrix was used for generating heat maps under R programming environment (Team 2015). Gene expression variations were analyzed for each insecticide relative to control. Genes showing > 4 fold changes (FC) ($Log_2FC > 2$) in RPKM with statistical significance ($p < 0.05$, $q < 0.05$) were considered to be affected by insecticide treatment. Among these, genes commonly over- or under-transcribed in all insecticide-treated larvae were selected for further characterization. If no detectable expression was observed in the control group, a pseudocount of 0.001 was used for the calculation of Log_2FC to avoid indefinite FC values.

The correlation coefficient was determined by Pearson's correlation analysis using SigmaPlot (Version 12.0, San Jose, CA, USA)

2.7 Quantitative real-time PCR (qPCR)

A total of eight genes with the criteria of > 4 × FC, $p < 0.05$ and $q < 0.05$ were selected for the qPCR-based confirmation of transcript amount. Based on the cDNA sequences of selected genes, sequence-specific primer sets were designed (Table 1-1). The qPCR reaction contained 25 ng cDNA, 5 pmol of each primer and 10 µl of SYBR I 2 × master mix (Takara, Shiga, Japan) in a total volume of 20 µl. Reactions were incubated at 95 °C for 30 s, followed by 40 cycles of thermal program (5 s at 95 °C, 15 s at 54 °C, and 15 s at 72 °C). A ribosomal protein gene (RPS13) was used as an internal reference gene. qPCR for each gene was conducted with three biological replicates, each replicate for five different insecticides. Relative transcript amounts of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). The transcript levels of the selected eight genes determined by qPCR were plotted against the corresponding Log_2FC values, and the linear regression was conducted using SigmaPlot (Version 12.0) to determine the degree of correlation between them.

Table 1-1. Sequence of primers used in qRT-PCR

Gene name	Primer name	Sequence	PCR amplicon (bp)
Ribosomal protein S13	S13F	GGTGTGATGCTCCGTGACTC	165
	S13R	GTTGCGTTCCAAGTGCTTCC	
Chorion peroxidase	PXT-F	CGTCCTAGACGAATACGTGA	137
	PXT-R	TGTTGAACGATACGAGGTCC	
Clavesin-1	Clvs1-F	TGTCGGACTTCGACTACAGT	136
	Clvs1-R	AGACTGCACCCTTTCATGTC	
Cuticle protein 19	LM19-F	ATGCCCACCAAGAATTCGTG	135
	LM19-R	ATTGACCTTTGACGACCTCT	
Glucose dehydrogenase	Gld-F	AAGAGCAAGCTGGACTGGAA	129
	Gld-R	GAGCATGGTGTTGAGTACTG	
Larval cuticle protein A3A	LCP-A3A-F	AGTACTTGGGACAACAGCAC	122
	LCP-A3A-R	GTACTGCTGCTGCTCTTGTT	
Nose resistant to fluoxetine protein 6	nrf6-F	GGAGGAATGTGCTTCAAGAG	134
	nrf6-R	TGGAAATCGGTCACGCAGAT	
Probable cytochrome P450 301a1	Cyp303A1-F	CGACTTCATCAGCTACATGG	141
	Cyp303A1-R	TCTGAAGACAGGCATCCTAG	
Probable cytochrome P450 9E2	Cyp9E2-F	GAGGAATGTGCTTCAAGAGG	133
	Cyp9E2-R	TGGAAATCGGTCACGCAGAT	

3. RESULTS

3.1 Determination of insecticide sublethal concentrations

The LC_{10} values at 24 h post-treatment were determined to be 0.32, 0.40, 16.1, 0.37 and 0.04 ppm for chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad, respectively (Table 1-2). All *p* values were > 0.05 for the chi-square, suggesting that the bioassay data well fit the probit model. After treating the sublethal concentrations of chlorantraniliprole, cypermethrin, indoxacarb and spinosad, treated DBM larvae were collected for RNA extraction at 10 h post-treatment as these insecticides caused rapid intoxication. In the case of dinotefuran, which showed a relatively slower intoxication response, treated larvae were collected for RNA extraction at 20 h post-treatment.

Table 1-2. LC_{10} and LC_{50} concentration determination of five different insecticides

Insecticide	**LC_{10}**		**LC_{50}**			
	Concentration (ppm)	**95% C.L (ppm)**	**Concentration (ppm)**	**95% C.L (ppm)**	**Slope**	**Chi-square**
Chlorantraniliprole	0.32	0.07-0.74	4.41	2.61-8.27	1.15	0.11
Cypermethrin	0.4	0.02-1.07	3.89	1.55-6.31	1.24	1.12
Dinotefuran	16.1	9.11-21.54	48.47	38.58-63.63	2.63	0.93
Indoxacarb	0.37	0.11-0.69	31.37	17.23-83.91	0.76	1.01
Spinosad	0.04	0.01-0.09	0.46	0.26-0.87	1.20	6.12

3.2 Determination of tolerance following sublethal pretreatment of insecticides

When DBM larvae were pretreated with sublethal concentrations (LC_{10}) of insecticides and then subsequently treated with medium lethal concentrations (LC_{50}), all pretreated larvae showed significantly reduced mortalities ($p < 0.05$) compared to un-pretreated controls except for the indoxacarb treatment ($p = 0.065$) (Fig. 1-1). The percent reductions in mortality responses were 30.4, 42.8, 54.5, 27.8 and 60.1% following the pretreatments with chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad, respectively.

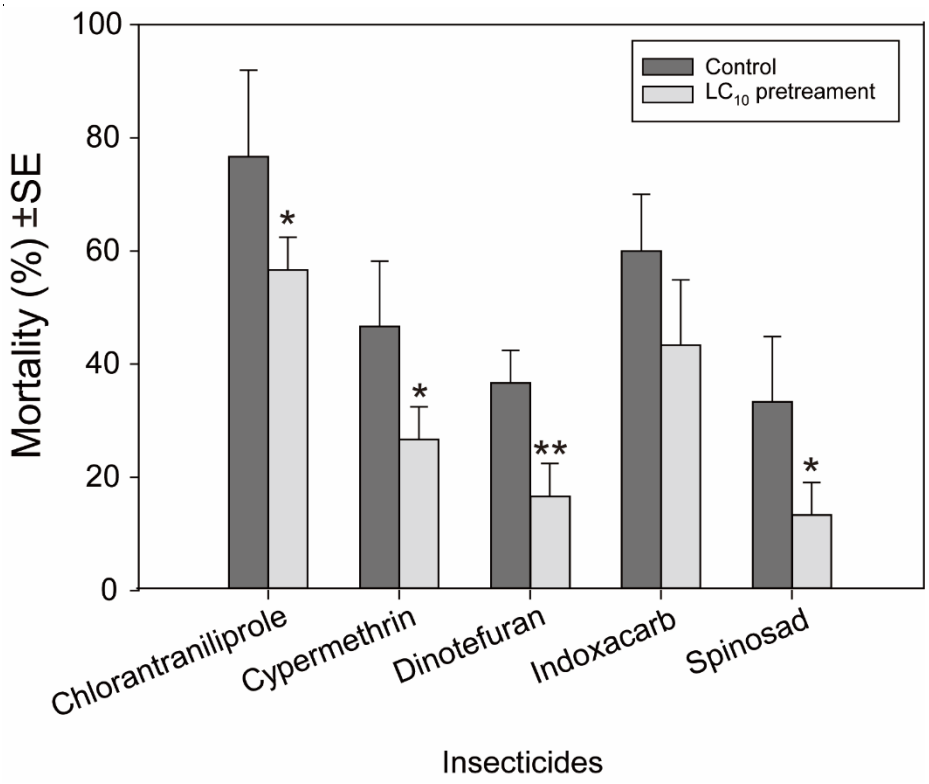

Fig. 1-1. Tolerance induction by pretreatment of sublethal concentrations (LC_{10}) of insecticides. The star marks indicate statistical significance between pretreatment vs. control (*, $p < 0.05$; **, $p < 0.01$).

3.3 De novo assembly of transcriptome data

Approximately, 70, 74, 92, 76.5, 75.3 and 80.7 million clear reads were generated from the control, chlorantraniliprole-, cypermethrin-, dinotefuran-, indoxacarb- and spinosad-treated DBM larvae, respectively. While de novo assembly generated a total of 117,181 unigenes with an average size of 662 bp, the reference-based assembly produced 18,073 unigenes, which only accounts for 15.4% of total unigenes obtained from the de novo assembly. Thus, subsequent analyses were conducted with the de novo-assembled unigene set. It should be noted that the number of unigenes estimated in this study does not necessarily indicate the actual number of genes but as multiple non-overlapping fragments of the same gene were likely counted as different unigenes in the de novo assembly, it is likely an overestimation. Among these unigenes, 41.6% were between 200 and 400 bp, 31.0% were between 400 and 600 bp, 9.6% were between 600 and 1000 bp and 17.8% had nucleotide lengths above 1000 bp (Fig. 1-2). The Blast results showed that 27,944 (23.8%) genes had homologies with other known genes whereas 89,237 (76.2%) genes have no homologies. Among the 27,944 unigenes, 15,944 genes (57.1%) have homologies with invertebrates (Fig. 1-3). A total of 63,202, 70,640, 70,641, 70,668, 71,183 and 70,382 genes had values of > 1.0 RPKM in the control,

chlorantraniliprole-, cypermethrin-, dinotefuran-, indoxacarb- and spinosad-treated samples, respectively. Among those genes, 26,537 (42.0%), 28,216 (39.9%), 28,029 (39.7%), 28,147 (39.8%), 28,085 (39.4%) and 28,552 (40.6%) genes were annotated to be known genes whereas the rest were unknown. A further GO classification showed 15,559 unigenes were annotated to GO terms, which consisted of three major categories: molecular function (F), cellular component (C) and biological process (P). DEG analysis revealed that a total of 705 unigenes exhibited differential expression patterns in the insecticide-treated larvae compared to the control. Among these DEGs, 125, 143, 182, 215 and 149 transcripts were determined to be up-regulated whereas 67, 45, 60, 60 and 38 transcripts were down-regulated following the treatment of chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad, respectively. GO analysis of the DEGs showed that genes affected by insecticide treatment were mainly found in the binding and catalytic activity in the molecular function; the cell, cell part and organelle in the cellular component and the cellular process, metabolic process and single organism process in the biological process (Fig. 1-4). When GO profiles were compared among different insecticide treatments, no apparent differences were noticed except for spinosad treatment (Fig. 1-4). Compared to other insecticide-treated groups, the spinosad treatment showed relatively higher proportions of genes in the categories of the

structural molecule activity and cellular process but lower proportions in the developmental process and multicellular organismal process.

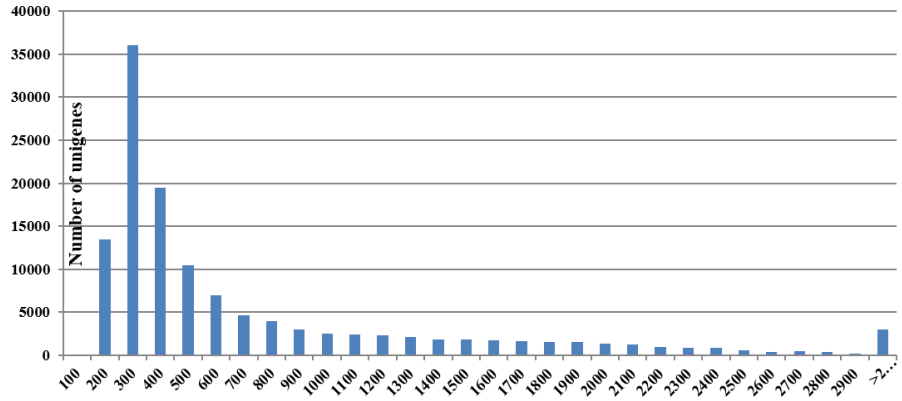

Fig. 1-2. Length distribution of unigenes generated from *de novo* assembly.

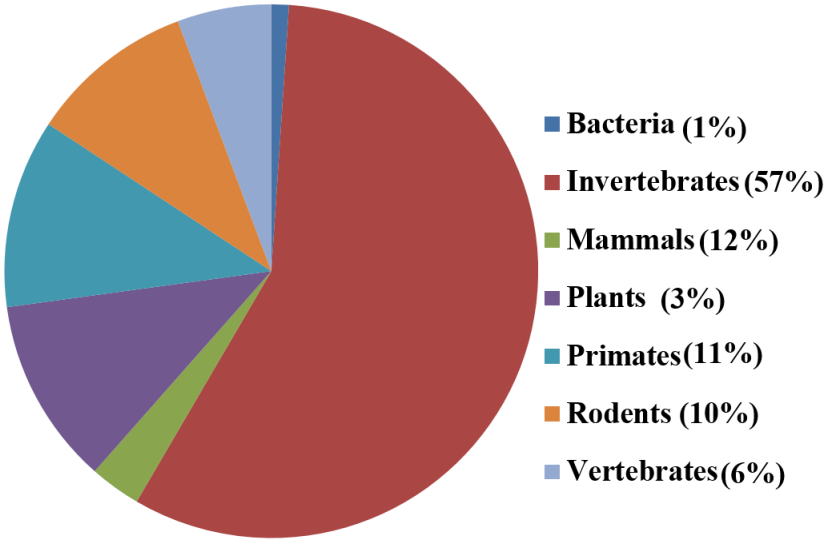

Fig. 1-3. Blast annotation of assembled unigenes which have homologies with other known genes.

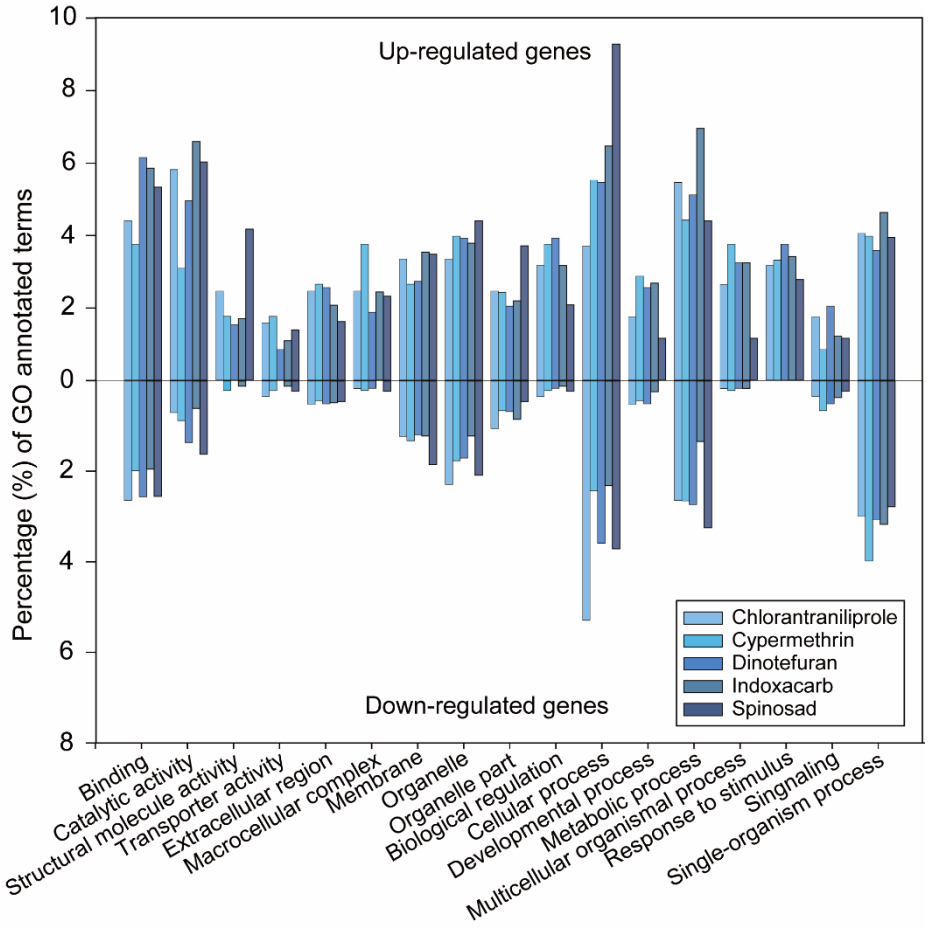

Fig. 1-4. Gene ontology of differentially expressed genes following the sublethal doses of insecticides.

3.4 DEGs following insecticide treatment

In the comparison between control and insecticide-treated larvae, a total of 32 genes were commonly over-transcribed in insecticide-treated larvae ($Log_2FC > 2$, $p < 0.05$, $q < 0.05$) (Table 1-3). The integrin alpha-PS1 gene showed the highest transcription levels (Log_2FC 14.7–16.9) in all insecticide treated groups, followed by the RNA-directed DNA polymerase from mobile element jockey and serine/threonine-protein kinase PRP4 homolog. However, the roles of these top three genes with high transcription levels in the induction of tolerance/resistance are unclear yet.

Table 1-3. Commonly over-transcribed genes following the treatment of sublethal concentrations of five different insecticides (p<0.05, q<0.05). The genes were arranged based on the order of average Log_2FC (fold change) values. The Cyp450 and cuticular protein genes were marked with light blue and pink colors, respectively. Genes that are tentatively associated with cuticle modification were marked with orange color

Gene ID	Gene name	Log_2FC relative to control/FPKM Value				
		Chlorantraniliprole	Cypermethrin	Dinotefuran	Indoxacarb	Spinosad
TBIU018746	Integrin alpha-PS1	15.2/384.5	15.9/737.8	16.9/1384.5	16.1/761.9	14.7/271.3
TBIU036569	RNA-directed DNA polymerase from mobile element jockey	14.9/307.9	15.0/390.8	13.6/140.0	14.4/225.6	13.8/149.9
TBIU038002	Serine/threonine-protein kinase PRP4 homolog	13.3/106.7	13.7/158.5	13.5/124.9	14.3/209.8	12.5/61.8
TBIU034838	Cuticular protein glycine-rich 24	7.76/12140	8.41/22097	8.91/28793	6.96/7164.7	7.48/10000
TBIG021872	Larval cuticle protein A3A	5.29/21.8	7.20/82.3	7.89/140.2	5.90/33.6	7.25/81.2
TBIU034804	Larval cuticle protein A1A	5.02/405.8	7.15/1870.4	7.92/2966.5	5.78/645.9	7.35/1860.7
TBIU008815	cuticular protein tweedle motif 3	5.61/6464.8	6.14/10806	6.38/11826	5.07/4588.2	5.52/6082.0
TBIU004200	cuticular protein hypothetical 20	6.03/407.9	6.72/758.4	6.11/460.4	4.27/123.8	5.33/249.9
TBIU016543	Cuticle protein 19	5.23/233.2	5.85/416.1	6.27/513.1	4.07/107.5	5.46/274.4
TBIU034868	C-type lectin 4	4.02/202.5	6.00/923.0	6.84/1527.9	5.47/568.7	4.53/288.1
TBIU005687	cuticular protein glycine-rich 14	4.69/7460.4	5.39/14066	5.65/15546	4.04/4892.4	4.78/7976.3
TBIU008814	cuticular protein tweedle motif 3	4.84/5251.6	5.29/8290.0	5.52/8985.5	4.31/3741.3	4.55/4295.0
TBIG024755	Cytochrome P450 9e2	3.63/2.25	5.35/7.45	6.20/14.2	4.27/3.52	4.71/4.52
TBIU009029	Alpha-tocopherol transfer protein-like	3.95/160.1	5.05/396.9	6.53/1023.7	3.90/159.8	4.28/202.2
TBIG014478	Acyl-CoA Delta(11) desaturase	3.21/39.1	5.13/148.1	5.87/261.2	3.81/59.8	3.98/64.0
TBIU027444	cuticular protein tweedle motif 2	4.23/3027.9	4.64/4652.0	5.03/5638.2	3.67/2113.1	4.36/3305.6
TBIU010954	Probable RNA-directed DNA polymerase from transposon X-element	4.10/124.7	4.58/201.5	5.22/290.5	3.89/111.2	3.25/69.4
TBIG000809	Nose resistant to fluoxetine protein 6	3.18/103.5	3.05/95.0	2.96/93.6	2.28/55.7	2.75/73.5
TBIG000319	Glucose dehydrogenase [FAD, quinone	1.35/3.67	3..21/13.4	3.80/21.2	2.01/5.86	2.28/6.72
TBIU043723	Peroxidasin homolog	3.34/600.8	4.70/1776.9	4.70/1655.5	3.80/850.7	3.34/599.7

TBIU013344	Protein Skeletor, isoforms B/C	4.29/546.8	4.21/598.3	4.68/770.9	3.38/300.8	3.31/278.4
TBIG018750	Clavesin-1	3.21/11.4	4.23/23.2	4.99/41.3	3.68/15.8	3.66/14.8
TBIG007646	Protein Skeletor, isoforms D/E	4.18/31.6	4.17/31.7	4.78/50.8	3.25/16.8	3.24/15.8
TBIG009971	Chorion peroxidase	3.21/27.8	4.56/71.1	4.67/81.1	3.72/39.9	3.11/24.8
TBIG011102	Serine proteinase stubble	4.13/51.7	4.02/48.1	4.07/52.4	2.90/22.1	3.58/33.6
TBIG012643	Probable nuclear hormone receptor HR3	1.30/5.97	5.70/126.5	6.26/196.1	3.71/32.0	1.59/6.98
TBIG008364	Circadian clock-controlled protein	3.67/57.4	3.49/50.8	3.38/49.7	2.66/28.6	2.43/23.2
TBIG004204	Laccase-4	2.84/37.4	3.39/55.1	3.66/70.2	2.65/33.2	2.53/29.0
TBIG008225	Protein yellow	2.74/10.9	2.83/11.7	4.00/27.8	2.03/6.71	3.02/12.7
TBIG009954	Trypsin-7	2.09/24.4	3.81/80.4	3.44/65.6	2.83/41.0	1.61/16.7
TBIG016852	Endocuticle structural glycoprotein SgAbd-2	3.15/274.8	2.78/214.1	2.83/234.3	1.92/118.0	2.89/219.5
TBIG017534	Cytochrome P450 301a1,	2.02/6.56	3.27/15.6	3.03/13.9	2.27/7.83	2.56/9.11
TBIG020903	Elongation of very long chain fatty acids protein 7	3.07/146.9	2.94/135.0	2.61/113.2	2.19/80.2	1.90/62.5
TBIG013156	Probable chitinase 3	2.02/93.4	2.92/174.4	2.78/166.8	1.72/76.5	2.47/121.8

Two Cyp450 genes (*Cyp9e2* and *Cyp301a1*) were significantly over-expressed in all treated larvae (Table 1-3, marked with light blue color). The Log_2FC values of *Cyp9e2* were 3.6, 5.4, 6.2, 4.3 and 4.7 in chlorantraniliprole-, cypermethrin-, dinotefuran-, indoxacarb- and spinosad-treated larvae, respectively. As for *Cyp301a1*, the Log_2FC values were 2.2, 4.0, 3.3, 3.0 and 3.6, respectively. Interestingly, a total of nine cuticle-related genes were up-regulated, including several cuticular protein glycine-rich genes, larval cuticle protein genes, cuticle protein 19, etc. (Table 1-3, marked with pink color). Three genes that are indirectly related with cuticle formation were also commonly over-transcribed in insecticide-treated groups were the glucose dehydrogenase, laccase-4 and chitinase 3 (Table 1-3, marked with orange color).

Table 1-4. Commonly under-transcribed genes following the treatment of sublethal concentrations of five different insecticides (P<0.05, q<0.05). The genes were arranged based on the order of average Log_2FC (fold change) values. Genes related with mitochondrial energy generation were marked with grey color and the Ryr-44F gene was marked with light blue color

Gene ID	Gene name	Log_2FC relative to control/FPKM Value				
		Chlorantraniliprole	Cypermethrin	Dinotefuran	Indoxacarb	Spinosad
TBIU113851	Putative humanin peptide	-17.7/0	-6.83/21.4	-7.57/11.8	-7.85/9.40	-17.7/0
TBIU050360	Protein NLRC3	-14.0/0	-6.81/1.71	-5.30/4.55	-6.51/1.88	-14.0/0
TBIU002125	NADH-ubiquinone oxidoreductase chain 5	-13.8/0	-4.78/6.00	-4.39/7.27	-5.29/3.76	-13.8/0
TBIU002972	NADH-ubiquinone oxidoreductase chain 4	-14.4/0	-3.38/24.0	-4.00/14.5	-4.30/11.3	-14.4/0
TBIU001137	Cytochrome b	-13.3/0	-3.80/8.57	-3.93/7.27	-4.24/5.64	-13.3/0
TBIU028516	Cytochrome c oxidase subunit 3	-14.4/0	-3.15/28.3	-3.19/25.5	-3.50/19.7	-6.82/1.93
TBIG015384	Malonate-semialdehyde dehydrogenase [acylating]	-2.37/582.8	-1.98/766.6	-3.59/264.7	-2.72/460.1	-2.97/368.2
TBIG024626	Ryanodine receptor 44F	-3.05/1075.3	-2.48/1599.5	-2.86/1298.1	-2.46/1629.4	-2.51/1501.0
TBIG004656	Cytochrome c oxidase subunit 6B	-2.56/8792.7	-2.00/13027	-2.65/8736.6	-2.34/10313	-2.28/10187

Besides these genes, some immune-related genes were also found to be over-transcribed, such as serine protease stubble, integrin alpha-PS1 (mew), C-type lectin gene, alpha-tocopherol transfer protein-like etc. Probable nuclear hormone receptor 3 (HR3), even though its ligand still remained unknown, was also found to be over-transcribed in all treated groups. A gene encoding uncharacterized protein LOC101736626 showed Log_2FC values of 6.9, 9.1, 9.1, 7.7 and 8.2 of over-transcription in the chlorantraniliprole-, cypermethrin-, dinotefuran-, indoxacarb- and spinosad-treated larvae, respectively. A variety of genes, which were also commonly up-regulated in all insecticide-treated groups but their potential roles in the acquisition of tolerance/resistance are unclear yet, include the acyl-CoA Delta (11) desaturase, peroxidasin homolog, protein skeletor, clavesin, chorion peroxidase, nose resistant to fluoxetine protein 6 (nrf6), etc.

While some genes were over-transcribed, a total of nine genes showed commonly decreased transcription levels in insecticide-treated larvae compared with control larvae ($Log_2FC < -2$, $p < 0.05$, $q < 0.05$) (Table 1-4). The most down-regulated gene was the putative humanin peptide gene (Log_2FC − 6.83 ~ − 17.7), and followed by the protein NLRC3 gene (Log_2FC − 5.30 ~ − 14.0). Except for a ryanodine receptor gene (Log_2FC − 2,46 ~ − 3.05), all other remaining genes that were

commonly down-regulated belonged to the gene groups composing the mitochondrial respiratory system, such as NADH-ubiquinone oxidoreductase chain 4/5, cytochrome b, cytochrome c oxidase subunit 3/6B and malonate-semialdehyde dehydrogenase (Table 1-4, marked with grey color).

3.5 Confirmation of DEG profiles by qPCR

To confirm the reliability of the DEG data, qPCR experiments were conducted. Cyp450s play vital roles in the detoxification of xenobiotics and cuticle proteins are key components of insect cuticle, which can be structurally altered when exposed to insecticides, thereby being closely involved in the induction of tolerance/resistance to insecticides. Because of this, two Cyp450 genes (*Cyp301a1*and *Cyp9e2*) and two cuticular protein genes (*TM-A3A* and *LM19*) were selected for validation along with other four genes (clavesin-1, chorion peroxidase, glucose dehydrogenase and *nrf6*) with $Log_2FC > 2$. The fold changes obtained from qPCR were plotted against the Log_2FC values estimated from transcriptome data (Fig. 1-5). The resulting correlation coefficient was 0.637, suggesting that DEG profiles obtained from the transcriptome data are moderately reliable

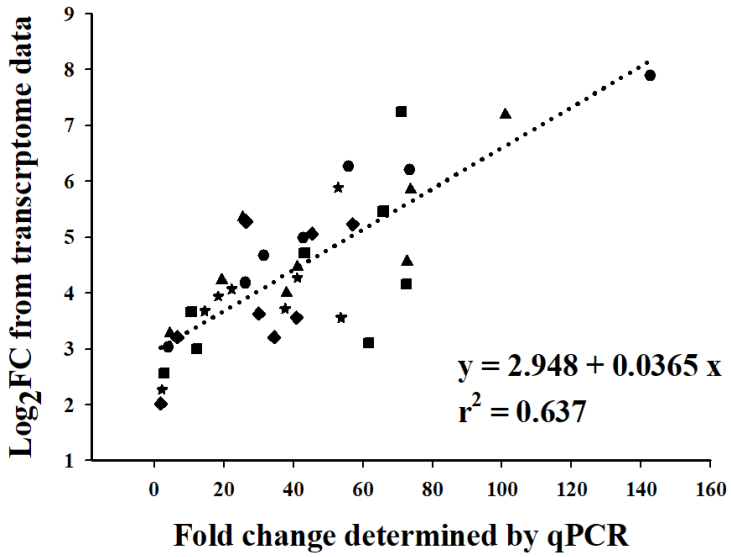

Fig. 1-5. The plot of the fold changes (FC) obtained from qPCR vs. the Log_2FC values estimated from transcriptome data. The dotted line indicates the linear regression line. Diamond indicates chlorantraniliprole treatment, triangle indicates cypermethrin treatment, circle indicates dinotefuran treatment, star indicates indoxacarb treatment, square indicates spinosad treatment.

4. DISCUSSION

4.1 Tolerance induction

Pretreatment of sublethal doses of insecticides induced the tolerance enhancement in *P. xylostella* larvae although its level by indoxacarb pretreatment was not significant ($p = 0.065$) (Fig. 1-1). A similar case of tolerance induction was also previously reported in body lice that were briefly exposed to a sublethal dose of ivermectin (Yoon et al. 2011). In current study, the tolerance enhancement was observed in all examined cases regardless of insecticide type, suggesting that the tolerance induction by sublethal treatment of insecticides is a rather general phenomenon. Since the induced overexpression of detoxification proteins, such as Cyp450s and ABCT, were determined to be responsible for tolerance in body lice (Yoon et al. 2011), differentially expressed genes upon the sublethal exposure to insecticides likely mediate the tolerance induction in *P. xylostella* as well.

4.2 GO profiles of DEGs

The overall GO profiles of the DEGs indicated that the genes classified in the categories of the binding (F), catalytic activity (F), transporter activity (F), metabolic process (P) and response to stimulus

(P) were all up-regulated (Fig. 1-4). As these GO categories may be crucial in the xenobiotics detoxification process, their up-regulation in the insecticide-treated larvae is consistent with the enhancement of tolerance. In the case of spinosad treatment, relatively higher proportions of DEG were found in the categories of structural molecule activity (F) and cellular process (C) but the physiological meaning of this finding is unclear at this point.

4.3 Commonly over-transcribed genes following the treatment of sublethal doses of insecticides

Two Cyp450 genes (*Cyp9e2* and *Cyp301a1*) were commonly over-expressed in all treated larvae. In particular, the *Cyp9e2* transcription increased significantly with Log_2FC values of 3.6–6.2. A similar case of induction was also reported in *Tribolium castaneum*, where the *Cyp9e2*, along with *Cyp6a14*, was overexpressed after exposure to sublethal doses of phosphine (Oppert et al. 2015). Likewise, the same *Cyp9e2* (*AcCyp9e2*) was also overexpressed in the forager compared to other worker groups and in the flumethrin-treated groups compared to untreated group in the Asian honey bee *Apis cerana cerana*, suggesting that AcCYP9E2 is likely involved in the xenobiotic metabolism and detoxification (Wujun 2016). Considering that the structural variety of insecticides examined [i.e., diamide (chlorantraniliprole), pyrethroid

(cypermethrin), neonicotinoid (dinotefuran), oxadiazine (indoxacarb) and macrocyclic lactone (spinosad)], the common responsiveness of *Cyp9e2* to all different groups of insecticides suggests that CYP9E2 is likely a generalist Cyp450 with a broad substrate specificity that can respond to various insecticides and xenobiotics.

CYP301A1 is known to be present in all insect genomes sequenced to date and be involved in the adult cuticle formation and perhaps ecdysone regulation in the cuticle in *Drosophila melanogaster* (Sztal et al. 2012). Along with CYP4G1, which is involved in water preservation via the cuticular hydrocarbon biosynthesis in terrestrial insects (Qiu et al. 2012) and is also a penetration factor in DDT resistance of fruit flies (Gellatly et al. 2015), CYP301A1 is expressed in the epidermis of cuticle, thus likely playing a crucial role in the regulation of cuticle permeability to ranges of molecules, including water and xenobiotics. Therefore, it is intriguing to speculate that overexpression of the cuticle-specific CYP301A1 upon the exposure to various insecticides may alleviate the water loss caused by intoxication process, thus increasing desiccation tolerance, or may reduce the penetration of treated insecticides, thereby increasing the tolerance of treated-larvae.

Some Cyp450 genes, particularly *Cyp6a20*, were also over-transcribed in most groups of treated DBM larvae though not in all

treatments. CYP6 groups are well known monooxygenases that oxidize a variety of insecticides and xenobiotics as demonstrated in many insect species, thus giving rise to tolerance/resistance. For examples, overexpression of CYP6BQ9 led to deltamethrin resistance in *T. castaneum* whereas overexpression of CYP6G1 induced resistance to DDT and imidacloprid in *D. melanogaster* (Zhu et al. 2010; Daborn et al. 2001). In addition, *Cyp6a14* and *Cyp303a1* were found to be over-transcribed in *Acyrthosiphon gossypii* when the plant host generated more secondary toxic metabolites under salinity stress (Wang et al. 2015). Taken together, overexpression of *Cyp6a20* likely indicates the induced detoxification mechanism of *P. xylostella* when exposed to sublethal doses of insecticides.

Following the treatment of sublethal doses of insecticides, many cuticular protein genes were commonly over-transcribed, including larval cuticle protein A1A/A3A, cuticle protein 19, cuticle protein glycine-rich 14/24, endocuticle structural glycoprotein, etc., which accounted for approximately 1/3 of the genes significantly over-transcribed. As discussed in earlier section, cuticle is the first barrier of insects against xenobiotics penetration and for water preservation. There are large bodies of information regarding the roles of cuticular protein in insecticide resistance and tolerance. A cuticle protein, CpCPLCG5, is a

critical factor in mosquito cuticle formation and involved in insecticide resistance (Fang et al. 2015). In a pyrethroid-resistant strain of *Cimex lectularius*, cuticle is significantly thicker than that of susceptible strain (Lilly et al. 2016). Cuticle proteins were also reported to contribute to insecticide resistance in *Anopheles gambiae*, in which CPLCG3/4 was suggested to increase the cuticle thickness, one component of insecticide penetration factors whereas CPF3 to increase desiccation resistance (Vannini et al. 2014). Although the physiological functions of the various cuticular proteins identified in this study remain to be elucidated, their significant and common overexpression upon the exposure to insecticides may indicate their role in tolerance/resistance to insecticide penetration and/or desiccation. With this in mind, the common overexpression of the glucose dehydrogenase that is essential for cuticular modification and a chitinase that digests or reshapes chitin in the cuticle appears to be accompanied by the over-transcription of the cuticular protein genes. In addition, a laccase gene (laccase-4) was also commonly up-regulated in all treatment groups. Laccase, a copper-containing oxidase, is known to be involved in a variety of physiological processes (Kunamneni et al. 2008). In insects, laccases, along with phenoloxydases, are implicated in cuticle sclerotization or tanning, which is essential to survival (Dittmer et al. 2010). Therefore, up-regulation of laccase also appears to contribute to cuticle structure

regulation. Taken together, modification of cuticular proteins appears to be a general defense mechanism against xenobiotics in DBM as well and to serve as a resistance factor when fixed in the population.

Besides the Cyp450 and cuticular protein genes, several genes that may contribute to the insecticide tolerance/resistance directly or indirectly were up-regulated. The Nose resistant to fluoxetine protein 6 (NRF6) was overexpressed in all treated groups (Log_2FC 3.6–5.0). It is known that NRF6 plays a role in the uptake and transport of a variety of molecules, including xenobiotic compounds and its expression is upregulated when aphids encounter xenobiotic stress (Bansal et al. 2014). Therefore, NRF6 is likely a potential generalist defense molecule when DBM is challenged with insecticides.

The probable nuclear hormone receptor 3 (HR3) gene was also commonly over-transcribed following insecticide treatments (Log_2FC of 1.3–6.3). Nuclear hormone receptors can work as transcription activators to initiate the expression of specific metabolic enzymes, such as Cyp450s, thereby boosting detoxification reactions (Tabb et al. 2006). Thus, up-regulation of specific nuclear hormone receptors such as HR3 may contribute to increase tolerance/resistance via enhancing detoxification in *P. xylostella*.

It is interesting that two RNA-directed DNA polymerase genes with retrotransposon origin were commonly over-transcribed following insecticide treatment (Table 1-3), but their implication in tolerance/resistance induction remains to be elucidated.

4.4 Commonly under-transcribed genes following the treatment of sublethal doses of insecticides

In contrast to the over-transcribed genes, some genes commonly exhibited reduced transcription levels following insecticide treatment. Most notably, an apparent down-regulation of mitochondrial energy generation system was observed in all insecticide-treated larvae (Table 1-4). It can be speculated that, when exposed to sublethal doses of insecticides, reduced energy generation may be beneficial to the intoxicated *P. xylostella* larvae in terms of increasing survival rate or acquiring tolerance; however, further investigation is required to elucidate the relations between the energy generation, intoxication, tolerance development and eventual survival of insects.

The ryanodine receptor 44F (Rya-r44F) gene also showed common down-regulation pattern in all treated insects (Table 1-4). As ryanodine receptor is an endoplasmic reticulum calcium channel that controls the excitation-contraction of insect muscle, it can be speculated that down-

regulation of Rya-r44F likely reduces muscle excitability, thereby counteracting against intoxication process by insecticides. Considering that all insecticides examined in this study exert depolarizing effects on excitable cells, reduced excitability via the down-regulation of Rya-r44F would contribute to the alleviation of intoxication. With this in mind, it is intriguing that the highest level of Rya-r44F down-regulation was observed in the treatment of chlorantraniliprole, of which target molecule is the ryanodine receptor (Cordova et al. 2006).

Putative humanin peptide was most down-regulated in all treated insects. As the mitochondrial humanin peptides are known to confer stress resistance in animals (Yen et al. 2013), its down-regulation may increase the susceptibility to stress, including intoxication. Thus, further investigation is needed to explain this seemingly contradictory phenomenon of down-regulation of humanin peptide in the insecticide-treated DBM.

NLRC3 belongs to a member of the nucleotide binding domain and leucine-rich repeat-containing protein (NLR) family of proteins and is known to function as a negative regulator of innate immune signaling (Zhang et al. 2014). Thus, the down-regulation of NLRC3 likely activates immune responses of the insecticide-treated *P. xylostella*, which would be beneficial to its survival.

5. CONCLUSIONS

Comparative transcriptome analysis followed by separate treatment of five insecticides enabled the identification of commonly responding genes to the sublethal challenge, thereby being involved in tolerance enhancement. The notable examples of over-transcribed genes include two Cyp450 genes and nine cuticular protein genes. Interestingly, many genes involved in the mitochondrial energy generation were down-regulated in all treated groups. Considering the physiological functions of these genes, their up- or down-regulation appears to be involved in direct or indirect detoxification processes. Furthermore, their common responsiveness to five different insecticides, regardless of their distinct structure and mode of action, suggests their roles as generalist defense molecules. The insecticide-induced detoxification factors likely confer tolerance to the exposed insects and eventually allow resistance development once they are fixed in the population although functional verification remains to be conducted. Therefore, systematic identification of such differentially expressed genes following exposure to different groups of insecticides could serve as a landmark for searching universal or specific metabolic factors, which would in turn enable the proactive resistance management of newly introduced insecticides prior to appearance of resistance.

CHAPTER II Transcriptomic identification and characterization of genes responding to sublethal concentrations of three different insecticides in the western flower thrips, *Frankliniella occidentalis*

ABSTRACT

Pretreatment with sublethal concentrations (LC_{10}) of three insecticides (chlorfenapyr, dinotefuran, and spinosad) enhanced tolerance to a lethal dose of the respective insecticide in the Western flower thrips, *Frankliniella occidentalis*. To identify genes responding to sublethal treatment with insecticides, transcriptome analysis was conducted for thrips treated with LC_{10} of the three insecticides. When based on a fold change >1.5 or < −1.5 as a selection criterion, 199 transcripts were commonly up-regulated, whereas 31 transcripts were commonly down-regulated following all three insecticide treatments. The differential expression levels of representative genes were validated by quantitative PCR. Most over-transcribed transcripts could be categorized as basic biological processes, such as proteolysis and lipid metabolism. Detoxification genes, such as one glutathione S transferase S1, three UDP-glucuronosyltransferases, four CYP450s, and one ABC transporter G family member 20, were commonly overexpressed in all three insecticide-treated groups. Knockdown of the five representative

commonly overexpressed genes via ingestion RNA interference increased mortalities to all the three test insecticides, supporting their common role in tolerance induction. In contrast, three C2H2-type zinc finger-containing proteins were significantly down-regulated in all insecticide-treated thrips groups. Since the tested insecticides have distinct structures and modes of action, the roles of commonly expressed genes in tolerance were discussed.

Keywords: Western flower thrips; Insecticide; Sublethal concentration; Tolerance; Transcriptome analysis; Cytochrome P450s

1. INTRODUCTION

The western flower thrips, *Frankliniella occidentalis* Pergande, is a serious polyphagous pest that causes severe damage to horticultural crops (Reitz et al. 2011; Woo 1988). *F. occidentalis* causes direct damages such as silvery scar, and also indirect damages by transmitting plant viruses such as tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV), leading to substantial economic loss (Webster et al. 2011; Zhao et al. 1995). This species has been widely distributed throughout the world since the 1970s, as the international trade in horticultural products expanded from Europe to Asia (Kirk et al. 2003). The use of insecticides with different modes of action is an important strategy for controlling *F. occidentalis* populations, but it has also resulted in the development of insecticide resistance because of the short life cycle and high fecundity of thrips, which are traits conducive to the evolution of resistance (Bielza et al. 2007; Brodsgaard 1994).

Given the continuous emergence of insecticide-resistant populations of *F. occidentalis*, a full understanding the resistance mechanisms is necessary for effective control this species. Cytochrome P450 monooxygenases (CYP450) have been reported to contribute to *F. occidentalis* resistance to various insecticides, including beniocarb, methiocarb, diazinon, acrinathrin, deltamethrin, fenvalerate, formetanate,

permethrin, imidacloprid, and abamectin. Glutathione-S-transferases (GSTs) are also known to be involved in resistance to methiocarb and endosulfan in *F. occidentalis* (Gao et al. 2012). Since enhanced metabolism and excretion of insecticide and its metabolites are major mechanisms for insecticide resistance (James et al. 2009), identification of these detoxification factors enables an understanding of the evolutionary processes behind metabolic resistance to insecticides.

To investigate which detoxification genes are involved in insecticide metabolism, previous researchers have used non-invasive induction assays (Kim et al. 2018; Yoon et al. 2011). Because lethal treatment with insecticides induces the expression of genes related to general physiological stress, as well as primary detoxification genes, treatment with a sublethal concentration (i.e. $<LC_{10}$) of insecticides could be a better strategy for identifying genes involved in the actual metabolism of the insecticide (Yoon et al. 2011). Because many genes associated with detoxification are inducible by sublethal insecticide treatment, which in turn results in tolerance, transcript profiling upon insecticide treatment enables identification of the major metabolic factors involved in insecticide resistance (Vontas et al. 2005; Willoughby et al. 2006; Yoon et al. 2011). Pretreatment with sublethal concentrations of five different insecticides (chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb

and spinosad) in the 3rd instar larvae of the diamondback moth, *Plutella xylostella*, resulted in enhanced tolerance, and transcriptome analyses of the treated larvae revealed that two CYP450 genes and nine cuticular protein genes were commonly over-expressed, suggesting these genes play a role in chemical defense at the early stage of intoxication (Gao et al. 2018).

The insecticides we selected for our study are chlorfenapyr, dinotefuran and spinosad. Chlorfenapyr (IRAC class: 13) belongs to a new class of chemicals- the pyrroles. This compound is a pro-insecticide and the activity depends on the oxidative removal of its N-ethoxymethyl group by mixed function oxidases (MFOs), and forms the compound CL303268, it is an uncoupler of oxidative phosphorylation via disruption of H proton gradient, therefore resulting in disruption of production of ATP, cellular death and ultimately organism mortality. Later CL303268 is degraded to the common metabolites include products of N-alkoxy side chain oxidation and removal and ring hydroxylation products (https://pubchem.ncbi.nlm.nih.gov/compound/Chlorfenapyr). Dinotefuran (IRAC class: 4A) is a broad-spectrum insecticide, which can be used for agricultural or residential pest management. It can control insect pests such as aphid, thrips, beetle and cockroach. Since dinotefuran is a member of neonicotinoids, it acts as an agonist of the insect nicotinic

acetylcholine receptors (nAChRs), disturbs the neurotransmission and leads to abnormal behavior and final death. Only less than 10% of this compound was metabolized to numerous minor metabolites, whereas most part was excreted unchanged in urine (C. Abstracts 2004). Spinosad is a mixture of spinosyn A and spinosyn D in a 17: 3 ratio. The spinosyns derived from a family of natural products obtained by fermentation of *S. spinosa*, belonged to a novel family of insecticidal macrocyclic lactones, which can be used to control a wide variety of insect pests such as Lepidopterans, Dipterans, Thysanoptrans and many others. The spinosyns mainly target binding sites on nAChRs and therefore disrupt acetylcholine neurotransmission. Spinosyns also have secondary effects as a γ-amino-butyric acid (GABA) neurotransmitter agonist and cause the hyperexcitation of insect nervous system and pest death eventually. In vivo metabolites of spinosyns involve N- and O-demethylation as well as conjugation with glutathione (Sparks et al. 2001).

In this study, genes commonly responding to sublethal concentrations of three different insecticides were identified by comparison of the transcriptome profiles among insecticide-treated and untreated *F. occidentalis*. Characterization of the differentially expressed genes in response to sublethal doses of insecticides may improve our understanding of the general chemical defense mechanisms in *F.*

occidentalis and different strategies used by different insects when they experience a similar xenobiotic stress.

2. MATERIAL AND METHODS

2.1 Insect strains and rearing

An insecticide-susceptible RDA strain of *F. occidentalis* was obtained from the Rural Development Administration, South Korea, and was reared on the cotyledons of kidney bean (*Phaseouls valgaris*) according to previously reported methods (Kwon et al. 2015). The RDA strain was originally collected from chrysanthemum plants in Suwon, Gyeonggi-do in 2001 and has been reared under laboratory conditions without any exposure to insecticide. Briefly, bean seeds were planted in sterilized soil for six days at 28 ± 1 °C, 55 ± 5% relative humidity, and a photoperiod of 16:8 (L: D) h. The thrips were fed on sprouted cotyledons in an insect breeding dish (91.4-mm diameter × 40-mm height; SPL Life Sciences, Korea) with water (5 ml)-soaked thin-layered cotton (Han-Chang Medic, Cheonan, Korea). In each breeding dish, 200–300 adults were maintained with 30–40 cotyledons. Because the RDA strain was collected from the field and reared in the laboratory without any insecticide exposure for more than 10 years, it was assumed to be relatively susceptible to the insecticides used in this study.

2.2 Insecticide treatment using the residual contact vial plus water (RCVpW) bioassay method, determination of sublethal doses, and tolerance bioassay

Three insecticides that have been recently widely used in Korea to control F. occidentalis were selected for this study. Technical grades of these three insecticides (>98.0% purity) were purchased from either Chem Service Inc. (West Chester, PA, USA) for spinosad or Sigma-Aldrich (St. Louis, MO, USA) for chlorfenapyr and dinotefuran. Stock solutions were obtained by dissolving the insecticide powder in acetone, and the working solutions were then serially diluted in acetone to their final test concentrations.

The insecticide-treated vials were prepared according to the methods noted above (Kwon et al. 2015). Briefly, a 1 μl aliquot of water was dropped onto a filter paper disc (0.5 × 0.5 mm) (Whatman, GE Healthcare, UK) fitted inside of the vial screw cap to maintain humidity inside the vial for at least 12 h in order to minimize thrips mortality caused by dessication, not by insecticide. For each insecticide, a 100 μl aliquot of a serially-diluted insecticide in acetone was coated onto the inner wall of a 5-ml glass vial (Taeshin Bio Science, Seoul, Korea) by placing the vial on its side on a roller mixer (Eberbach, Ann Arbor, MI, USA) for 1 h in a fume hood until the acetone was completely evaporated.

Using a custom-made aspirator, 15 to 20 females were transferred to each insecticide-coated vial in the triplicate, and the number of dead thrips was counted at 8 h post-treatment. Thrips showing immobility for 3 s were considered dead. The LC_{10} and LC_{50} were determined by Probit analysis using IBM SPSS Statistics software 20.0 (IBM Crop., NY, USA).

To determine whether thrips exposed to low (LC_{10}) concentrations of insecticides developed tolerance, females were pretreated with LC_{10} of insecticides via RCVpW as described above. For the control group, vials were coated with acetone only. After 8 h post-treatment, the pretreated or control insects were transferred to new vials coated with lethal concentrations (LC_{50}) of the same insecticides, and mortalities were evaluated after another 8 h post-treatment. The tolerance bioassay was conducted with three replicates, each with 30 females in the LC_{10} pretreatment and 20 females in the second LC_{50} treatment. Statistical differences in mortality responses were determined by Student's *t*-test (Sigmaplot 12.0, San Jose, CA, USA).

2.3 Insecticide treatment and total RNA extraction

To administer sublethal doses of the three insecticides to thrips, the same RCVpW bioassay method was used. Fifty females were treated with LC_{10} concentrations of each insecticide for 8 h and collected into a

1.5 ml tube. The treated thrips were homogenized in TRIzol reagent (MRC, Cincinnati, OH, USA) by using a glass plunger homogenizer (Fisher Scientific, Göteborg, Sweden), and total RNA was extracted according to the manufacturer's instructions.

2.4 Library construction and sequencing

Prepared total RNA was processed by using RNase-free DNase I (Takara, Shiga, Japan) to remove any possible genomic DNA contaminants. Concentration and integrity of the RNA samples were determined using a NanoDrop 8000 spectrophotometer (Thermo, Waltham, MA, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Only RNA samples that met the requirements of an OD260/280 value ≥1.8 and integrity number ≥ 7.0 were selected for further processing. The qualified total RNA samples were used for mRNA preparation and cDNA library construction using the Illumina TruSeq standard mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Multiple cDNA libraries were then paired-end sequenced by using an Illumina NovaSeq 6000 Sequence System (Illumina, San Diego, CA, USA).

2.5 Sequence processing and annotation

Before analyzing the data, quality control was conducted to check the overall read quality, total bases, total reads, and GC percents. To reduce the bias of the analysis results, the obtained paired-end sequences were subjected to the Trimmomatic program (Illumina, San Diego, CA, USA) with default parameters to remove artifacts such as the adapter, contaminant DNA, and PCR duplicates. For quality trimming, qualified reads with Phred quality >20 were obtained, and the reads with minimum read length of <36 bp were trimmed. The aligned reads were generated by using HISAT2 (CCB, Johns Hopkins University, USA) and mapped to the western flower thrips reference genome (Focc_2.1, https://www.ncb-i.nlm.nih.gov/nuccore/JMDY00000000.2/). The aligned reads were then assembled through StringTie (CCB), which generated the information on known/novel/alternative spliced transcripts.

2.6 Reference-based differentially expressed gene (DEG) analysis

In order to have a full view of the UniGene expression patterns of our insecticide-treated samples, we aligned reads from each sample with the reference transcriptome using bowtie2 with custom parameters. Transcript abundances were estimated as fragments per kilobase per

million reads mapped (FPKM). After filtering the transcripts that were of low quality based on pre-process and quality control, quantile normalization (using preprocessCore R library) was conducted with Log_2 (FPKM+1). In addition, statistical analysis was conducted for the fold change of each sample. Gene expression levels were compared between control groups and the other insecticide-treated samples. Genes that showed a fold change of >1.5 or < −1.5 were considered to have been up or down regulated by the insecticide treatments. Among these, genes commonly over- or under-transcribed in all insecticide-treated thrips samples were selected for subsequent characterization. The correlation coefficient was determined by Pearson's correlation analysis using SigmaPlot (Version 12.0, San Jose, CA, USA).

2.7 Quantitative real-time PCR (qPCR)

Transcription levels of two proteolytic genes (transmembrane protease serine 9 like and cathepsin L1-like) and three detoxification genes (*Cyp6k1*, *GstS1*, and *UGT2b17-like*) were selected for validation along with two other genes (lipase 3-like and vacuolar H [+] ATPase 100kD subunit 2) with a fold change >1.5. Sequence-specific primer sets were designed based on the cDNA sequences of selected genes (Table 2-1). Total RNA was extracted from tolerance-induced thrips by using the same methods described above and were treated with DNase I (Takara)

to remove any gDNA contamination. cDNA was synthesized using the Superscript IV kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Each 20 µl qPCR reaction contained 25 ng cDNA, 2 pmol of each primer, and 10 µl of SYBR I 2 × master mix (Takara, Shiga, Japan) and was performed using the Roche LightCycler 96 system (Roche, Basel, Swiss). Reactions were incubated at 95 °C for 30 s, followed by 40 cycles of thermal program (5 s at 95 °C, 15 s at 56 °C, and 15 s at 72 °C). A ribosomal protein L32 (*RPL32*) was used as an internal reference gene (Yang et al. 2015). qPCR for each gene was conducted with three biological replicates per insecticide treatment. Relative transcription levels of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001)

Table 2-1. Sequence of primers used in qPCR

Gene Name	Primer Name	Sequence	GC Content (%)	Tm (°C)	Amplicon Length (bp)
Ribosomal Protein L32	RPL32-F	GCCAAGACCAGGCATATGCT	55	60.5	132
	RPL32-R	GCTTCTTAGCAGATACAGCGT	47.6	59.5	
Transmembrane protease serine 9 like	TPS9-F	GTCCATCCTAAGCGCAAGCT	55	60.5	146
	TPS9-R	ATGGCTTGACGGGAACCGAT	55	60.5	
Cathepsin L1-like	CP1-F	TCGGCTACAACCAGTACGCT	55	60.5	144
	CP1-R	CTCCAGTCGACCTTCTTGCT	55	60.5	
Cytochrome P 450 6k1	CYP6k1-F	CCGGAGATCATCAAGCACGT	55	60.5	142
	CYP6k1-R	ATCTTAGCGCGGAGCTCCTT	55	60.5	
Glutathione S transferase S1	GSTS1-F	CCTGTTCGCCTATGGCAATAT	47.6	59.5	153
	GSTS1-R	ACGGTATCGACTGATTGCAGT	47.6	59.5	
UDP-glucuronosyltransferase 2B17 like	UGT2B17-F	ACCGACAAGATGACCCTCCT	55	60.5	158
	UGT2B17-R	GCTGACGTTCTTGGTGAGCT	55	60.5	
Lipase 3-like isoform X2	LIP3-F	GCCTGATCTATGTCGGCCAT	55	60.5	163
	LIP3-R	CACGCCCTTGGAGATGATCT	55	60.5	
Vacuolar H[+] ATPase 100kD subunit 2	V100-F	GAGCTCATGACGTGGAAGGT	55	60.5	133
	V100-R	AGGTTCTGGAGCTCGTCCTT	55	60.5	

2.8 dsRNA synthesis

Five representative genes [*cytochrome P 450 6a2* (*Cyp6a2*), *glycine-rich cell wall structural protein 1.8-like* (*Gcwp 1.8*), *glutathione S transferase S1* (*GstS1*), *maltase B1* (*Mb1*) and *transmembrane protease serine 9 like* (*Tps9*)] were selected from commonly overexpressed DEGs as the targets of ingestion RNA interference (RNAi) to confirm their roles in tolerance induction. The DNA templates for dsRNA synthesis were PCR-amplified using primer sets containing T7 promoter sequence from the previously mentioned cDNA (Table 2-2). The templates of *Cyp6a2*, *Mb1* and *Tps9* were amplified by ExTaq DNA polymerase (Takara) with following thermal cycle condition: 95 °C for 3 min, 5 cycles of 95 °C for 30s, 56 °C for 30s, 72 °C for 1 min and 35 cycles of 95 °C for 30s, 70 °C for 30s, 72 °C for 1 min. The templates of *Gcwp 1.8* and *GstS1* were amplified by Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA) with the following thermal condition: 98 °C for 1 min, 5 cycles of 98 °C for 10s, 56 °C for 30s, 72 °C for 20s and 35 cycles of 98 °C for 10s, 70 °C for 30s, 72 °C for 20s. The resulting PCR products were then purified with a DNA cleanup kit (New England Biolabs) and used as the templates for in vitro transcription using the Megascript T7 transcription kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. dsRNA was dissolved in

nuclease-free water and diluted to a final concentration of 100 ng/μl. The diluted dsRNA was used immediately or stored in a 150 μl aliquot at −80 °C. The dsRNA of *Apis mellifera* acetylcholinesterase (*Amace1*, 551 bp) was synthesized by Genolution Inc. (Seoul, Korea) and used as the negative control after verifying that Amace1 dsRNA does not induce any target-specific mortality (Han et al. 2019).

Table 2-2. Sequence of primers used for dsRNA preparation

Gene Name	Primer Name	Sequence	GC Content (%)	Tm (°C)	Amplicon Length (bp)
Maltase B1	dsMb1-F	taatacgactcactatagggGGAAGGTCAATGCACAGCCT	48.0	78.1	557
	dsMb1-R	taatacgactcactatagggTCGTTCCATGTCCATCCTGAT	44.0	76.9	
Cytochrome P 450 6a2	dsCyp6a2-F	taatacgactcactatagggAACTTCGCCGACAGCATCCT	48.0	78.1	627
	dsCyp6a2-F	taatacgactcactatagggCGGAACAGCCTCATGATGGT	48.0	78.1	
Transmembrane protease serine 9 like	dsTps9-F	taatacgactcactatagggGTGCGCATGGACAGCAACTT	48.0	78.1	603
	dsTps9-R	taatacgactcactatagggTGTTGATACACCAAGCCGTAG	44.0	76.9	
Glutathione S transferase S1	dsGstS1-F	taatacgactcactatagggGTGGTGTGGACGGTCGGAAT	50.0	78.9	390
	dsGstS1-F	taatacgactcactatagggGCCTTAACGGTATCGACTGAT	44.0	76.9	
Glycine-rich cell wall structural protein 1.8-like	dsGcwp1.8-F	taatacgactcactatagggGCATAGTGGCCCAATCGCAT	48.0	78.1	538
	dsGcwp1.8-R	taatacgactcactatagggAAGACCTCCGTTGAGTGCTG	48.0	78.1	

2.9 Ingestion RNAi and insecticide bioassay

The ingestion RNAi was conducted using the three-unit feeding chamber as described previously (Han et al. 2019). Briefly, 15 μg dsRNA (150 μl of 100 ng/μl) was added to the bottom chamber and covered with a 16-mm diameter kidney bean leaf disc. Then, 15 females, which emerged within 3 days, were transferred over the leaf disc, and the middle and top units were covered. The leaf disc and dsRNA were replaced every 24 h. The survived thrips were collected after 48 h, among which five individuals were used for qPCR analysis and the rest were transferred to glass vials precoated with LC_{50} of the test insecticide. The RNAi for each representative gene in conjunction with bioassay was conducted three times, and the number of female individuals in each bioassay varied from 6 to 10. The mortality was recorded at 8 h-posttreatment, and the statistical differences in mortality between control and dsRNA-treated thrips were determined by Student's *t*-test.

3. RESULTS

3.1 Increased tolerance following sublethal pretreatment with insecticides

The determination of the LC_{10} and LC_{50} concentrations of the three insecticides are given in Table 2-3. To investigate whether tolerance to the test insecticides occurred in thrips, bioassays at the LC_{50} concentrations were conducted following pretreatment at the LC_{10} concentrations of the three insecticides. All pre-treated thrips showed reduced mortality compared to the control thrips. The mortality responses of insecticide-pretreated samples were significantly lower (~1.27-fold lower for chlorfenapyr; 1.40-fold lower for dinotefuran; and 1.62-fold lower for spinosad, as judged by the final % mortality value) than those of control thrips (p = .019 for chlorfenapyr; p = .035 for dinotefuran; p = .010 for spinosad) (Fig. 2-1).

Table 2-3. LC_{10} and LC_{50} concentration determination of three different insecticides via RCVpW method

IRAC group[a]	Insecticide	N	LC_{10} (ppm)	LC_{10} 95% C.L[b]	LC_{50} (ppm)	LC_{50} 95% C.L[b]
13	Chlofenapyr	240	0.48	0.04-0.89	1.41	0.66-2.59
4A	Dinotefuran	403	5.31	2.82-7.93	29.7	21.7-44.1
5	Spinosad	577	0.48	0.29-0.68	2.44	1.99-2.96

[a] Group number classified by the mode of action according to the Insecticide Resistance Action Committee (IRAC).
[b] C.L : Confident Limit

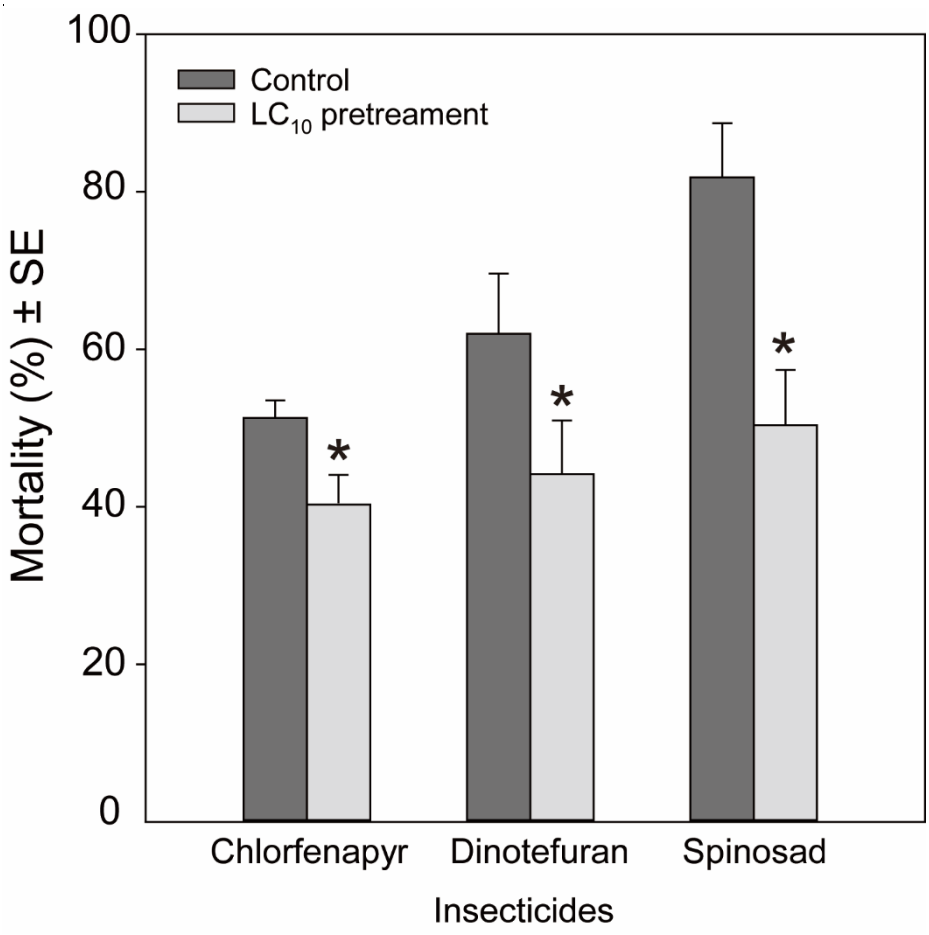

Fig. 2-1. Tolerance induction by pretreatment with sublethal concentrations (LC_{10}) of insecticides. *, $p < 0.05$.

3.2 Transcriptome data analysis

Approximately 55.4, 64.8, 59.2, and 49.8 million reads were generated in the control, chlorfenapyr-, dinotefuran-, and spinosad-treated thrips, and 91.2%, 91.6%, 90.8%, and 91.4% of these reads were mapped, respectively. With these mapped reads, a total of 17,553 transcripts were assembled; 5607 of these were later filtered out because of extremely low FPKM values. The remaining 11,946 transcripts were further analyzed. Among these transcripts, 13.6% were between 100 and 500 bp, 27.5% were between 500 and 1000 bp, 35.3% were between 1000 and 2000 bp, and 23.6% had nucleotide lengths less than 100 bp or above 2000 bp (Fig. 2-2.). Values of >1.0 FPKM occurred in 10,077 genes in the control group, 10,379 genes in the chlorfenapyr-treated group, 10,481 genes in the dinotefuran-treated group, and 10,496 genes in the spinosad-treated group. GO analysis classified the GO items of 10,881 transcripts into three major categories: molecular function (F), cellular component (C), and biological process (P). DEG analysis showed that a total of 2057 transcripts exhibited at least a 1.5-fold expression difference in the insecticide-treated thrips compared to controls. Among these DEGs, 404 genes were up-regulated with chlorfenapyr, 386 genes were up-regulated with dinotefuran, and 756 genes were up-regulated with spinosad. In contrast, 124 genes were

down-regulated with chlorfenapyr, 107 genes were down-regulated with dinotefuran, and 169 genes were down-regulated with spinosad. GO analysis revealed no apparent differences in annotated GO items among the three major categories (Fig. 2-3).

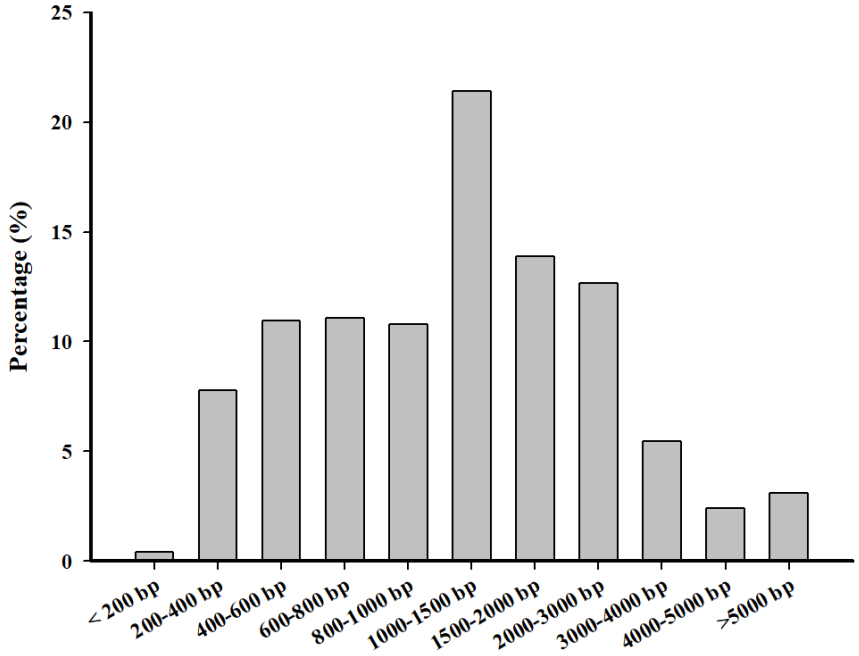

Fig. 2-2. Length distribution of overall analyzed transcripts.

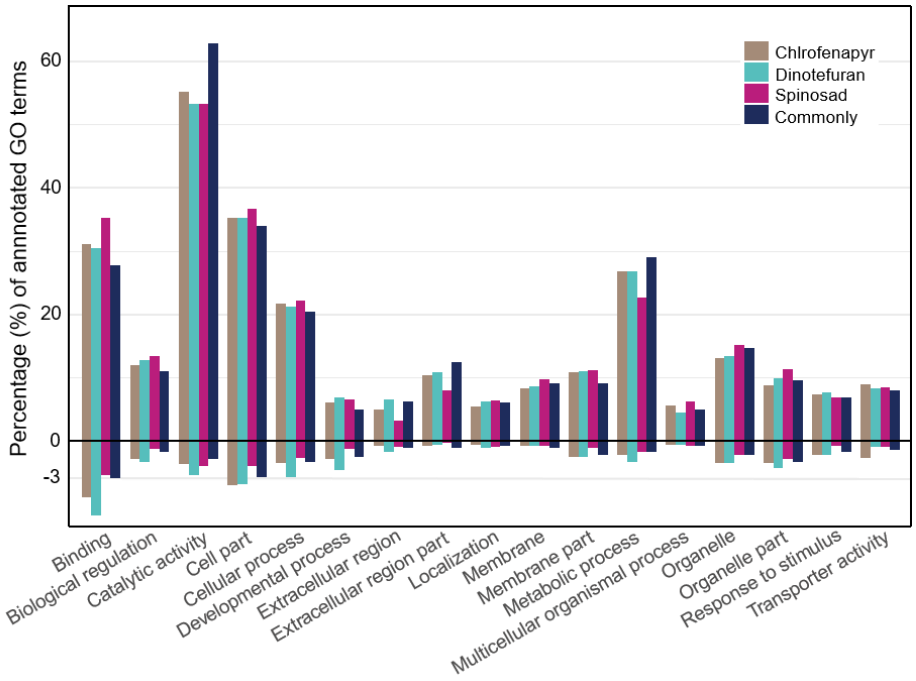

Fig. 2-3. Gene ontology (GO) distribution of differentially expressed genes (DEGs) following treatment with sublethal concentrations of three insecticides.

3.3 DEGs following insecticide treatment

A total of 230 out of 2057 genes that showed more than a 1.5-fold expression difference compared to controls were identified in all three insecticide-treated thrips. Of these 230 genes, 199 transcripts were up-regulated, whereas 31 were down-regulated. Forty-two transcripts showed no matches in the reference database, and 32 were uncharacterized proteins. Transcripts associated with protease activity (15.2%) showed the highest proportion among the commonly responding DEGs, followed by glycosidase (7.8%) and detoxification (6.1%) transcripts. All genes up- or down-regulated are listed in Table 2-4.

Table 2-4. Overall commonly responded DEGs in all three insecticide treatments

Gene ID	Gene Name	Fold change/FPKM Value		
		Chlorfenapyr	Dinotefuran	Spinosad
FOCC005273-RA	Transmembrane protease serine 9 like	2.21/145.9	1.96/129.6	3.05/213.9
FOCC015893-RA	Transmembrane protease serine 9 like	1.79/16.5	1.98/17.9	2.53/23.5
FOCC003002-RA	Transmembrane protease serine 9 like	1.71/3.88	1.53/3.40	2.02/5.12
FOCC017129-RA	Transmembrane protease serine 9 like	1.96/88.2	1.89/84.3	3.27/153.5
FOCC016146-RA	Transmembrane protease serine 9 like	1.75/71.4	1.83/73.9	3.08/127.5
FOCC003507-RA	Chymotrypsin-like	1.96/429.4	1.76/392.8	3.34/737.1
FOCC010748-RA	Chymotrypsin-like	1.87/28.9	1.85/28.1	2.7/42.3
FOCC016846-RA	Chymotrypsin-like	1.67/44.3	1.92/49.8	2.72/71.8
FOCC008020-RA	Chymotrypsin-like	1.5/83.3	1.61/87.7	2.19/121.9
FOCC005270-RA	Chymotrypsin-like	1.71/21.3	1.74/21.3	2.55/31.5
FOCC008019-RA	Chymotrypsin-like	1.64/18.1	1.57/17.1	2.29/25.5
FOCC014665-RA	Chymotrypsin-like	1.55/25.8	1.71/28.0	2.55/43.1
FOCC010472-RA	Chymotrypsin-2-like	1.58/21.2	1.68/21.7	2.24/29.9
FOCC005119-RA	Thyrotropin-releasing hormone-degrading ectoenzyme-like	2.05/6.20	2.12/6.39	4.43/14.6
FOCC005117-RA	Thyrotropin-releasing hormone-degrading ectoenzyme-like	1.79/9.72	1.92/10.3	2.4/13.7
FOCC011470-RA	Trypsin-1-like	1.94/1096.7	1.95/1103.8	3.77/1925.3
FOCC002031-RA	Trypsin-1-like	2/708.7	1.57/546.4	2.9/967.5
FOCC016547-RA	Trypsin-1-like	1.86/29.4	2.1/32.9	1.79/28.3
FOCC017250-RA	Trypsin-1	1.6/3.98	1.5/3.66	2.71/7.73
FOCC011695-RA	Trypsin-2	1.63/17.4	1.93/20.3	2.34/25.0
FOCC006955-RA	Trypsin 3A1-like	1.67/216.7	1.74/221.6	1.86/258.2
FOCC005301-RA	Trypsin-7-like	1.54/22.6	1.71/24.7	1.9/27.7
FOCC007230-RA	Trypsin-7-like	1.72/54.5	1.7/52.9	1.91/60.7
FOCC005903-RA	Cathepsin L1-like	2.13/3337.9	2.08/3277.3	4.4/6679.1
FOCC009718-RA	Cathepsin L1-like	1.57/28.1	1.67/29.5	1.58/28.3

FOCC016260-RA	Cathepsin L1-like isoform X2	1.97/764.8	1.82/714.9	4.18/1494.0
FOCC005907-RA	Cathepsin L1-like isoform X3	1.87/837.8	1.77/796.8	2.54/1071.9
FOCC008837-RA	Cathepsin B-like	1.7/424.6	1.8/447.9	2.36/553.6
FOCC005265-RA	Carboxypeptidase A1	1.83/70.2	1.65/62.9	2.76/106.6
FOCC016528-RA	Carboxypeptidase B-like	1.64/28.1	1.65/27.8	1.78/30.4
FOCC003634-RA	Serine protease 3-like	1.86/343.6	1.77/325.1	3.48/638.7
FOCC005272-RA	Granzyme M-like	1.77/44.1	1.87/45.5	2.04/50.5
FOCC017468-RA	Aminopeptidase	1.76/17.8	1.68/16.6	2.55/25.8
FOCC016926-RA	neuroendocrine convertase 2	1.73/11.3	2/12.9	2.05/13.9
FOCC013211-RA	Inter-alpha-trypsin inhibitor heavy chain H4	1.79/67.0	2.23/82.8	7/281.9
FOCC012969-RA	Myrosinase 1-like	1.8/32.3	2.01/35.4	3.3/59.2
FOCC014245-RA	Myrosinase 1-like	1.8/20.1	1.68/18.6	5.1/57.1
FOCC012754-RA	Myrosinase 1-like	1.93/10.3	1.58/8.33	3.34/18.6
FOCC015589-RA	Myrosinase 1-like	1.92/14.0	1.84/13.0	6.85/50.3
FOCC013450-RA	Myrosinase 1-like	1.63/26.2	1.5/24.0	2.37/38.7
FOCC014762-RA	Myrosinase 1-like	1.81/54.1	1.8/52.6	2.69/81.3
FOCC003511-RA	Myrosinase 1-like	1.74/24.6	1.91/26.6	1.96/27.6
FOCC012970-RA	Myrosinase 1-like	1.6/77.1	1.75/83.9	1.74/85.8
FOCC006088-RA	Maltase B1	2.25/59.2	2.67/69.7	8.21/226.7
FOCC006090-RA	Maltase A6, isoform C	1.62/167.0	1.98/198.5	2.74/296.1
FOCC006089-RA	Maltase A1	1.53/191.4	1.64/202.1	2.43/321.4
FOCC012064-RA	Trehalase	1.67/15.5	1.82/16.7	3.17/29.5
FOCC003635-RA	Alpha-amylase B	1.81/197.7	1.65/179.3	4.09/439.8
FOCC007995-RA	Levanase	2.45/219.3	1.8/156.3	6.38/546.5
FOCC008033-RA	Beta-glucosidase 42-like	1.54/15.5	1.61/16.0	1.95/20.1
FOCC016201-RA	Cytosolic beta-glucosidase-like	1.61/223.0	1.82/247.4	1.89/273.9
FOCC015912-RA	Glucosylceramidase	1.78/11.4	2.07/13.1	2.26/14.9
FOCC009361-RA	Myogenesis-regulating glycosidase	1.54/159.7	1.65/167.8	1.68/180.2
FOCC005019-RA	Lysozyme C-3-like	1.69/57.3	1.79/59.7	1.72/58.2
FOCC008839-RA	lipase 3-like isoform X2	1.85/74.2	2.02/80.8	4.2/178.3

FOCC013474-RA	fatty acid synthase isoform X1	1.78/9.36	1.84/9.59	3.98/21.7
FOCC005111-RA	lambda-crystallin homolog	1.9/45.2	1.88/44.0	3.22/76.6
FOCC001955-RA	Very-long-chain 3-oxoacyl-CoA reductase	1.67/47.3	1.51/41.6	2.25/63.6
FOCC009651-RA	Delta(7)-sterol 5(6)-desaturase erg32-like	1.75/88.0	1.83/90.8	3.08/163.0
FOCC017273-RA	Pancreatic lipase-related protein 3-like	1.69/10.0	1.66/9.66	2.44/15.1
FOCC016660-RA	Long-chain-fatty-acid--CoA ligase 5 isoform X1	1.85/5.21	1.65/4.53	2.11/6.50
FOCC001625-RA	Inactive pancreatic lipase-related protein 1-like	2.08/7.82	1.89/6.89	2.3/9.02
FOCC015494-RA	Receptor-type tyrosine-protein phosphatase N2	1.52/6.52	1.86/8.08	2.75/12.7
FOCC000160-RA	Putative phospholipase B-like 2	1.7/29.5	1.66/28.3	1.84/31.8
FOCC016061-RA	Lipase member H-like isoform X3	1.95/4.99	1.7/4.23	1.56/4.11
FOCC013203-RA	glucose dehydrogenase [FAD, quinone]-like	2.92/8.29	1.67/4.33	3.29/9.70
FOCC008953-RA	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase	1.76/29.8	1.69/28.0	3.14/53.4
FOCC006709-RA	Alcohol dehydrogenase [NADP(+)]-like	1.92/39.2	1.73/34.9	2.49/51.0
FOCC006708-RA	Alcohol dehydrogenase [NADP(+)]-like	1.71/36.4	1.57/32.8	2.11/45.4
FOCC016759-RA	Mitochondrial enolase superfamily member 1	1.6/141.9	1.55/135.0	1.8/164.8
FOCC003072-RA	Phosphoenolpyruvate carboxykinase [GTP]	1.79/171.2	1.62/150.2	1.96/195.5
FOCC016865-RA	UDP-glucuronosyltransferase 2B17 like	2/8.34	2.27/9.39	6.08/25.9
FOCC003406-RA	UDP-glucuronosyltransferase 2B2 like	1.98/23.3	1.93/22.4	4.48/52.7
FOCC001729-RA	UDP-glucuronosyltransferase 2c1	1.66/9.48	2.1/11.9	2.41/14.5
FOCC002672-RA	UDP-glucuronosyltransferase 2B15 like	1.53/22.2	1.61/23.1	1.81/26.2
FOCC016845-RA	cyp6k1	2/228.7	1.7/185.9	3.35/376.2
FOCC004908-RA	cyp6a13	2.25/29.4	1.71/22.0	2.2/28.6
FOCC016890-RA	cyp6a2	2.12/46.6	1.57/33.7	2.33/50.7
FOCC016704-RA	cyp6a14	1.65/16.2	1.76/17.0	2.59/25.5
FOCC007810-RA	Glutathione S transferase S1	2.69/64.4	1.99/47.2	2.96/71.5

FOCC016012-RA	Alpha-Esterase-5	2.03/2.63	2.13/2.91	2.87/4.48
FOCC016132-RA	Carboxylic ester hydrolase	1.69/22.0	2.15/27.5	2.72/35.5
FOCC004400-RA	Gut esterase 1	2.26/35.7	1.64/25.3	2.42/38.5
FOCC011405-RA	Alpha-Esterase-1	1.75/5.93	1.68/5.60	2.34/8.57
FOCC012231-RA	ABC transporter G family member 20	1.69/20.0	1.72/20.1	2.47/29.0
FOCC015180-RA	Vacuolar H[+] ATPase 100kD subunit 2	1.74/17.0	1.77/16.9	2.48/24.4
FOCC005842-RA	Cytochrome oxidase subunit I	2.5/2886.0	1.59/1937.0	4.3/4759.6
FOCC013317-RA	V-type proton ATPase 16 kDa proteolipid subunit	1.79/18.2	1.63/16.3	2.05/21.2
FOCC006758-RA	V-type proton ATPase 16 kDa proteolipid subunit	1.74/24.2	1.72/23.6	1.9/26.4
FOCC009723-RA	Sodium/potassium-transporting ATPase subunit beta-2	1.52/17.9	1.98/22.9	2.2/25.8
FOCC009724-RA	Tyrosine aminotransferase	1.81/9.29	1.75/8.86	2.97/15.9
FOCC010397-RA	Proline dehydrogenase 1, mitochondrial	1.63/28.0	1.72/29.2	2.84/49.4
FOCC001417-RA	4-hydroxyphenylpyruvate dioxygenase	2.04/110.8	1.67/88.4	2.55/141.2
FOCC014418-RA	Glycine dehydrogenase (decarboxylating), mitochondrial	1.76/115.0	1.51/95.8	2.08/138.2
FOCC007375-RA	Aromatic-L-amino-acid decarboxylase	1.77/37.9	1.5/31.7	1.73/37.1
FOCC011624-RA	Mucin-2-like	1.54/4.91	2.14/7.10	7.57/26.5
FOCC017295-RA	Mucin-2-like	1.78/31.3	2.02/35.1	1.57/27.3
FOCC010854-RA	Ankyrin repeat domain 17	1.53/20.5	1.71/22.4	2.11/28.1
FOCC008259-RA	Calmodulin	1.71/2.84	1.58/2.62	3.33/6.84
FOCC003089-RA	Galectin	1.52/23.1	1.78/26.5	2.58/39.4
FOCC003922-RA	Phenoloxidase 2	1.99/20.8	1.77/18.1	1.99/21.1
FOCC005163-RA	Inositol-trisphosphate 3-kinase homolog	1.66/28.9	1.56/26.8	1.76/30.4
FOCC016590-RA	Calcium-binding protein P-like isoform X4	1.59/44.0	1.72/46.2	1.8/49.7
FOCC017112-RA	Superoxide dismutase [Cu-Zn]	1.57/39.4	1.51/37.2	1.69/42.9
FOCC006731-RA	Proton-coupled amino acid transporter-like protein pathetic	1.75/3.76	1.53/3.18	4.67/11.8
FOCC013155-RA	Solute carrier family 15 member 1-like isoform X2	1.76/22.7	1.86/23.7	3.76/49.1

FOCC006835-RA	Major facilitator superfamily domain-containing protein 12	1.96/21.3	1.59/16.9	3.09/33.6
FOCC017387-RA	Sialin	1.79/2.72	1.59/2.03	2.16/3.32
FOCC017382-RA	Facilitated trehalose transporter Tret1-like	1.76/2.12	1.54/1.78	1.86/2.54
FOCC002259-RA	Vitellogenin-2-like	1.65/26.3	1.69/26.8	8.78/145.2
FOCC002665-RA	Vitellogenin-1-like	2.15/148.5	1.85/127.6	6.13/412.9
FOCC005500-RA	Vitellogenin-1-like	1.64/17.0	1.86/19.1	3.1/32.3
FOCC009176-RA	Apolipophorins	1.62/27.2	1.82/30.2	5.33/91.6
FOCC011998-RA	Aquaporin-2-like isoform X1	2.25/33.6	1.6/23.4	2.13/31.4
FOCC012817-RA	Apolipoprotein D-like	1.5/2476.4	1.54/2565.4	1.85/3006.3
FOCC016038-RA	glycine-rich cell wall structural protein 1.8-like	2.28/21.1	2.03/18.5	2.95/27.1
FOCC011799-RA	Homeobox protein 2-like	1.51/6.11	2.09/8.67	2.25/9.87
FOCC011770-RA	Protein BTG2-like	2.23/70.3	1.7/53.0	3.57/114.0
FOCC009162-RA	Paramyosin, long form	1.62/41.5	1.9/48.2	2.4/61.9
FOCC017319-RA	Paramyosin, long form	1.62/84.6	1.88/97.3	1.88/98.8
FOCC006811-RA	Patronin	1.75/9.44	1.71/9.14	1.87/10.6
FOCC001674-RA	Myosin heavy chain, muscle	1.55/110.3	1.79/127.9	1.96/141.8
FOCC013372-RA	TPPP family protein CG45057	1.54/39.0	1.72/37.9	1.73/44.4
FOCC016575-RA	Myosin-9-like isoform X1	1.55/13.0	1.5/12.4	1.62/14.1
FOCC004790-RA	cAMP-specific 3',5'-cyclic phosphodiesterase, isoform I	1.74/16.7	1.5/13.9	2.71/25.9
FOCC007045-RA	Ankyrin repeat and SOCS box protein 14-like	1.66/10.5	1.69/10.5	2.18/14.4
FOCC006649-RA	Alkaline phosphatase	2.14/88.0	2.12/86.4	2.75/114.3
FOCC006348-RA	Extracellular serine/threonine protein CG31145	1.76/20.1	1.54/17.1	2.87/32.5
FOCC003429-RA	Apyrase-like	1.6/16.8	1.66/17.1	2.81/29.4
FOCC014595-RA	Twitchin isoform X1	1.51/6.93	1.75/8.10	2.34/11.6
FOCC004333-RA	cGMP-dependent protein kinase	1.77/0.94	1.77/0.92	1.7/0.94
FOCC014267-RA	Tribbles homolog 3-like	1.56/22.9	1.51/21.9	2.03/29.6

FOCC003906-RA	ADP-sugar pyrophosphatase-like isoform X2	1.98/288.4	1.5/210.5	2.21/326.8
FOCC017436-RA	O-methyltransferase family protein	2.37/114.5	1.52/71.8	1.72/83.9
FOCC001704-RA	Four and a half LIM domains protein 2 isoform X6	1.5/19.4	1.82/23.3	2.22/28.5
FOCC005676-RA	Transferrin-like	1.74/15.5	1.76/15.4	1.68/15.5
FOCC003916-RA	Voltage-dependent calcium channel subunit alpha-2/delta-3-like	1.65/1.94	1.51/1.77	1.79/2.44
FOCC013785-RA	Farnesol dehydrogenase	1.52/24.1	1.56/24.5	1.85/29.3
FOCC003375-RA	Muscle LIM protein Mlp84B-like	1.57/600.6	1.57/595.5	1.74/666.6
FOCC002827-RA	ITG-like peptide	1.71/13.6	2.13/16.7	2.26/18.3
FOCC005969-RA	Longitudinals lacking	-2.03/3.56	-2.5/2.71	-1.86/4.20
FOCC001594-RA	Diptericin A	-3.35/11.5	-5.47/6.68	-1.5/25.8
FOCC005923-RA	Zinc finger protein 32-like	-4.2/0.25	-4.19/0.27	-4.56/0.13
FOCC006858-RA	Putative zinc finger and SCAN domain-containing protein 5C	-1.62/1.78	-1.58/1.91	-1.94/1.41
FOCC006813-RA	Laccase-2-like	-2.36/0.43	-1.74/1.05	-2/0.76
FOCC007944-RA	Dynein regulatory complex subunit 4	-1.68/2.38	-1.51/2.85	-1.97/2.05
FOCC005330-RA	Dynein regulatory complex protein 8-like isoform X1	-1.78/0.20	-1.5/0.49	-1.69/0.29
FOCC009666-RA	Beta-1,3-galactosyltransferase brn	-1.67/3.87	-1.67/3.82	-1.93/3.42
FOCC007243-RA	B9 domain-containing protein 1	-1.83/9.10	-1.81/9.08	-1.94/8.87
FOCC008345-RA	Mediator of DNA damage checkpoint protein 1-like isoform X2	-1.87/20.3	-1.56/24.1	-2.23/17.3
FOCC016854-RA	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1-like	-1.93/1.54	-1.89/1.65	-2.13/1.40
FOCC012227-RA	Putative ferric-chelate reductase 1 homolog	-1.63/4.60	-2.54/2.65	-1.68/4.80
FOCC013510-RA	Transposable element tc3 transposase	-4.36/2.68	-1.72/8.15	-2.62/8.33
FOCC016188-RA	Transposable element tc3 transposase	-1.64/0.31	-1.53/0.44	-1.71/0.25
FOCC003940-RA	Venom serine protease-like	-1.52/0.79	-1.52/0.85	-1.66/0.70

FOCC010451-RA	Chromatin accessibility complex subunit 1	-1.57/2.71	-1.57/2.79	-1.67/2.75
FOCC013344-RA	Poly [ADP-ribose] polymerase 12-like isoform X2	-1.59/3.16	-1.55/3.29	-1.54/3.52
FOCC005880-RA	Small lysine-rich protein 1	-2.03/0.68	-1.66/1.17	-1.94/0.85
FOCC008281-RA	Cysteine-rich DPF motif domain-containing protein 1	-1.63/5.02	-1.59/5.18	-2.67/3.86
FOCC011204-RA	Uncharacterized protein LOC113212699	1.66/10.4	1.96/12.2	2.83/18.4
FOCC015419-RA	Uncharacterized protein LOC113218109	1.57/7.03	1.69/7.53	4.17/19.84
FOCC006311-RA	Uncharacterized protein LOC113215160	1.59/879.3	1.58/879.1	1.51/837.4
FOCC005108-RA	Uncharacterized protein LOC113211650	1.86/15.1	1.64/12.9	3.36/27.1
FOCC001627-RA	Uncharacterized protein LOC113204194	1.85/254.8	1.97/271.0	1.64/232.4
FOCC008401-RA	Uncharacterized protein LOC113215429	2.09/48.5	1.52/34.6	2.7/62.7
FOCC006310-RA	Uncharacterized protein LOC113215158	1.56/9.22	1.86/10.9	2.54/15.7
FOCC007632-RA	Uncharacterized protein LOC113206112	1.74/49.3	1.85/51.5	2.56/72.4
FOCC016794-RA	Uncharacterized protein LOC113212790	1.92/18.1	1.99/18.5	3.94/32.3
FOCC012798-RA	Uncharacterized protein LOC113218437	1.53/127.0	2.13/173.8	2.95/261.0
FOCC008480-RA	Uncharacterized protein LOC113210130	1.53/148.3	1.65/155.9	2.83/288.4
FOCC012151-RA	Uncharacterized protein LOC113206195	1.53/128.6	1.67/139.1	1.72/147.1
FOCC006312-RA	Uncharacterized protein LOC113215160	1.68/545.5	1.56/487.4	1.79/549.6
FOCC012635-RA	Uncharacterized protein LOC113218471	1.69/4.07	1.53/3.59	1.9/5.04
FOCC012340-RA	Uncharacterized protein LOC113213843	1.74/15.1	1.63/13.7	1.72/14.4
FOCC007801-RA	Uncharacterized protein LOC113213094	1.94/44.4	1.76/39.4	2.53/57.6
FOCC009827-RA	Uncharacterized protein LOC113207276	2.33/69.9	1.73/51.5	2.44/73.9
FOCC014766-RA	Uncharacterized protein LOC113207108	2.54/48.7	2.29/43.2	3.84/73.8
FOCC004272-RA	Uncharacterized protein LOC113206549	1.51/0.77	1.78/1.19	2.18/1.79
FOCC017087-RA	Uncharacterized protein LOC113212336	1.63/11.2	1.63/11.0	1.86/20.3
FOCC015488-RA	Uncharacterized protein LOC113207877	2.05/42.0	1.84/37.0	7.31/155.8
FOCC013833-RA	Uncharacterized protein LOC113208133	1.63/23.8	1.79/25.6	1.96/28.5
FOCC004298-RA	Uncharacterized protein LOC113207078	1.97/36.1	2/35.8	4.66/86.1
FOCC003568-RA	Uncharacterized protein LOC113203428	1.51/1.30	1.57/1.48	1.7/1.79
FOCC013134-RA	Uncharacterized protein LOC113215581	2.18/56.7	1.76/45.1	1.65/43.1

FOCC017259-RA	Uncharacterized protein LOC113213952	1.55/15.8	1.72/17.2	1.75/18.1
FOCC017462-RA	Uncharacterized protein LOC113216855	-2.75/1.50	-1.81/2.88	-3.07/1.32
FOCC015182-RA	Uncharacterized protein LOC113212632	-1.83/0.49	-2/0.33	-1.62/0.74
FOCC007422-RA	Uncharacterized protein LOC113214071	-1.5/4.03	-1.51/3.92	-1.82/3.35
FOCC016335-RA	Uncharacterized protein LOC113203934	-1.71/0.54	-1.54/0.77	-1.8/0.48
FOCC005172-RA	Uncharacterized protein LOC113203205	-2.49/8.83	-2.52/8.57	-2.63/8.64
FOCC017296-RA	No hit	1.75/1.09	2.51/2.13	3.33/3.36
FOCC003390-RA	No hit	1.91/5.92	2.06/6.42	3.01/10.2
FOCC008720-RA	No hit	1.82/876.1	1.89/915.3	3.05/1341.7
FOCC001497-RA	No hit	1.93/24.0	1.91/23.5	4.17/52.0
FOCC015980-RA	No hit	1.64/60.4	1.69/61.9	2.16/80.7
FOCC010261-RA	No hit	1.6/14.1	1.67/14.4	2.29/20.6
FOCC009860-RA	No hit	1.86/2.70	1.63/2.32	3.47/6.33
FOCC015489-RA	No hit	1.92/190.4	1.63/159.8	2.03/215.3
FOCC011564-RA	No hit	1.6/66.6	1.63/67.2	2.37/100.2
FOCC017417-RA	No hit	1.81/4.37	1.83/4.37	3.19/8.63
FOCC006422-RA	No hit	2.21/18.4	1.96/19.7	3.05/22.6
FOCC015852-RA	No hit	1.58/3.75	1.8/4.35	2.21/5.95
FOCC011699-RA	No hit	1.75/58.1	1.85/60.6	2.5/84.5
FOCC013669-RA	No hit	2.31/14.8	2.01/12.6	2.58/16.8
FOCC014246-RA	No hit	1.61/53.5	1.58/51.5	2.28/75.7
FOCC011433-RA	No hit	1.78/58.1	1.82/59.0	2.75/91.1
FOCC007529-RA	No hit	1.79/10.7	2.12/12.6	2.24/14.1
FOCC004840-RA	No hit	1.79/213.8	1.81/214.0	2.64/327.1
FOCC006452-RA	No hit	2.34/75.7	1.55/50.2	2.41/79.6
FOCC016396-RA	No hit	1.74/83.1	1.59/74.2	2.51/120.2
FOCC008721-RA	No hit	1.69/71.6	1.69/70.9	2.23/95.5
FOCC014169-RA	No hit	2.27/74.4	1.76/57.1	2.7/90.4
FOCC004432-RA	No hit	2.28/33.5	1.85/26.7	5.89/87.5
FOCC009415-RA	No hit	1.56/42.0	1.5/39.8	2.57/69.6
FOCC013188-RA	No hit	1.5/11.0	1.55/11.2	1.7/13.0

FOCC017518-RA	No hit	1.51/1.28	1.73/1.71	1.96/2.22
FOCC007805-RA	No hit	1.55/45.1	1.71/48.9	1.71/50.0
FOCC016781-RA	No hit	1.57/29.2	1.73/31.8	1.68/31.0
FOCC007799-RA	No hit	1.58/135.1	1.65/138.4	1.65/142.2
FOCC017237-RA	No hit	1.61/13.5	1.63/13.4	1.6/14.0
FOCC016899-RA	No hit	1.72/6.31	1.5/5.35	1.69/6.59
FOCC007800-RA	No hit	1.76/161.9	1.76/157.0	1.7/161.4
FOCC016885-RA	No hit	1.79/24.8	2.3/31.7	1.79/25.0
FOCC009207-RA	No hit	1.84/97.0	1.74/90.1	1.96/104.2
FOCC011256-RA	No hit	-2.59/8.22	-1.88/11.2	-3.87/5.61
FOCC007993-RA	No hit	-1.72/2.53	-1.57/2.94	-2.22/1.87
FOCC006468-RA	No hit	-1.71/7.28	-2.27/5.21	-2.51/5.10
FOCC012294-RA	No hit	-2.04/11.8	-1.79/13.2	-4.24/5.68
FOCC000124-RA	No hit	-1.5/0.28	-1.63/0.18	-1.82/0.04
FOCC008587-RA	No hit	-1.79/3.21	-1.51/3.96	-1.89/3.23
FOCC012324-RA	No hit	-1.62/0.37	-1.64/0.37	-1.72/0.28
FOCC013098-RA	No hit	-1.5/3.75	-2.22/2.24	-1.69/3.43

In the 35 transcripts related to proteolysis, all were up-regulated compared to the control, except for a serine protease-like LD47230p gene. The average fold change of the transcripts varied from 1.56 to 2.66, and the cathepsin L1-like and thyrotropin-releasing hormone-degrading ectoenzyme-like genes showed the highest expression difference compared to the control group, with an average fold change of 2.87. Interestingly, the transcription levels of one protease inhibitor gene and inter-alpha-trypsin inhibitor heavy chain H4 increased in all insecticide-treated thrips. Another five transcripts involved in the amino acid metabolic process, tyrosine aminotransferase, 4-hydroxyphenylpyruvate dioxygenase, proline dehydrogenase 1, glycine dehydrogenase, and aromatic-L-amino-acid decarboxylase, were also over-transcribed.

A total of 18 of the commonly up-regulated transcripts were related to glycosidase (Table 2-4), and eight of them were identified as myrosinase 1-like genes. Among the genes, maltase B1 showed the highest average fold change (4.38), followed by myrosinase-1-like gene (3.54). Seventeen transcripts were found to be involved in carbohydrate and lipid metabolic processes, and they all showed significantly higher expression levels compared to the control sample. In that category, a lipase-3-like gene showed the highest fold change of 2.69, followed by glucose dehydrogenase-like (2.63), fatty acid synthase 1 (2.53), and

lambda-crystallin homolog (2.33).

A total of 14 detoxification-related genes were significantly over-expressed in all insecticide-treated thrips, including four CYP450 genes (*Cyp6a2*, *Cyp6a13*, *Cyp6a14*, and *Cyp6k1*), four UDP-glycosyltransferase genes (*UGT2b2-like*, *UGT2b15-like*, *UGT2b17-like*, and *UGT2c1*), four esterase genes (carboxylic ester hydrolase, gut esterase 1, alpha-esterase-1, and alpha-esterase-5), one GST, and one ATP-binding cassette transporter (ABC transporter G20). The *UGT2b17-like* gene showed the greatest difference, with an average fold change of 3.45. The fold changes of other detoxification genes varied from 1.65 to 2.80 (Table 2-5).

Table 2-5. Over-transcribed genes commonly responded in all three insecticides treatments

Gene ID	Gene Name	Fold Change relative to Control/FPKM Value		
		Chlorfenapyr	Dinotefuran	Spinosad
Proteolysis related				
FOCC005273-RA	Transmembrane protease serine 9 like	2.21/148.8	1.96/119.3	3.05/333.5
FOCC003507-RA	Brachyurin-like	1.96/429.4	1.76/392.8	3.34/737.1
FOCC005119-RA	Thyrotropin-releasing hormone-degrading ectoenzyme-like	2.05/6.20	2.12/6.39	4.43/14.6
FOCC011470-RA	Trypsin-1-like	1.94/1096.7	1.95/1103.8	3.77/1925.3
FOCC011695-RA	Trypsin-2	1.63/17.4	1.93/20.3	2.34/25.0
FOCC006955-RA	Trypsin 3A1-like	1.67/216.7	1.74/221.6	1.86/258.2
FOCC007230-RA	Trypsin-7-like	1.72/54.5	1.70/52.9	1.91/60.7
FOCC005903-RA	Cathepsin L1-like	2.13/3337.9	2.08/3277.3	4.40/6679.1
FOCC008837-RA	Cathepsin B-like	1.64/424.6	1.65/447.9	1.78/553.6
FOCC005265-RA	Carboxypeptidase A1	1.83/70.2	1.65/62.9	2.76/106.6
FOCC005272-RA	Granzyme M-like	1.77/44.1	1.87/45.5	2.04/50.5
FOCC003634-RA	Serine protease 3-like	1.86/343.6	1.77/325.1	3.48/638.7
FOCC017468-RA	Aminopeptidase	1.76/17.8	1.68/16.6	2.55/25.8
FOCC010472-RA	Chymotrypsin-2-like	1.73/11.3	2.00/12.9	2.05/13.9
FOCC016926-RA	Neuroendocrine convertase 2	1.79/67.0	2.23/82.8	7.00/281.9
FOCC013211-RA	Inter-alpha-trypsin inhibitor heavy chain H4	1.79/67.0	2.23/82.8	7.00/281.9
Amino acid metabolism				
FOCC009724-RA	Tyrosine aminotransferase	1.81/9.29	1.75/8.86	2.97/15.9
FOCC001417-RA	4-hydroxyphenylpyruvate dioxygenase	2.04/110.8	1.67/88.4	2.55/141.2
FOCC010397-RA	Proline dehydrogenase 1, mitochondrial	1.63/28.0	1.72/29.2	2.84/49.4
FOCC014418-RA	Glycine cleavage system P protein	1.76/115.0	1.51/95.8	2.08/138.2
FOCC007375-RA	Aromatic-L-amino-acid decarboxylase	1.77/37.9	1.50/31.7	1.73/37.1
Glycosidase activity				
FOCC015589-RA	Myrosinase 1-like	1.92/14.0	1.84/13.0	6.85/50.3

FOCC006088-RA	Maltase B1	2.25/59.2	2.67/69.7	8.21/226.7
FOCC012064-RA	Trehalase	1.67/15.5	1.82/16.7	3.17/29.5
FOCC006089-RA	Maltase A1	1.53/191.4	1.64/202.1	2.43/321.4
FOCC003635-RA	Alpha-amylase B	1.81/197.7	1.65/179.3	4.09/439.8
FOCC005019-RA	Lysozyme C-3-like	1.69/57.3	1.79/59.7	1.72/58.2
FOCC006090-RA	Maltase A6, isoform C	1.62/167.0	1.98/198.5	2.74/296.1
FOCC015912-RA	Glucosylceramidase	1.78/11.4	2.07/13.1	2.26/14.9
FOCC016201-RA	Cytosolic beta-glucosidase-like	1.61/222.9	1.82/247.4	1.89/273.9
FOCC007995-RA	Uncharacterized protein LOC113213115 (levanse)	2.45/219.3	1.80/156.3	6.38/546.5
Lipid metabolism				
FOCC008839-RA	Lipase 3-like isoform X2	1.85/74.2	2.02/80.8	4.20/178.3
FOCC013474-RA	Fatty acid synthase isoform X1	1.78/9.36	1.84/9.59	2.98/21.7
FOCC005111-RA	Lambda-crystallin homolog	1.90/45.2	1.88/44.0	3.22/76.6
FOCC009651-RA	Delta(7)-sterol 5(6)-desaturase erg32-like	1.75/88.0	1.83/90.8	3.08/163.0
FOCC001625-RA	Inactive pancreatic lipase-related protein 1-like	2.08/7.80	1.89/6.89	2.30/9.02
FOCC015494-RA	Receptor-type tyrosine-protein phosphatase N2	1.52/6.52	1.86/8.08	2.75/12.7
FOCC001955-RA	Very-long-chain 3-oxoacyl-CoA reductase	1.67/47.3	1.51/41.6	2.25/63.6
FOCC000160-RA	Putative phospholipase B-like 2	1.70/29.5	1.66/28.2	1.84/31.8
FOCC016660-RA	Long-chain-fatty-acid--CoA ligase 5 isoform X1	1.85/5.20	1.65/4.53	2.11/6.50
Carbohydrate metabolism				
FOCC013203-RA	Glucose dehydrogenase [FAD, quinone]-like	2.92/8.29	1.67/4.33	3.29/9.70
FOCC008953-RA	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase	1.76/29.8	1.69/28.0	3.14/53.4
FOCC006709-RA	Alcohol dehydrogenase [NADP(+)]-like	1.92/39.2	1.73/34.9	2.49/51.0
FOCC003072-RA	Phosphoenolpyruvate carboxykinase [GTP]	1.79/171.2	1.62/150.2	1.96/195.5
Detoxification related				
FOCC016890-RA	Cytochrome P 450 6a2	2.12/46.6	1.57/33.7	2.33/50.7
FOCC004908-RA	Cytochrome P 450 6a13	2.25/29.4	1.71/22.0	2.20/28.6
FOCC016704-RA	Cytochrome P 450 6a14	1.65/16.2	1.76/17.0	2.59/25.5
FOCC016845-RA	Cytochrome P 450 6k1	2.00/228.7	1.70/185.9	3.35/376.2
FOCC003406-RA	UGT2b2like	1.98/23.3	1.93/22.3	4.48/52.7

FOCC002672-RA	UGT2b15 like	1.53/22.2	1.61/23.1	1.81/26.2
FOCC016865-RA	UGT2b17 like	2.00/8.34	2.27/9.39	6.08/25.9
FOCC001729-RA	UGT2c1	1.66/9.48	2.10/11.9	2.41/14.5
FOCC016132-RA	Carboxylic ester hydrolase	1.69/22.0	2.15/27.4	2.72/35.5
FOCC004400-RA	Gut esterase 1	2.26/35.6	1.64/25.3	2.42/38.5
FOCC016012-RA	Alpha-Esterase-5	2.03/2.63	2.13/2.91	2.87/4.48
FOCC011405-RA	Alpha-Esterase-1	1.75/5.93	1.68/5.60	2.34/8.57
FOCC007810-RA	Glutathione S transferase S1	2.69/64.4	1.99/47.2	2.96/71.5
FOCC012231-RA	ABC transporter G family member 20	1.69/19.9	1.72/20.1	2.47/29.0
Energy metabolism				
FOCC013317-RA	V-type proton ATPase 16 kDa proteolipid subunit	1.79/18.2	1.63/16.3	2.05/21.2
FOCC015180-RA	V-type proton ATPase 116 kDa subunit a isoform X2	1.74/17.0	1.77/16.9	2.48/24.4
FOCC009723-RA	Sodium/potassium-transporting ATPase subunit beta-2-like	1.52/17.9	1.98/22.9	2.20/25.8
FOCC005842-RA	Cytochrome oxidase subunit I	2.50/2886.0	1.59/1936.9	4.30/4759.6
Immune response				
FOCC011624-RA	Mucin-2-like	1.54/4.91	2.14/7.10	7.57/26.5
FOCC010854-RA	Ankyrin repeat domain 17	1.53/20.5	1.71/22.4	2.11/28.1
Stress response				
FOCC008259-RA	Calmodulin	1.71/2.84	1.58/2.62	3.33/6.84
FOCC003089-RA	Galectin-4-like	1.52/23.1	1.78/26.5	2.58/39.4
FOCC003922-RA	Phenoloxidase 2-like	1.99/20.8	1.77/18.1	1.99/21.1
FOCC016590-RA	Calcium-binding protein P-like isoform X4	1.59/44.0	1.72/46.2	1.80/49.7
FOCC005163-RA	Inositol 1,4,5-triphosphate kinase 1	1.66/28.9	1.56/26.8	1.76/30.4
Transport				
FOCC002259-RA	Vitellogenin-2-like	1.65/26.3	1.69/26.8	8.78/145.2
FOCC002665-RA	Vitellogenin-1	2.15/148.5	1.85/127.6	6.13/412.9
FOCC009176-RA	Apolipophorins	1.62/27.2	1.82/30.2	5.33/91.6
FOCC006731-RA	Proton-coupled amino acid transporter-like protein pathetic	1.75/3.76	1.53/3.18	4.67/11.7

FOCC013155-RA	Solute carrier family 15 member 1-like isoform X2	1.76/22.7	1.86/23.7	3.76/49.1
FOCC006835-RA	Major facilitator superfamily domain-containing protein 12	1.96/21.3	1.59/16.9	3.09/33.6
FOCC017387-RA	Sialin	1.79/2.72	1.59/2.03	2.16/3.32
FOCC017382-RA	Facilitated trehalose transporter Tret1-like	1.76/2.12	1.54/1.78	1.86/2.54
FOCC012817-RA	Apolipoprotein D-like	1.50/2476.4	1.54/2565.4	1.85/3006.3
FOCC005676-RA	Transferrin-like	1.74/15.5	1.76/15.4	1.68/15.5
Cytoskeleton regulation				
FOCC009162-RA	Paramyosin, long form	1.62/41.5	1.90/48.2	2.40/61.9
FOCC006811-RA	Patronin isoform X6	1.62/9.43	1.88/9.14	1.88/10.6
FOCC001674-RA	Myosin heavy chain, muscle	1.55/110.3	1.79/127.8	1.96/141.8
FOCC013372-RA	TPPP family protein CG45057	1.54/39.0	1.52/37.9	1.73/44.4
Others				
FOCC016038-RA	Glycine-rich cell wall structural protein 1.8-like	2.28/21.1	2.03/18.5	2.95/27.1
FOCC004790-RA	cAMP-specific 3',5'-cyclic phosphodiesterase, isoform I	1.74/16.7	1.50/13.9	2.71/25.9
FOCC011998-RA	Aquaporin-2-like isoform X1	2.25/33.6	1.60/23.4	2.13/31.4
FOCC003906-RA	ADP-sugar pyrophosphatase-like isoform X2	1.98/288.4	1.50/210.5	2.21/326.8
FOCC017436-RA	Putative caffeoyl-CoA O-methyltransferase At1g67980	2.37/114.5	1.52/71.8	1.72/83.9
FOCC001704-RA	Four and a half LIM domains protein 2 isoform X6	1.50/19.4	1.82/23.3	2.22/28.5
FOCC003375-RA	Muscle LIM protein Mlp84B-like	1.57600.6	1.57/595.5	1.74/666.6

While most DEGs were over-transcribed, 31 genes were commonly under-transcribed following treatment with low (LC_{10}) concentrations of these three insecticides. The most down-regulated gene was a zinc finger protein 32-like gene, a probable transcription factor, with fold changes of − 4.20, − 4.19, and − 4.56 by chlorfenapyr, dinotefuran, and spinosad, respectively. In addition, two more transcription-related genes were also down-regulated; the transcription levels of longitudinal lacking and zinc finger and SCAN domain-containing protein 5C were decreased 2.13- and 1.71-fold, respectively. Another significant down-regulated gene was diptericin A, an antimicrobial peptide with fold changes of − 3.35 for chlorfenapyr, − 5.47 for dinotefuran, and − 1.50 for spinosad (Table 2-6).

Table 2-6. Under-transcribed genes commonly responded in all three insecticides treatments

Gene ID	Gene Name	Fold Change relative to Control/FPKM Value		
		Chlorfenapyr	Dinotefuran	Spinosad
Transcriptional regulation				
FOCC005923-RA	zinc finger protein 32-like	-4.20/0.25	-4.19/0.27	-4.56/0.13
FOCC005969-RA	longitudinals lacking	-2.03/3.56	-2.50/2.71	-1.86/4.20
FOCC006858-RA	putative zinc finger and SCAN domain-containing protein 5C	-1.62/1.78	-1.58/1.91	-1.94/1.41
Motor activity				
FOCC007944-RA	Dynein regulatory complex subunit 4	-1.68/2.38	-1.51/2.85	-1.97/2.05
FOCC005330-RA	Dynein regulatory complex protein 8-like isoform X1	-1.78/0.20	-1.50/0.49	-1.66/0.29
Immune response				
FOCC001594-RA	Diptericin A	-3.35/11.5	-5.47/6.68	-1.50/25.8
Cuticle related				
FOCC006813-RA	laccase-2-like	-2.36/0.43	-1.74/1.05	-2.00/0.76
DNA damage response				
FOCC008345-RA	mediator of DNA damage checkpoint protein 1-like isoform X2	-1.87/20.3	-1.56/24.1	-2.23/17.3
FOCC013344-RA	poly [ADP-ribose] polymerase 12-like isoform X2	-1.59/3.16	-1.55/3.29	-1.54/3.52
Others				
FOCC009666-RA	Beta-1,3-galactosyltransferase brn	-1.67/3.87	-1.67/3.82	-1.93/3.42
FOCC007243-RA	B9 domain-containing protein 1	-1.83/9.10	-1.81/9.08	-1.94/8.87
FOCC010451-RA	chromatin accessibility complex protein 1-like	-1.52/0.79	-1.52/0.85	-1.66/0.70
FOCC016188-RA	transposable element tc3 transposase	-1.57/2.71	-1.57/2.79	-1.67/2.75
FOCC012227-RA	putative ferric-chelate reductase 1 homolog	-1.64/0.31	-1.53/0.44	-1.71/0.25

3.4 Validation of DEG profiles by qPCR

To confirm the reliability of the DEG data, qPCR experiments were conducted. The fold changes of seven major genes obtained from qPCR were plotted against the fold change values estimated from transcriptome data (Fig. 2-4). The resulting correlation coefficient was 0.774, suggesting that DEG profiles obtained from the transcriptome data were moderately reliable.

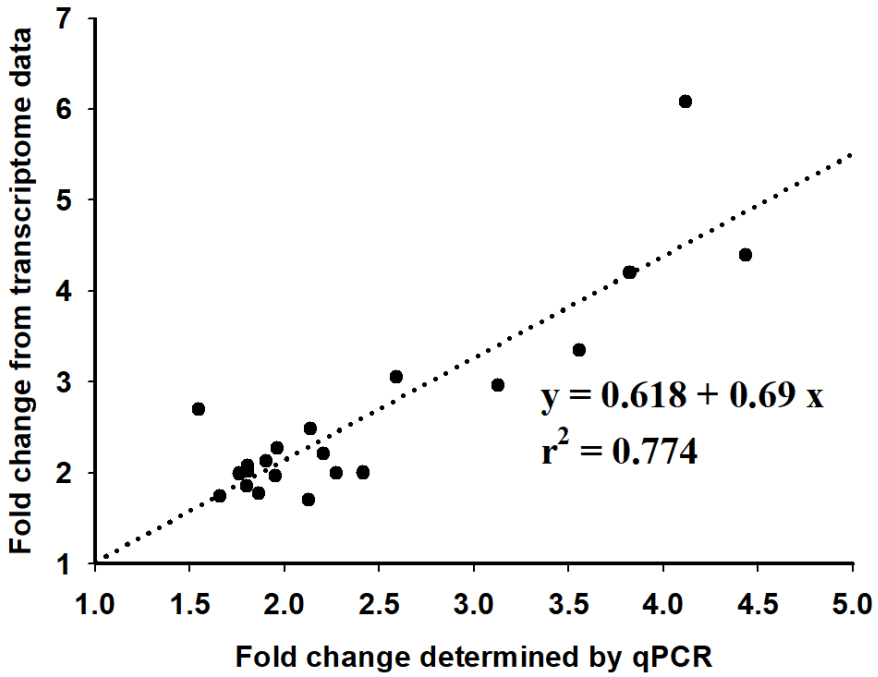

Fig. 2-4. The plot of the fold changes (FC) obtained from qPCR vs. the FC values estimated from transcriptome data. The dotted line indicates the linear regression line. The qPCR was conducted with three biological replicates, and the mean FC value was used for plotting.

3.5 Effects of target gene RNAi on the insecticide toxicity

The qPCR results showed that ingestion RNAi induced 59.3%, 80.5%, 42.8% 42.4% and 49.6% knockdown of all genes (*Cyp6a2*, *Gcwp1.8*, *GstS1*, *Mb-1* and *Tps9*) tested when compared with control (Fig. 2-5). When treated with LC_{50}s of chlorfenapyr, dinotefuran and spinosad, mortality increased 31.2%–65.1%, 16.9%–31.9% and 13.4%–29.5%, following knockdown of the representative target genes except for the thrips treated with dsMb1 followed by dinotefuran (Fig. 2-6). Among them, thrips treated with the combinations of ds*Cyp6a2*-chlorfenapyr, ds*Cyp6a2*-spinosad and dsMb1-spinosad exhibited significant mortality elevations ($p < .05$).

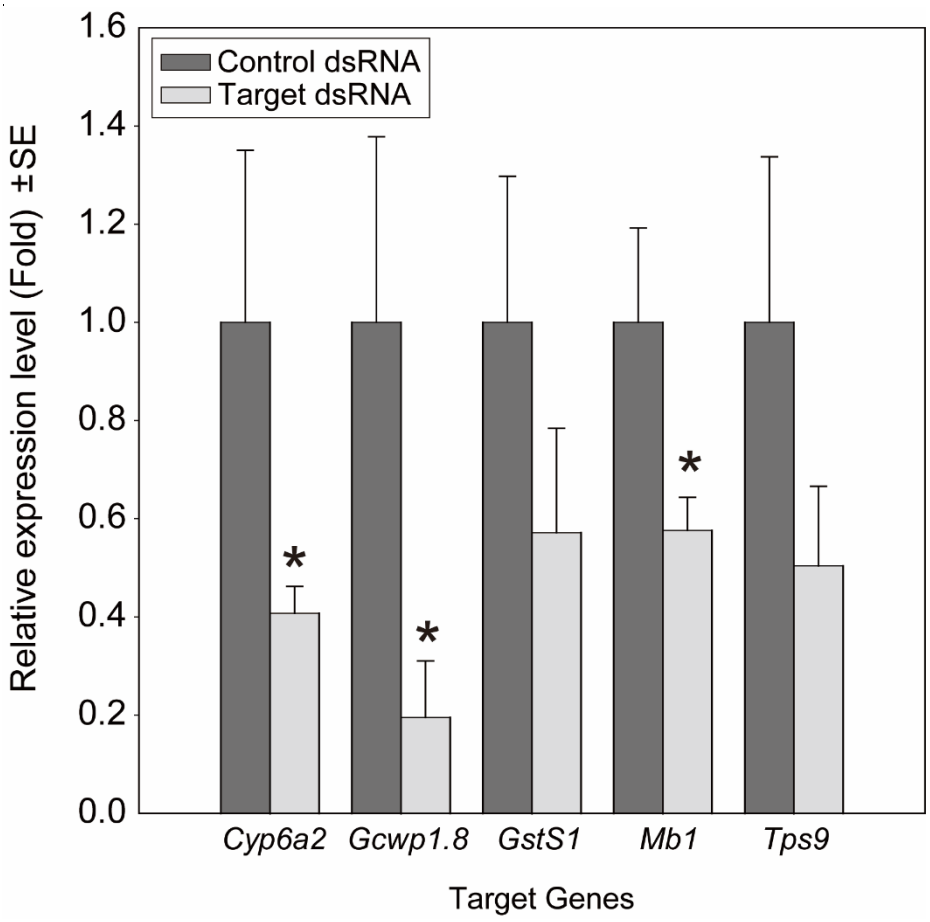

Fig. 2-5. Reduced transcription levels of representative target genes followed by ingestion RNAi. The star marks indicate significant differences ($p < 0.05$, Student's *t*-test) in transcription level between control (dsAce1-treated) and target dsRNA-treated thrips.

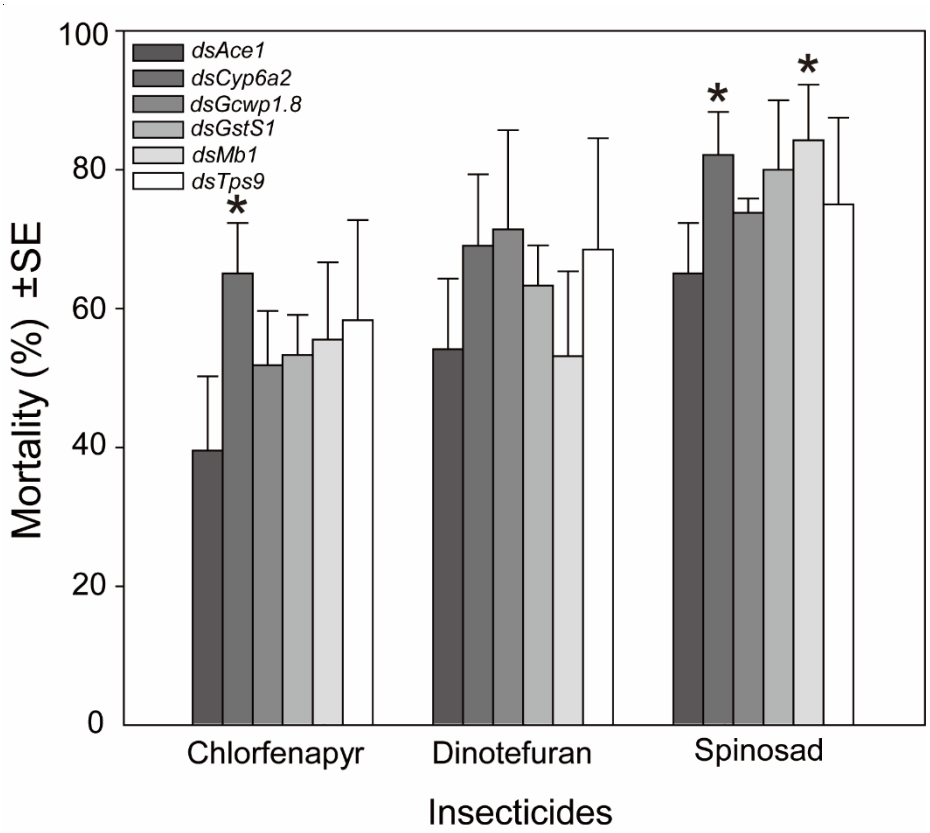

Fig. 2-6. Enhanced mortalities in the thrips treated with dsRNA of representative target genes. Mortality was evaluated at 8 h post-treatment of LC_{50} of the test insecticide. The star marks indicate significant differences ($p < 0.05$, Student's *t*-test) in mortality between control (dsAce1-treated) and target dsRNA-treated thrips.

4. DISCUSSION

4.1 Tolerance induction

Compared to some other published data, the LC_{10} and LC_{50} of the RDA susceptible strains seem to be somewhat higher. Although the RDA strain has not been exposed to any insecticide for almost 20 years after field collection, some general resistant traits may be still maintained in this strain, thus reducing the baseline susceptibility to the test insecticides. Pretreatment with concentrations of five insecticides, which result in only low levels of lethality or were sublethal, has been shown to increase tolerance in *P. xylostella* larvae (Gao et al. 2018). Similar cases of tolerance induction have been reported in body lice (Yoon et al. 2011) and *Drosophila melanogaster* (Kim et al. 2018) that were briefly exposed to a sublethal dose of ivermectin. In the current study, tolerance enhancement was also observed in all examined cases regardless of insecticide type, suggesting that tolerance induction by sublethal or low lethal treatments with insecticides is a rather general phenomenon. Since the induced overexpression of detoxification proteins, such as Cyp450s and ABC transporters, are responsible for tolerance in body lice (Yoon et al. 2011), differentially expressed genes upon sublethal/low lethal (LC_{10}) exposures to insecticides likely mediate tolerance induction in *F. occidentalis*, as well.

4.2 GO profiles of DEGs

The overall GO profiles of the majority of DEGs in every category were up-regulated following pretreatments with LC_{10} concentrations of insecticides. In contrast, under-transcribed DEGs with complete annotation only accounted for 10.3% in chlorfenapyr-treated thrips, 12.2% in dinotefuran-treated thrips, and 14.4% in spinosad-treated thrips. In the categories of metabolic process (P), catalytic activity (F), and transporter activity (F), the ratios of these items in up-regulated DEGs were significantly higher than in down-regulated DEGs. As these GO categories are likely crucial components in the xenobiotics detoxification process, their up-regulation in the insecticide-treated thrips may be connected to the increase in tolerance. The commonly responding DEGs also showed slightly higher ratios in metabolic process (P), extracellular region (C), extracellular region part (C), and catalytic activity (F) suggesting that the general chemical defense-related DEGs may be more common in these categories.

4.3 Comparison of DEG profiles between *F. occidentalis* and *P. xylostella*

When comparing *F. occidentalis* transcriptome with previously reported data from *P. xylostella* (Gao et al. 2018), overall DEG profiles

were completely different, suggesting that the gene sets involved in tolerance induction at the initial stage of intoxication can vary greatly depending on the insect species, developmental stage and its physiology. One main difference was that there were no up-regulated cuticular protein genes in *F. occidentalis*, but these genes were commonly overexpressed in the treated *P. xylostella* larvae and were suggested to be associated with desiccation resistance. Since *F. occidentalis* is a sucking pest and likely consumes more liquid than *P. xylostella*, water preservation may be less crucial in this species, so induced expression of cuticular proteins may not be required in *F. occidentalis*.

Another main difference can be found in the mitochondrial respiratory chain system, which was not affected in F. occidentalis in the current study. In contrast, its several components were down-regulated in all treated *P. xylostella* larvae following sublethal treatment with insecticides, where reduced energy generation was proposed to be beneficial for acquiring tolerance. In *F. occidentalis*, however, insecticide treatment induced the overexpression of several genes involved in carbohydrate and lipid metabolic processes, thereby resulting in elevated metabolism. It can be speculated that the role of energy metabolism in the tolerance induction process differs between these two species when exposed to sublethal doses of insecticides. It is also worthy

to note that the insecticide treatment methods were different between the two studies. Only contact intoxication is possible in the RCVpW bioassay method for *F. occidentalis* whereas both contact and ingestion intoxication is possible in the leaf dipping bioassay used for *P. xylostella*, which can be affected by more variable factors. Further study is warranted to identify the associations between energy metabolism and tolerance induction in different species of insects.

4.4 Commonly over-transcribed genes following treatment with sublethal concentrations of insecticides

A total of 34 protein degradation-related genes were up-regulated in tolerance-induced thrips. Several studies have reported elevated proteolytic activity in insects after exposure to various kinds of insecticides (Ahmed et al. 1998; Wilkins et al. 1999). For example, fenitrothion-treated house fly (*Musca domestica*) and ethion-treated silkworm (*Bombyx mori*) showed increased activities of most proteases (Nath et al. 1997; Wilkins et al. 1999). Eight chymotrypsin-like protein genes were up-regulated in insecticide-treated thrips (Zhang et al. 2013). Chymotrypsins have been found to be involved in deltamethrin metabolism in the mosquito *Culex pipiens pallens* (Lv et al. 2016) and the degradation of deltamethrin (Yang et al. 2008). In addition, knockdown of chymotrypsin-like genes significantly enhanced *Bacillus*

thuringiensis insecticidal efficiency in Asian corn borer *Ostrinia furnacalis* (Guan et al. 2017). Taken together, these findings suggest that chymotrypsin-like protein likely responds to intoxication either by bacterial endotoxins or neurotoxic insecticides, as shown in the current study. Eight of 34 trypsin-like genes were overexpressed following sublethal treatment with insecticides. Trypsins, as a group of metabolic enzymes, are also known to be involved in the insecticide stress response (David et al. 2010; Wilkins 2017). The expression level of trypsins has been reported to be affected by insecticide treatments in various insect species (Silva et al. 2010; Zhang et al. 2012; Zhou et al. 2018). Five cathepsin genes, which had cysteine-type peptidase activity, were up-regulated in the current study. A similar case has been reported in green peach aphid *Myzus persicae*, where cathepsins were overexpressed in both susceptible and resistant strains following pirimicarb treatment (Silva et al. 2012).

A total of 18 glycosidase genes, including myrosinase genes, were up-regulated by sublethal treatment with the three insecticides. Myrosinase plays an important part in the plant/insect response to insecticides as a component of the defense system (Zhu et al. 2015). When the brown planthopper *Nilaparvata lugens* and silk worm *B. mori* were treated with different insecticides, both the activity and expression

level of soluble trehalase increased significantly. Maltase B1, a glycosidase, was also up-regulated significantly in a fruit fly *D. melanogaster* strain exposed to DDT (Seong et al. 2017). Feeding the Egyptian cotton leafworm *Spodoptera littoralis* an artificial diet supplemented with a sublethal concentration of cyfluthrin enhanced the activity of maltase and amylase (Bernard et al. 1993). These findings may indicate that xenobiotic stress caused by insecticides destroys the homeostasis of energy metabolism, thus inducing glycogenolysis to meet the required energy demands.

Four Cyp450 genes (*Cyp6k1*, *Cyp6a13*, *Cyp6a2*, and *Cyp6a14*) were commonly over-expressed in response to the three insecticides. CYP6 groups are well known to be involved in metabolism of various insecticides and xenobiotics, thus conferring tolerance/resistance (Feyereisen 2006). Multiple reports have found that various CYP6 groups, such as Cyp6g1, Cyp6p4, and Cyp6p9, are involved in insecticide resistance in different species (Bass et al. 2011). The commonly up-regulated nature of CYP6 family genes following sublethal exposure to insecticides with distinct structures (i.e., chlorfenapyr – pyrrole; dinotefuran – neonicotinoid; and spinosad – macrocyclic lactone) suggests that the Cyp6 family is involved in the detoxification of a broad range of insecticides and xenobiotics.

Another Cyp450 gene, *Cyp304a1*, was also overexpressed in spinosad-treated thrips, and this finding is consistent with a previously reported study of spinosad-treated diamondback moths (Gao et al. 2018). Except in the spinosad-treated sample, *Cyp304a1* did not respond to any other tested insecticides (chlorantraniliprole, chlorfenapyr, cypermethrin, dinotefuran, indoxacarb), indicating *Cyp304a1* is somewhat specific to spinosad.

In the current study, four UGT genes were commonly overexpressed following sublethal exposure to the three insecticides. UGTs are phase II detoxification enzymes that can catalyze the conjugation of small lipophilic molecules with uridine diphosphate (UDP) sugars, thus increasing their water solubility for efficient elimination. Overexpression of UGT2b17 has been reported to be responsible for chlorantraniliprole resistance in the diamondback moth, and its expression level was shown to increase 30.7- to 77.3-fold in four resistant strains (Li et al. 2017).

GSTs also play important roles in phase II metabolism of detoxification and protection from the oxidative stress caused by exposure to insecticides. In the current study, only one GST S1 was found to be overexpressed in all three insecticide treatments. Similar cases have also been reported in *D. melanogaster* and the beet armyworm *S. exigua* when treated with an insecticidal extract of *Piper nigrum* and

chlorantraniliprole; in these studies, the relative expression level of GST S1 increased 2.22- and 2.31- fold, respectively (Jensen et al. 2006; Liu J 2013).

The ATP binding cassette transporters (ABC transporters) mostly function as primary active transporters that use ATP while transporting substrates across lipid membranes. The ABC transporter gene family is associated with resistance of carbamates, macrocyclic lactones, neonicotinoids, organophosphates, and pyrethroids in more than 20 insect species (Wannes Dermauw 2014). In the spinosad-resistant house fly, all three transcripts of ABC transporter G20 were over-transcribed, indicative of possible involvement in resistance (Hojland et al. 2017).

4.5 Mortality increase in the thrips with representative target genes knocked down

All combined treatments of dsRNA and insecticides showed tendencies of increased mortality when compared with the control combination except for the ds*Mb1*-dinotefuran treatment although not all results were supported by statistical analysis. This finding supports that the commonly overexpressed genes, at least the five genes tested, are likely involved in tolerance induction to the three insecticides in *F. occidentalis*.

5. CONCLUSION

When adult western flower thrips were treated with low and medium lethal doses of three different insecticides consecutively using the RCVpW method, tolerance was significantly increased following all insecticide treatments. Through comparative transcriptome analysis, genes that may commonly contribute to insecticide tolerance were characterized. Four Cyp450 genes, four UGT genes, and another six detoxification-related genes were commonly overexpressed. In addition, genes involved in the catabolism of protein, lipid, and carbohydrate were up-regulated. In contrast, three transcriptional regulators were under-scribed. Considering the physiological functions of these genes, their up- or down-regulation appears to be involved in direct or indirect detoxification processes. Furthermore, their common responsiveness to three insecticides with distinct structures and modes of action indicates their potential roles in the general defense system. Detoxification-related genes that can be induced by sublethal or low lethal doses of insecticides are highly likely to be fixed in the population when insects are selected further. Therefore, systematic identification of such differentially expressed genes would help our understanding of both universal and specific metabolic resistance factors, which further benefits the proactive management of resistance.

CHAPTER III Transcriptomic identification and characterization of genes responding to sublethal concentrations of six different insecticides in the common fruit fly, *Drosophila melanogaster*

ABSTRACT

Pretreatment of sublethal concentrations (LC_{10}) of six insecticides (chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad) significantly elevated tolerance of the common fruit fly, *Drosophila melanogaster*, to the lethal concentration of respective insecticide. Commonly responding genes to sublethal treatments of the six insecticides were identified by transcriptome analysis based on a fold change > 1.5 or < −1.5 and $p < 0.05$ as selection criterions. Following all six insecticide treatments, 26 transcripts were commonly over-transcribed, whereas 30 transcripts were commonly under-transcribed. The reliability of the transcriptome data was confirmed by quantitative PCR. The majority groups of overexpressed transcripts included genes related with olfactory behavior, such as odorant-binding proteins, and immune related genes, including attacin, diptericin and immune induced molecule 18 In contrast, genes belonging to the mitochondrial respiratory chain were commonly underexpressed, such as mitochondrial NADH-ubiquinone oxidoreductase chain 1/3/4/5 and mitochondrial cytochrome b/c. Genes related to eggshell formation and motion were also under-

transcribed, which may indicate the energy trade-off happened when the *D. melanogaster* dealt with xenobiotic stress. Since the tested insecticides have distinct structures and modes of action, the roles of commonly expressed genes in tolerance and different responding gene pool in different insect species were discussed.

Keywords: Common fruit fly; Insecticide; Sublethal concentration; Tolerance; Transcriptome analysis; Immune response; Olfactory behavior; Respiratory chain

1. INTRODUCTION

The common fruit fly, *Drosophila melanogaster,* is one of the most intensively studied organisms in biology and serves as a model system for the investigation of many developmental and cellular process because of its rapid life cycle and relative simple genome (Adams et al. 2000). Based on these characteristics, *D. melanogaster* has also been used as a model insect for insecticide resistance studies (Wilson 1988).

One of the most important mechanisms for insecticide resistance is enhanced metabolism, which can be acquired by either overexpression or structural alteration of detoxification genes such as cytochrome P450s (CYPs), glutathione S-transferases (GSTs), ATP-binding cassette transporters (ABCTs) and UDP-glucuronosyltransferases (UGTs), etc (James et al. 2009; Yoon et al. 2011). The well-studied genome of *D. melanogaster* has facilitated discovering the metabolic and toxicodynamic mechanisms related with insecticide resistance (Scott et al. 2019; Daborn et al. 2007). Understanding how the detoxification factors respond to insecticide stress may help us to have a full view of the process how insecticide-induced tolerance factors are fixed as metabolic resistance trait in insects.

The insecticides used for this study are chlorantraniliprole,

cypermethrin, dinotefuran indoxacarb, ivermectin and spinosad. Chlorantraniliprole (IRAC class: 13) is an anthranilic diamide developed by DuPont, it targets the muscle calcium channel ryanodine receptor, caused the uncontrolled calcium ion release and the rapid paralysis and death of insects (https://pubchem.ncbi.nlm.nih.gov/ compound /Chlorantraniliprole). Cypermethrin (IRAC class: 3A) is a synthetic pyrethroid and mainly prolongs the opening of voltage-gated sodium channel and cause the hypo-polarization and hyperexcitation of the cell, and finally insect death (https://pubchem.ncbi.nlm.nih.gov/ compound/2912). Dinotefuran (IRA-C class: 4A) is a member of neonicotinoids, it acts as an agonist of the insect nicotinic acetylcholine receptors therefore disrupts the acetylcholine mediated neurotransmission (C. Abstracts 2004). Indoxac-arb (IRAC class: 22A) is an oxadiazine pesticide, and blocks the ion conductance of the neuronal sodium channel and nicotinic receptors (https://pubchem.ncbi.nlm.nih.gov/compound/107720). Ivermectin (IR-AC class: 6) is a macrocyclic lactone derived from *Streptomyces avermitilis*, it binds and activates glutamate-gated chloride channels (GluCls) on neurons and pharyngeal muscle cells (https://pubchem.n-cbi.nlm.nih.gov/compound/6321424). Spinosad (IRAC class: 5) is a macrocyclic lactone which derived from *Saccharopolyspora spinose*. The spinosyns mainly target binding sites on nAChRs and therefore

disrupt acetylcholine neurotransmission. Spinosyns also have secondary effects as a γ-amino-butyric acid (GABA) neurotransmitter agonist (Sparks et al. 2001).

To study the detoxification genes that are involved in insecticide metabolism, the non-invasive induction assays were used (Kim et al. 2018; Yoon et al. 2011). Compared with lethal treatment, treatment with a sublethal concentration (i.e. < LC_{10}) of insecticides could be a better strategy for identifying genes involved in tolerance the actual metabolism of the insecticide and avoid some noises from general physiological stress (Yoon et al. 2011). Previous studies using the similar approach showed some cuticular protein genes, detoxification genes and proteolysis-related genes may be involved in the initial tolerance induction in *P. xylostella* and *F. occidentalis* (Gao et al. 2018; Gao et al. 2020).

In this study, inducible metabolic factors that can contribute to the insecticide tolerance/resistance were identified by comparing the transcript profiles between insecticide-treated and untreated *D. melanogaster*. Characterization of the commonly responding genes to sublethal concentrations of different insecticides may improve our understanding of the general chemical defense mechanisms in *D. melanogaster.* In addition, cross-comparison between the three insect

species [*P. xylostella*, *F. occidentalis* (Gao et al. 2018; Gao et al. 2020) and *D. melanogaster*] treated with the same condition would provide the basic information on the similarities and species-specific differences in the way of insect's responses to xenobiotic stress.

2. MATERIAL AND METHODS

2.1 Insect strains and rearing

The wild type Canton-S strain of *D. melanogaster* was obtained from the Lab of Insect Physiology (Seoul National University, Seoul, South Korea) and was reared on the standard *Drosophila* media. Briefly, for 200 ml water, the media contains 1.84 g agar, 12.5 g dry yeast, 8.16 g corn meal, 16.8 g dextrose, 1 ml honey dew, 2.92 ml mold inhibitor and 1.15 ml antibiotics. The flies were kept in fly vial (25-mm diameter × 95-mm height; Hansol Tech, Seoul, Korea) under the condition of 25 ± 1 °C, 60 ± 5% relative humidity, and a photoperiod of 16:8 (L: D) h. In each fly vial, 100-150 adults were maintained.

2.2 Determination of sublethal concentrations and tolerance bioassay

Six technical grade insecticides of chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Chem Service Inc. (West Chester, PA, USA). Insecticides were dissolved in acetone and then serially diluted with acetone to obtain different concentrations of each insecticide. Ten of newly emerged female adult files (within 2 days) were used in each sample, 0.2 μl of insecticide was applied to the junction area

between head and thorax of the individual fly by using a microliter syringe (Hamilton Company Inc. Reno, NV, USA), then the treated flies were placed in the rearing incubator, and the mortality was calculated at 16 h post-treatment. For each concentration of all insecticides, three replications were conducted. The LC_{10} and LC_{50} were determined by Probit analysis using IBM SPSS Statistics software 20.0 (IBM Crop., NY, USA).

To determine whether *D. melanogaster* exposed to sublethal (LC_{10}) concentrations of insecticides developed tolerance, female adults were pretreated with LC_{10} of insecticides. For the control group, female adults were treated with acetone only. After 8 h post-treatment, the pretreated or control flies were treated again with medium lethal concentrations (LC_{50}) of the same insecticides, and mortalities were evaluated after 16 h post-treatment. The tolerance bioassay was conducted with three replicates, each with 30 females in the LC_{10} pretreatment and 27-30 females in the second LC_{50} treatment. Statistical differences in mortality responses were determined by Student's *t*-test (Sigmaplot 12.0, San Jose, CA, USA).

2.3 Insecticide treatment and total RNA extraction

Sublethal concentrations of the six insecticides were treated to thirty

newly emerged female adults using the same method as mentioned above. Any survived flies were subjected to RNA extraction after 16 h treatment. The treated flies were homogenized in TRIzol reagent (MRC, Cincinnati, OH, USA) with stainless steel beads (JC Bio, Seoul, Korea), and total RNA was extracted according to the manufacturer's instructions.

2.4 Library construction and sequencing

RNase-free DNase I (Takara, Shiga, Japan) was added to the prepared total RNA for avoid any contamination from genomic DNA. Concentration and integrity of the RNA samples were determined using a NanoDrop 8000 spectrophotometer (Thermo, Waltham, MA, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. The RNA samples which met the requirements of an $OD_{260/280}$ value ≥1.8 and integrity number ≥ 7.0 were used for further steps. The qualified total RNA samples were used for mRNA preparation and cDNA library construction by the Illumina TruSeq stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Multiple cDNA libraries were then paired-end sequenced by using an Illumina NovaSeq 6000 Sequence System (Illumina, San Diego, CA, USA).

2.5 Sequence processing and annotation

Before analyzing the data, quality control was conducted to check the overall read quality, total reads, and GC rate. The raw sequence data coming from previous pipelines were imported to FastQC (Babraham Bioinformatics, Cambridge, UK) to have a quick check and then subjected to Trimmomatic program (Illumina, San Diego, CA, USA) with default parameters to remove artifacts such as the adapter, contaminant DNA, and PCR duplicates. For quality trimming, qualified reads with Phred quality > 30 were obtained, and the reads with minimum read length of < 100 bp were trimmed. The aligned reads were generated by using HISAT2 (CCB, Johns Hopkins University, Baltimore, MD, USA) and used Ensembl (https://asia.ensembl.org/index.html) as the reference.

2.6 Reference-based differentially expressed gene (DEG) analysis

The overall UniGene expression pattern of individual samples were obtained by aligning reads with the reference transcriptome using bowtie2 with custom parameters (Langmead et al. 2012). Transcript abundances in reads per kilobase per million reads mapped (RPKM) were estimated using RSEM (RNA-Seq by Expectation Maximization)

through the Trinity plug-in, run_RSEM.pl (Li et al. 2011; Grabherr et al. 2011). In order to identify the differential expression patterns of transcripts, the TMM-normalized RPKM matrix was used for generating heat maps under R programming environment (Team 2015). Gene expression levels were compared between control groups and the other insecticide-treated samples and that showed a fold change of > 1.5 or < −1.5 were considered to have been up or down regulated by the insecticide treatments. Among these, genes commonly over- or under-transcribed in all insecticide-treated *D. melanogaster* samples were selected for subsequent characterization. The correlation coefficient was determined by Pearson's correlation analysis using SigmaPlot (Version 12.0, San Jose, CA, USA).

2.7 Quantitative real-time PCR (qPCR)

Transcription levels of ten DEGs were selected for validation through qPCR, included three olfactory genes (odorant-binding protein 19a, odorant-binding protein 99b and antennal protein 10), three immune related genes (diptericin, immune induced molecule 18 and attacin-C), one detoxification gene glutathione S transferase E4, one proteolytic gene lysosomal aspartic protease, one eggshell structure protein chorion protein 16 and one cytochrome b5 with a fold change >1.5. Sequence-specific primer sets were designed based on the cDNA sequences of

selected genes (Table 3-1). Total RNA was extracted from sublethal concentrations treated flies by using the same methods described above and were treated with DNase I (Takara) to remove any gDNA contamination. cDNA was synthesized using the Superscript IV kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Each 20 µl qPCR reaction contained 25 ng cDNA, 5 pmol of each primer, and 10 µl of SYBR I 2 × master mix (Takara, Shiga, Japan) and was performed using the Roche LightCycler 96 system (Roche, Basel, Swiss). Reactions were incubated at 95 °C for 30 s, followed by 40 cycles of thermal program (5 s at 95 °C, 15 s at 56 °C, and 15 s at 72 °C). A ribosomal protein L32 (*DMRPL32*) was used as an internal reference gene (Ponton et al. 2011). qPCR for each gene was conducted with three biological replicates for control and each insecticide treatment. Relative transcription levels of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

2.8 Common DEGs identification of dinotefuran and spinosad treatments in three species

Among seven insecticides we used in the whole study, both dinotefuran and spinosad were treated to all three insect species. Therefore, analyzing the common DEGs of these two insecticide treatments may facilitate the understanding of specific insecticide

tolerance induction in different insect species. Since the gene IDs or symbols in each individual species are different, a blastp method was used to identify the homolog genes in these three species. Briefly, I collected the protein sequences of all DEGs from dinotefuran- or spinosad- treated of *P. xylostella* (http://iae.fafu.edu.cn/DBM/), *F. occidentalis* (Focc_2.1, https://www.ncbi.nlm.nih.gov/nuccore/JMDY00000000.2/) and *D. melanogaster* (https://flybase.org/). *D. melanogaster* was used as reference because it is the most well-annotated, whereas the other two species were used as queries to perform blastp. The genes showed in both query lists were identified as common DEGs across all three species.

Table 3-1. Sequence of primers used in qPCR

Gene Name	**Primer Name**	**Sequence**	**GC Content (%)**	**Tm (°C)**	**Amplicon Length (bp)**
Ribosomal Protein L32	DMRPL32-F	ATGCTAAGCTGTCGCACAAATG	45	60.1	107
	DMRPL32-R	GTTCGATCCGTAACCGATGT	50	58.4	
Attacin-C	DMAttC-F	TTGGACCTAAGCAAGGCCGT	55	60.5	130
	DMAttC-R	GATTGTTGTAGCCCAGGGTG	55	60.5	
Antennal protein 10	DMA10-F	AAGAATGGTGGAGCAGGCCT	55	60.5	105
	DMA10-R	GCCCTCCAGGCACTTTATGT	55	60.5	
Glutathione S transferase E4	DMGstE4-F	GGACGATGATGCCTGCATCT	55	60.5	140
	DMGstE4-R	ACACCCGACTCGAAGTGCAT	55	60.5	
Odorant-binding protein 99b	DMOBP99b-F	TGGCCGATCACCATCACCAT	55	60.5	118
	DMOBP99b-R	GTACTTCTCCACGAGCTCCT	55	60.5	
Diptericin	DMDPT-F	CCGCAGTACCCACTCAATCT	55	60.5	123
	DMDPT-R	TCAGTCCAATCTCGTGGCGT	55	60.5	
Immune induced molecule 18	DMIM18-F	AGGAAACGGATCGGGATCTG	55	60.5	100
	DMIM18-R	TGGTTTCCCAATCGGAGCGT	55	60.5	
Odorant-binding protein 19a	DMOBP19A-F	AAAGCTGATGCGCGACGTCT	55	60.5	112
	DMOBP19A-R	TGATGTAGCAGTTGGTGTCCT	47.6	59.5	
CG31928	DMCG31928-F	GACTGCAAGAAGGTGTCTCAT	47.6	59.5	113
	DMCG31928-R	CTGATACGCAGGTTCCACTG	55	60.5	
CG6870	DMCG6870-F	CAGTAATCAGGTGGTCGTTGT	47.6	59.5	124
	DMCG6870-R	CGTAGATTACCACCCAGCAAT	47.6	59.5	
Chorion protein 16	DMCP16-F	GCTACGGCGATGTGGTTAAG	55	60.5	123
	DMCP16-R	CGGTTGAGGGAATTCCAGTC	55	60.5	

3. RESULTS

3.1 Determination of insecticide sublethal concentrations and tolerance induction

LC_{50} concentrations of chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad were 0.73, 0.07, 2.78, 1595.8, 0.64 and 0.85 ppm, respectively (Table 3-2). The LC_{10} concentrations were estimated as 0.066, 0.008, 0.05, 173.5, 0.049 and 0.089 ppm, respectively. To investigate whether tolerance to the test insecticides are inducible in *D. melanogaster*, bioassays using LC_{50} concentrations were conducted following pretreatment with the LC_{10} concentrations of the six insecticides. All pre-treated flies showed reduced mortality compared to the control flies. Mortality reduction rate in each insecticide treatment varies from 33.3% to 57.1%. Among them, the mortality reduction rates were significantly high in chlorantraniliprole-, dinotefuran- and ivermectin-pretreated flies (38.9%, $p = 0.025$; 50.0%, $p = 0.047$; 57.1%, $p = 0.039$, respectively). The mortalities of cypermethrin-, indoxacarb- and spinosad-pretreated flies were also decreased but their reduction rates were not significant ($p = 0.070$, 0.069 and 0.101, respectively) (Fig. 3-1).

Table 3-2. LC_{10} and LC_{50} concentration determination of six different insecticides by topical treatment

IRAC group[a]	Insecticide	N	LC_{10} (ppm)	LC_{10} 95% C.L[b]	LC_{50} (ppm)	LC_{50} 95% C.L[b]
28	Chlorantraniliprole	180	0.066	0.009-0.169	0.73	0.33-1.60
3A	Cypermethrin	186	0.008	0.001-0.020	0.07	0.03-0.16
4A	Dinotefuran	177	0.05	0.002-0.217	2.78	0.83-14.06
22A	Indoxacarb	192	173.48	22.70-385.4	1595.8	782.2-6209.9
6	Ivermectin	180	0.049	0.004-0.149	0.64	0.25-1.43
5	Spinosad	150	0.089	0.010-0.231	0.85	0.37-2.01

[a] Group number classified by the mode of action according to the Insecticide Resistance Action Committee (IRAC).
[b] C.L : Confident Limit

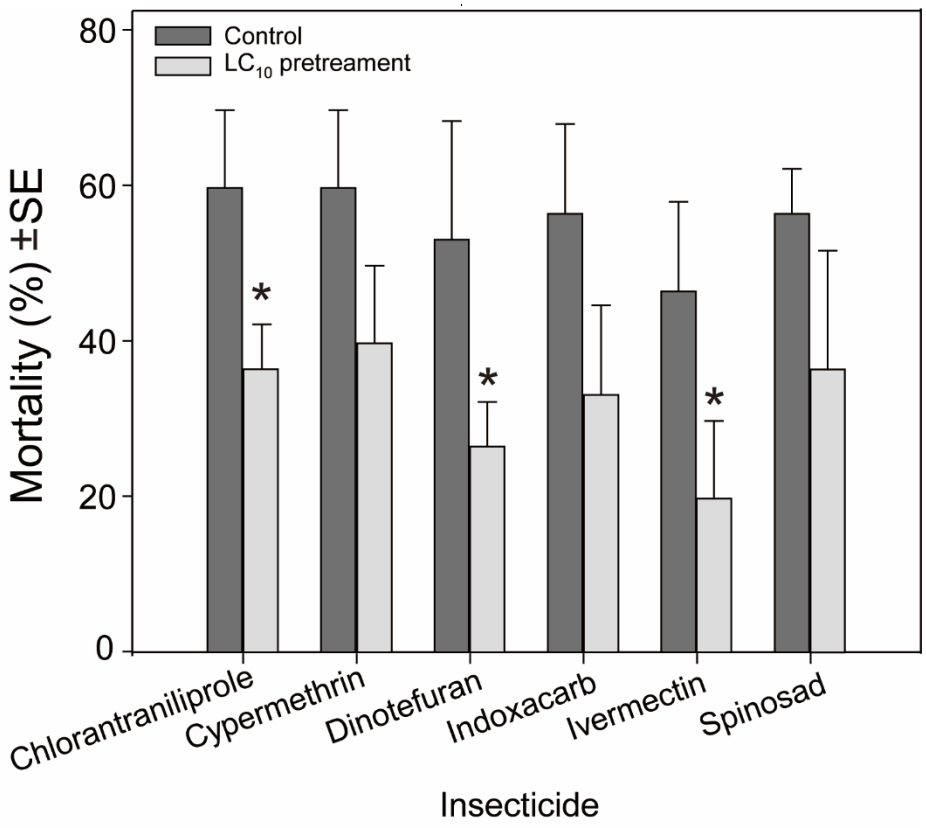

Fig. 3-1. Tolerance induction by pretreatment with sublethal concentrations (LC_{10}) of insecticides. *, $p < 0.05$.

3.2 Transcriptome data analysis

Approximately 42.8, 46.3, 51.5, 53.1, 49.0, 47.5 and 39.7 million reads were generated in the control, chlorantraniliprole-, cypermethrin-, dinotefuran-, indoxacarb-, ivermectin- and spinosad-treated flies, and 92.8, 92.2, 92.5, 92.3, 92.2, 92.4 and 92.5% of these reads were mapped, respectively. A total of 12,202, 12,167, 12,263, 12,086, 12,160, 12,233 and 12,021 genes were assembled from the mapped reads, respectively. Pearson correlation coefficient between FPKM of genes of different samples varied from 0.92 to 1, which indicated a very solid genetic background among these six insecticide-treated samples. DEG analysis showed that there were 260, 281, 277, 328, 427 and 271 genes were significantly differentially regulated. GO enrichment analysis classified the majority of DEGs found in all insecticide-treated groups into three major categories: molecular function, biological process and cellular component. The ratio GO items were 26.0% to 45.5%, 45.1 % to 57.4 % and 9.1% to 16.6% in molecular function, biological process and cellular component, respectively. No apparent differences in annotated GO items were found between different insecticides except dinotefuran-treatment, in which the ratios of enriched GO items in molecular function and cellular component were lower and higher compared to other insecticide-treated groups, respectively (Fig. 3-2).

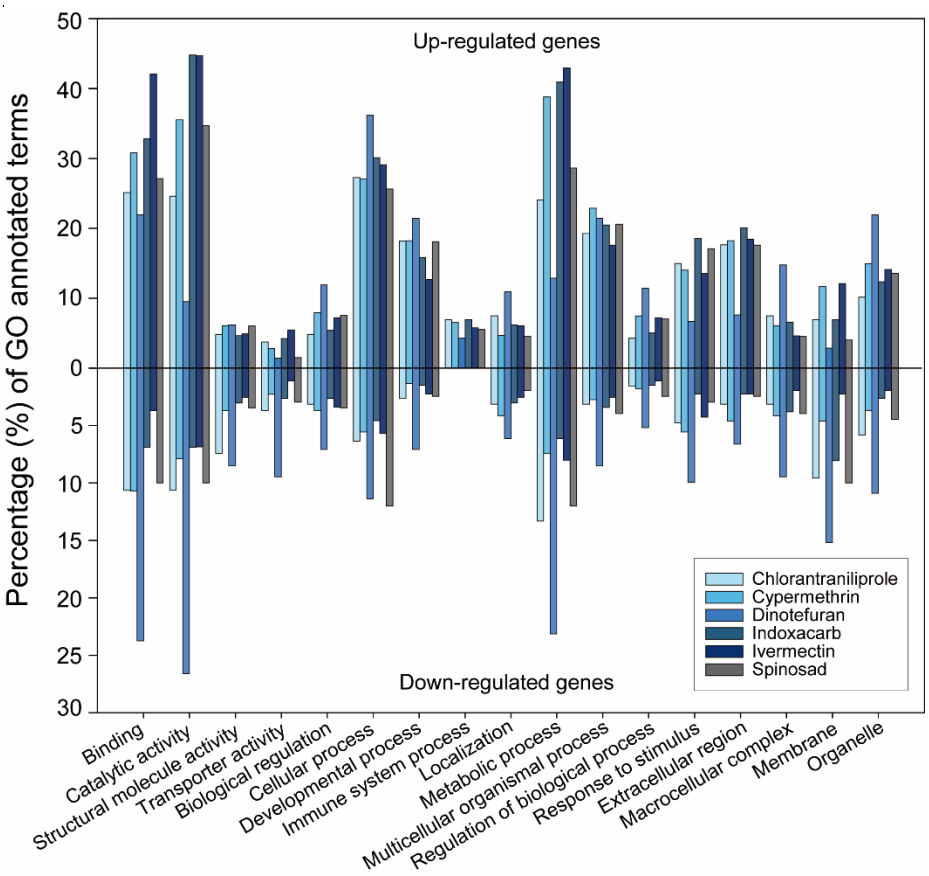

Fig. 3-2. Gene ontology (GO) distribution of differentially expressed genes (DEGs) following treatment with sublethal concentrations of three insecticides.

3.3 DEGs following insecticide treatment

When using a fold change > 1.5 or < −1.5 and $p < 0.05$ as selection criterions, DEG analysis showed that 123, 173, 75, 245, 368 and 145 genes were overexpressed whereas 137, 108, 202, 83, 59 and 126 genes were underexpressed following the treatment of chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad, respectively. All genes overexpressed or underexpressed are listed in Table 3-3 and Table 3-4.

A total of 56 genes that showed more than a 1.5-fold expression difference compared to controls were identified in all six insecticide-treated flies. Among these 56 genes, 26 genes were overexpressed, whereas 30 were underexpressed. The overexpressed genes included ten genes related with olfactory behavior, seven immune response related genes and one detoxification gene.

In the olfactory behavior related genes, all ten genes were overexpressed compared to the control, among which seven were odorant-binding proteins (OBPs) with the average Log_2FC varied from 1.3 (Obp83a) to 3.0 (Obp19c). The other three genes were 3-beta-hydroxysteroid 3-dehydrogenase (antdh), antennal protein 10 and odorant receptor co-receptor (Orco), and their Log_2FC varied from 1.1

to 1.4. A total of seven DEGs were immune response related, including attacin-C, drosocin, diptericin and immune induced molecule 18, etc. All of them showed significantly higher expression level than control samples, with an average Log_2FC varied from 1.2 to 2.4. Two transcription factors [ventral nervous system defective (*vnd*) and extra-extra (*exex*)], one glycosidase maltase-A4 (Mal-A4) and one detoxification-related gene glutathione S transferase E4 (GstE4) were also commonly overexpressed when the flies exposed to sublethal concentrations of all the six insecticides.

While almost half of the DEGs were overexpressed, the other half were commonly under-expressed following the treatment with sublethal concentrations of these six insecticides. Genes encoding eight components of mitochondrial membrane respiratory chain were significantly underexpressed. Four genes encoded the subunit of complex I (NADH-ubiquinone oxidoreductase chain 1/3/4/5), two complex III subunit genes (cytochrome b/b5) and two complex IV subunit genes (cytochrome c oxidase subunit II and subunit 7A1). The greatest expression reduction was found in the mitochondrial NADH-ubiquinone oxidoreductase chain 3 gene (Log_2FC = -3.4), with other genes exhibiting the average Log_2FC from -1.4 to -2.8. Six genes related with eggshell formation were underexpressed at relative high levels with

the average Log_2FC value ranged from -2.9 to -4.7, including chorion proteins CP15/16/19/36, CG32642 and CG12716. However, while all other insecticide treatment caused expression reduction, the sublethal concentration of cypermethrin induced overexpression of chorion protein 16/19/36. In addition, the cuticular protein 72Ec, troponin C isoform 4 and flightin, together with a long non-coding RNA CR43459 were also underexpressed (Table 3-4).

Table 3-3. Over-transcribed genes commonly responded in all six insecticides treatments

Gene ID	Gene Name	Log_2FC relative to Control/FPKM Value					
		Chlorantraniliprole	Cypermethrin	Dinotefuran	Indoxacarb	Ivermectin	Spinosad
Olfactory behavior related protein							
FBgn0031111	Odorant-binding protein 19c	3.98/18.7	3.90/17.2	3.67/14.9	2.55/6.80	-0.89/0.59	0.89/2.21
FBgn0039685	Odorant-binding protein 99b	1.79/194.7	1.23/129.0	2.07/234.3	1.80/191.2	2.02/221.4	1.10/118.4
FBgn0031109	Odorant-binding protein 19a	1.17/5.81	2.12/10.9	0.94/4.92	1.62/7.79	2.06/10.5	1.49/7.10
FBgn0010401	Os-C	0.92/18.7	1.61/29.4	0.98/19.4	1.91/36.4	2.05/39.9	1.46/26.7
FBgn0011283	Pheromone-binding protein-related protein 5	1.17/13.6	1.72/19.5	0.71/9.82	1.76/20.1	1.62/18.1	1.51/17.0
FBgn0011293	antennal protein 10	1.04/20.6	1.49/27.4	1.14/21.8	1.57/29.0	1.83/34.5	1.28/23.9
FBgn0010403	Olfactory-specific E	1.24/28.1	1.54/33.5	1.08/24.8	1.52/33.3	1.75/38.7	1.17/26.2
FBgn0011281	Pheromone-binding protein-related protein 3	0.98/18.1	1.60/27.1	0.79/15.7	1.56/26.3	1.66/28.0	1.33/22.5
FBgn0037324	Odorant receptor co-receptor	1.15/3.68	1.41/4.28	0.85/3.03	1.67/5.21	1.33/4.07	1.26/3.92
FBgn0026268	Antdh	0.73/8.11	1.15/10.6	0.65/7.60	1.26/11.5	1.71/15.5	1.12/10.4
Immune response							
FBgn0041579	Attacin-C	3.11/153.0	1.62/52.9	2.51/99.8	2.99/137.3	2.53/99.2	1.36/44.6
FBgn0010388	Drosocin	1.68/90.1	2.11/117.8	1.88/102.0	2.13/119.7	1.64/84.9	2.27/132.5
FBgn0067903	Immune induced molecule 18	1.96/12.5	1.89/11.6	2.06/13.2	1.86/11.4	2.06/13.0	1.87/11.5
FBgn0034407	Diptericin B	1.99/93.8	2.07/96.4	1.68/74.7	2.39/120.8	2.33/114.8	1.08/48.7
FBgn0004240	Diptericin	1.40/38.9	1.84/51.2	1.35/36.9	1.41/38.2	1.48/39.7	1.92/54.5
FBgn0022355	Transferrin 1	1.13/381.0	0.97/332.2	1.83/613.0	1.97/668.0	1.96/657.3	0.55/250.5
FBgn0043578	PGRP-SB1	1.19/47.0	1.35/50.9	0.90/37.9	1.29/49.1	1.69/64.4	0.95/39.0
Homeodomain transcription factor							

FBgn0041156	extra-extra	1.20/3.21	1.71/4.40	0.78/2.33	1.33/3.41	1.68/4.29	1.19/3.10
FBgn0261930	ventral nervous system defective	0.91/2.03	1.34/2.60	0.66/1.61	1.60/2.08	1.35/2.61	1.17/2.33
Metabolic process							
FBgn0033294	Maltase A4	1.26/26.6	0.81/18.9	1.26/26.2	1.25/25.6	1.15/23.8	1.22/25.2
FBgn0261575	target of brain insulin	1.20/181.0	0.44/103.6	1.62/239.1	1.95/295.3	1.37/197.1	0.87/140.2
FBgn0039769	Sphingomyelin phosphodiesterase	1.54/14.6	1.32/12.2	0.90/9.20	0.80/8.51	1.25/11.5	1.33/12.3
Detoxification related							
FBgn0063496	Glutathione S transferase E4	1.62/7.54	2.07/9.88	1.20/5.52	1.90/8.91	1.91/8.92	1.70/7.80
Others							
FBgn0000644	Follicle cell protein 3C	5.39/50.0	1.98/4.59	3.38/12.3	4.16/20.8	-1.48/0.39	3.34/11.9
FBgn0261675	Niemann-Pick type C-1b	1.70/3.42	0.65/1.60	1.03/2.11	1.33/2.59	1.81/3.55	1.14/2.30
FBgn0035768	CG14834	3.53/11.2	4.20/17.4	3.33/9.64	2.89/7.03	-0.72/0.61	2.42/5.11
FBgn0030666	CG12708	2.29/3.91	1.24/1.82	2.44/4.28	1.49/2.22	1.55/2.33	2.14/3.42

Table 3-4. Under-transcribed genes commonly responded in all six insecticides treatments

Gene ID	Gene Name	Log_2FC relative to Control/FPKM Value					
		Chlorantraniliprole	Cypermethrin	Dinotefuran	Indoxacarb	Ivermectin	Spinosad
Mitochondrial respiratory chain							
FBgn0013681	Mitochondrial NADH-ubiquinone oxidoreductase chain 3	-4.07/85.6	-4.23/74.9	-3.30/144.1	-2.94/183.1	-2.67/219.3	-3.12/162.5
FBgn0013679	Mitochondrial NADH-ubiquinone oxidoreductase chain 1	-2.79/340.3	-3.13/261.0	-3.19/255.5	-2.66/363.4	-2.51/401.6	-2.64/370.4
FBgn0013678	Mitochondrial Cytochrome b	-3.15/955.0	-3.39/787.8	-2.64/1341	-2.33/1645	-2.28/1690	-2.61/1363
FBgn0262952	Mitochondrial NADH-ubiquinone oxidoreductase chain 4	-2.83/503.5	-2.91/464.0	-2.79/513.0	-2.18/770.1	-2.14/789.8	-2.44/649.9
FBgn0013684	Mitochondrial NADH-ubiquinone oxidoreductase chain 5	-2.78/274.0	-2.93/240.9	-2.65/297.1	-2.29/376.3	-2.08/433.9	-2.33/367.6
FBgn0032652	Cytochrome b5	-1.96/7.61	-1.33/11.3	-1.52/10.1	-1.47/10.3	-1.56/9.69	-1.53/9.87
FBgn0013675	mitochondrial Cytochrome c oxidase subunit II	-1.48/4723	-1.59/4253	-1.71/3956	-1.12/5911	-1.00/6381	-1.28/5296
FBgn0085201	Cytochrome c oxidase subunit 7A1, mitochondrial	-1.30/203.4	--1.34/192.7	-2.89/66.5	-0.58/326.7	-0.42/364.0	-0.85/272.7
Eggshell formation							
FBgn0052642	CG32642	-1.09/8.61	-1.79/5.13	-6.00/0.28	-8.84/0.04	-8.72/0.04	-1.62/5.79
FBgn0000358	Chorion protein 19	-2.95/22.6	1.41/448.5	-4.17/9.52	-6.35/2.13	-8.30/0.54	-4.82/6.02

FBgn0000359	Chorion protein 36	-3.38/12.7	0.63/198.2	-2.07/31.0	-5.21/3.48	-8.32/0.40	-6.54/1.37
FBgn0000356	Chorion protein 16	-2.31/17.5	1.63/261.5	-5.54/1.85	-6.00/1.33	-7.54/0.45	-3.23/9.05
FBgn0000355	Chorion protein 15	-0.95/963.3	-4.54/77.5	-4.28/94.4	-4.87/62.2	-5.74/33.7	-1.44/671.1
FBgn0030439	CG12716	-2.41/0.43	-2.11/0.52	-3.99/0.14	-2.69/00.35	-2.5/0.39	-3.86/0.16
Muscle and cytoskeleton							
FBgn0005633	Flightin	-2.59/42.5	-4.58/10.4	-6.84/2.21	-1.46/90.7	-0.78/144.4	-1.59/83.3
FBgn0033027	Troponin C isoform 4	-1.94/63.3	-4.05/14.2	-5.33/5.96	-1.34/93.6	-0.98/118.6	-1.57/79.7
FBgn0000047	Actin 88F	-1.52/86.3	-2.44/44.1	-3.96/15.7	-0.86/132.9	-0.30/194.8	-0.94/126.2
FBgn0034151	CG15617	-2.30/1.42	-2.82/0.96	-4.34/0.34	-0.90/3.64	-0.20/5.90	-1.16/3.07
FBgn0036935	CG14186	-1.63/4.67	-1.41/5.31	-0.86/7.88	-0.34/11.2	-1.14/6.37	-1.38/5.46
Catabolic process							
FBgn0051928	Lysosomal aspartic protease	-1.55/7.33	-1.97/5.32	-3.16/2.37	-4.64/0.84	-5.13/0.59	-2.15/21.0
FBgn0051661	Lysosomal aspartic protease	-0.85/7.11	-2.04/3.03	-2.55/2.17	-3.86/0.86	-2.12/2.86	-1.19/5.51
FBgn0036997	L-threonine 3-dehydrogenase, mitochondrial	-0.87/6.49	-1.62/3.75	-1.29/4.80	-1.09/5.41	-1.38/4.41	-1.33/4.62
Cuticular protein							
FBgn0036619	Cuticular protein 72Ec	-1.26/6.06	-1.03/6.92	-1.64/4.61	-1.04/6.93	0.16/15.7	-1.21/6.16
Ribosomal protein and non-coding RNA							
FBgn0085810	28S ribosomal protein S5, mitochondrial	-1.25/5.38	-1.29/5.06	-0.87/6.89	-1.42/4.64	-1.05/5.97	-1.50/4.42
FBgn0263413	CR43459	-1.31/1.00	-0.72/1.47	-1.85/0.68	-0.98/1.23	-1.08/1.13	-1.49/0.87
Others							
FBgn0037292	Mpv17-like protein	-1.41/13.2	-1.17/15.1	-3.03/4.23	-0.70/21.0	-0.41/25.5	-1.34/13.5
FBgn0261504	Signal recognition particle 7SL RNA CR42652	-1.49/19.6	-1.62/17.4	-1.95/14.1	-1.71/16.4	-0.15/48.1	-1.64/17.3
FBgn0040637	CG11458	-1.88/2.83	-1.80/2.90	-2.26/2.15	-0.62/6.61	-0.35/7.93	-1.26/4.26

FBgn0040699	CG15024	-inf/0	-2.06/0.16	-inf/0	-inf/0	-0.87/0.36	-inf/0
FBgn0036044	PDZ and LIM domain protein Zasp	-2.40/0.8	-3.14/0.5	-5.06/0.1	-1.08/2.0	-0.51/3.0	-1.38/1.6

3.4 Validation of DEG profiles by qPCR

The reliability of the DEG data were verified using qPCR. The Log_2FC of ten selected DEGs obtained from qPCR were plotted against those values estimated from transcriptome data (Fig. 3-3). The resulting correlation coefficient was 0.823, suggesting that DEG profiles obtained from the transcriptome data were reliable.

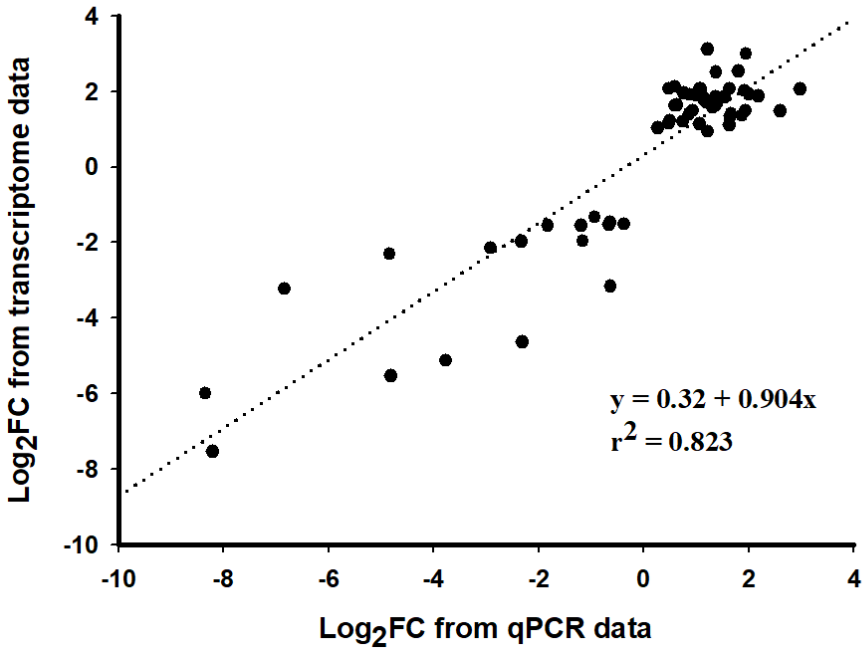

Fig. 3-3. The plot of the fold changes (FC) obtained from qPCR vs. the FC values estimated from transcriptome data. The dotted line indicates the linear regression line. The qPCR was conducted with three biological replicates, and the mean FC value was used for plotting.

3.5 Common DEGs identified in dinotefuran- and spinosad-treated three species

A total of 12 genes were identified in dinotefuran treatments in all three species (Table 2). Among the 12 common DEGs in dinotefuran treatments, only two of them showed consistent over/underexpression. Three genes belonged to CYPs (i.e. Cyp4d21, Cyp6a18 and Cyp6w1) showed a common differential expression patter in all three species treated with dinotefuran, but their expression patterns were distinct each other. All the three CYPs were all underexpressed in *P. xylostella*, whereas they were overexpressed in *F. occidentalis*. In *D. melanogaster*, however, one was overexpressed but other two were underexpressed. Five genes, which have serine-type endopeptidase activity, were identified in dinotefuran-treated samples, among which only the hypodermin-B was consistently underexpressed. In *P. xylostella* and *F. occidentalis*, four and three out of five proteolysis-related genes were overexpressed, respectively. However, these five genes were all underexpressed in *D. melanogaster*.

A total of 22 genes were identified in spinosad-treated three species, among which four genes were CYPs (Cyp4d1, Cyp4e3, Cyp6a2 and Cyp6a8) (Table 3). All the four CYPs were commonly overexpressed in the three species when treated them with sublethal concentrations of

spinosad. In addition, three genes related with lipid metabolism were commonly overexpressed in all three spinosad-treated species.

Table 2. Differentially expressed genes commonly responded in all three insect species in dinotefuran treatments

Gene ID	Gene Name	Log_2FC relative to Control/FPKM Value		
		P. xylostella	***F. occidentalis***	***D. melanogaster***
Detoxification				
FBgn0031925	Cytochrome P450-4d21	-1.87/2.59	0.59/37.2	-inf/0
FBgn0039519	Cytochrome P450-6a18	-1.81/11.0	0.81/10.8	-1.43/7.03
FBgn0033065	Cytochrome P450-6w1	-1.46/5.29	0.59/37.2	1.43/64.9
Proteolysis				
FBgn0039778	Jonah 99Fi	5.64/6.83	1.24/4.52	-1.98/39.1
FBgn0039777	Jonah 99Fii	5.59/6.58	2.02/17.9	-1.58/30.4
FBgn0042186	Trypsin epsilon	5.64/6.83	1.97/16.4	-2.11/6.25
FBgn0034807	Hypodermin-B	-1.75/5.42	-0.60/0.89	-1.73/10.3
FBgn0031406	Spermathecal endopeptidase 1	2.17/53.8	-0.97/2.51	-3.83/3.18
Muscle regulation				
FBgn0005666	Bent	-2.60/2.13	0.77/22.0	-1.13/18.0
FBgn0053519	Unc-89	-2.60/2.13	0.77/22.0	-1.24/10.1
Cytoskeleton regulation				
FBgn0002466	Sticky	1.58/13.1	0.72/179.3	1.12/13.2
Others				
FBgn0038395	Protein takeout	-2.37/7.90	-0.63/3.30	1.42/19.4

Table 3. Differentially expressed genes commonly responded in all three insect species in spinosad treatments

Gene ID	Gene Name	Log_2FC relative to Control/FPKM Value		
		P.xylostella	***F. occidentalis***	***D. melanogaster***
Detoxification				
FBgn0005670	Cytochrome P450-4d1	2.03/2.90	0.91/42.8	1.14/7.68
FBgn0015035	Cytochrome P450-4e3	2.03/2.90	1.12/15.6	0.80/64.6
FBgn0000473	Cytochrome P450-6a2	2.56/9.11	0.87/12.0	1.21/45.6
FBgn0013772	Cytochrome P450-6a8	2.56/9.11	0.91/42.8	1.91/17.4
Proteolysis				
FBgn0031653	Jonah 25Biii	1.61/16.7	0.81/36.7	0.75/777.0
FBgn0003357	Jonah 99Ciii	3.58/33.6	0.81/36.7	0.71/340.0
FBgn0051681	Hypodermin-B	Inf/0.53	-0.79/3.16	-1.35/7.96
FBgn0035670	Chymotrypsin BI	1.63/2.27	1.65/9.33	0.70/302.0
FBgn0259998	Trypsin	1.61/16.7	0.81/36.7	0.97/83.6
FBgn0042186	Trypsin epsilon	-1.07/248.0	-0.79/3.16	-1.15/12.1
FBgn0036738	Serine proteases 1/2	3.58/33.6	0.81/36.7	1.18/86.2
FBgn0031406	Spermathecal endopeptidase 1	-1.34/6.50	-0.79/3.16	-2.23/9.61
FBgn0002926	Nudel	1.63/2.27	0.99/97.9	0.92/8.26
Lipid metabolism				
FBgn0051091	Lipase 3	2.73/7.41	1.02/215.3	1.06/6.22
FBgn0029831	Pancreatic triacylglycerol lipase	1.44/10.8	0.80/3.66	0.82/107
FBgn0032055	Putative fatty acyl-CoA reductase CG5065	2.21/5.69	1.67/39.2	1.45/20.6
Muscle regulation				
FBgn0013348	Troponin C at 41C	Inf/0.63	0.89/7.49	-0.66/191
Cytoskeleton regulation				

FBgn0000047	Actin 88F	-1.30/2.45	0.84/1.94	-0.94/126.0
FBgn0002466	Sticky	1.20/35.4	2.03/439.8	0.66/9.52
Sensory behavior				
FBgn0011293	Antennal protein 10	1.47/33.5	0.96/172.1	1.28/23.9
FBgn0019830	Congested-like trachea	2.29/12.0	0.86/1.31	0.66/95.4
Others				
FBgn0003499	Stripe	-2.33/0.94	1.11/12.8	-1.08/1.17

4. DISCUSSION

4.1 Tolerance induction

Pretreatment with sublethal concentrations of different insecticides has been shown to increase tolerance in *P. xylostella* larvae and female *F. occidentalis* (Gao et al. 2018; Gao et al. 2020). Along with the cases of body lice (Yoon et al. 2011) and *Drosophila melanogaster* (Kim et al. 2018), regardless the insecticide type and insect species, suggesting that tolerance induction by sublethal or low lethal insecticide treatments is a rather general phenomenon. Thus, the commonly responding DEGs may be involved in the process of tolerance induction in *D. melanogaster*.

4.2 GO profiles of DEGs

The overall GO profiles in three major categories had no apparent differences between five insecticide treatments, whereas dinotefuran-treatment showed a relatively different GO profile, in which its enriched GO items in molecular function were lower but those in cellular component were higher. Since there were more underexpressed DEGs in dinotefuran treated sample, the underexpressed GO items also took a major part. Additionally, the most noteworthy GO item was immune

system process, belonging to biological process, it was only found in overexpressed DEGs, indicated the commonly upregulation in the all six insecticide treatments.

4.3 Commonly over-transcribed genes following treatment with sublethal concentrations of insecticides

A total of ten olfactory behavior related genes were overexpressed in the flies treated with sublethal concentrations of all insecticides including seven general odorant-binding proteins (OBPs), one odorant receptor, one antennal protein and one steroid dehydrogenase. In insects, OBPs are thought to be responsible to provide the initial molecular interactions for chemical signals to the olfactory receptors (ORs) (Zhou et al. 2009). When selecting an *Anopheles gambiae* population with a mixture containing pesticide and herbicides for 20 generations, genes encoding six OBPs and one antennal carrier protein, along with the genes related to detoxification, protein and lipid metabolism, were significantly overexpressed compared to the unselected strains (Nkya et al. 2014). Odorant binding protein 2 was found to be significantly induced when exposed to imidacloprid in *Diaphorina citri*. RNAi knockdown of this gene increased the susceptibility of *D. citri* to imidacloprid, and its recombinant protein showed a strong binding property to imidacloprid *in vitro* (Liu et al. 2020). Another study revealed that two general OBPs,

AlepGOBP1 and *AlepGOBP2*, have distinct binding affinity to sex pheromones, plant volatiles and insecticides in *Athetis lepigone* (Zhang et al. 2020). Since these six OBPs in our transcriptome analysis were commonly overexpressed when exposed to six different insecticides, they might have a broad binding spectrum to chemicals, thereby likely functioning as non-specific bioscavengers in *D. melanogaster*. Since no similar genes related with olfactory behavior were identified in either *P. xylostella*, or *F. occidentalis* (Gao et al. 2018; Gao et al. 2020) following the sublethal exposure to insecticides, overexpression of the genes in olfactory system appears to be specific to *D. melanogaster*. In addition to OBPs, other overexpressed components in the olfactory system may be involved in the initial recognition of insecticides penetrated into body, thus initiating the signaling cascades that lead to the downstream detoxification and tolerance induction in *D. melanogaster*.

A total of seven immune related transcripts were commonly overexpressed in all six insecticide treatments, including six antimicrobial peptides (AMPs) and one peptidoglycan recognition protein. Innate immune response is the only response system which insects rely on to defense against foreign pathogens since insects lack an adaptive immune system. AMPs are induced when the recognition proteins identify microbes or non-self objects, and these responses are

mostly non-specific. Although chemical pesticides were proposed not to affect AMPs production previously (James et al. 2012), many studies have already showed that exposure to insecticides significantly alter the expression level of some AMPs. Overexpressed AMPs had been reported when insects exposed to sublethal concentrations of insecticides. In honey bee *Apis mellifera* and *Apis cerana*, the expression level of *defensin1/2* was significantly up-regulated when treated with two neonicotinoids imidacloprid and clothianidin (Li et al. 2017). In *P. xylostella*, vitamin C and acetylsalicylic acid enhanced insecticide resistance and induced the elevated expression of gloverin, indicating that insecticide resistance may somehow depend on effects on the immune system (Xia et al. 2018). A resistant strain of *Culex pipiens* responded greater than the susceptible strain when they were challenged by injection of lipopolysaccharide (LPS), and the expression level of gambicin, defensin, transferrin and NO synthase were all up-regulated (Vezilier et al. 2013). When exposed the *D. melanogaster* to acetic acid, ethanol and 2-phenylethanol, several AMPs regulated by Toll and IMD pathway were upregulated (Seong et al. 2020).

While several studies showed the up-regulated immune system via overexpression of AMPs when insects received sublethal insecticide stress, a few cases of AMP underexpression had been reported when

insects exposed to sublethal concentrations of insecticides. For example, a low dose of imidacloprid significantly inhibited the expression level of *diptericin A* and *drosomysin-like 2* in *D. melanogaster* (Martelli et al. 2020). *Diptericin-A* was also commonly underexpressed when *F. occidentalis* was exposed to three different insecticides (Gao et al. 2020). When fed the *Bombyx mori* with phoxim dipped leaves, four AMPs were found to be underexpressed in the fat body (Gu et al. 2015).

There were two homeodomain transcription factors, ventral nervous system defective (*vnd*) and extra-extra (*exex*) were commonly overexpressed in the six insecticide treatments. *Vnd* plays an essential role during the development of embryonic nervous system, and is involved in apoptosis via NK2-specific domain (Lee et al. 2014). *Exex* is the homolog of the vertebrate HD proteins *MNR2/Hb9* and functions as a transcriptional repressor during CNS development (Broihier et al. 2002). Considering that transcription factors such as *Maf-S* and *cap n collar* have been reported to regulate insecticide resistance in *Abpheles gambiae* and *Tribolium castaneum* (Ingham et al. 2017; Kalsi et al. 2017), these two transcription factors can be involved in the detection of insecticides, thus activating downstream detoxification pathways.

Another commonly overexpressed DEG is *maltase A4*, a glycosidase, which was also overexpressed in a Nora virus-infected *D.*

melanogaster strain (Cordes et al. 2013). In a previous study, another three maltases, *maltase A1*, *maltase A6* and *maltase B1* were found to be overexpressed in *F. occidentalis* treated with sublethal concentrations of insecticides (Gao et al. 2020). In addition, *maltase A1*, *maltase A2* and *maltase B2* were overexpressed, though not in all, but in at least four different insecticide treatments in this work. Up-regulation of these glycosidases seems to affect glycogenolysis, which appears a general response to the xenobiotic stress caused by different insecticides.

Glutathione S transferase E4 (*GstE4*) was the only commonly overexpressed detoxification gene. GSTs play important roles in phase II metabolism of detoxification and protection from the oxidative stress caused by exposure to insecticides. In *Anopheles arabiensis*, although *GstE4* does not have a role in insecticide detoxification, it has significant higher expression level in resistant strain and found to be responsible for insecticide sequestration (Wilding et al. 2015; Abdalla et al. 2014). Therefore, it can be speculated that GstE4 is likely involved in the early stage of detoxification either via phase II reaction or sequestration in *D. melanogaster*.

No Cyp450 genes were commonly over-transcribed in all six insecticide treatments. Nevertheless, *Cyp6a2* was significantly overexpressed in indoxacarb-, ivermectin- and spinosad-treatments

whereas *Cyp4d1* was significantly overexpressed in cypermethrin-, ivermectin- and spinosad-treatments. CYP6 groups are known to be involved in metabolism of insecticides and xenobiotics, thus conferring tolerance/resistance (Feyereisen 2006). Multiple reports have found that CYP6 groups are involved in insecticide resistance in different species (Bass and Field 2011). Cyp6a2 was commonly overexpressed under three different insecticide treatment in *F. occidentalis* (Gao et al. 2020). When used deltamethrin to treat *D. melanogaster*, *Cyp4d1* was also over-transcribed (Liu et al. 2020). The commonly overexpressed nature of these two Cyp450 genes following sublethal exposure to insecticides with distinct structures suggests they may have a broad substrate specificity for universal detoxification of various insecticides and xenobiotics.

4.4 Commonly under-transcribed genes following treatment with sublethal concentrations of insecticides

On the contrary to the over-transcribed genes, some genes were commonly under-transcribed following insecticide treatment. Seven components of mitochondrial respiratory chain were identified and counted as a major part of overall under-transcribed gene (Table 3-4). A similar down regulation of these components was also observed in *P. xylostella* treated with different sublethal concentrations of insecticides

(Gao et al. 2018). In total, five genes belonging to mitochondrial respiratory chain were commonly underexpressed, such as NADH-ubiquinone oxidoreductase 4/5, cytochrome c oxidase subunit 3 and subunit 6B and a cytochrome b. This apparent down-regulation of mitochondrial energy generation may indicate a total decrease on energy consumption when the flies suffered from xenobiotic stress. It can be explained that overall reduced energy generation and consumption may be necessary for the intoxicated flies to conserve energy resource, increase survival rate and acquire tolerance when exposed to sublethal concentrations of insecticides; or sublethal concentrations of insecticides exposure may lead to mitochondrial dysfuction (Martelli et al. 2020).

Six genes related with eggshell formation were found to be commonly underexpressed except chorion protein 16/19/36 in the cypermethrin-treated files. The chorion is the outermost membrane and the interface between the embryo and the environment, thereby serving to protect the egg (Spradling et al. 1980). It is unclear yet how these chorion genes were underexpressed in the treated flies. However, considering that chorion is required for egg formation but not for adult survival under insecticide stress, but it can be speculated that down-regulation of chorion formation may be the primary option for resource trade-off in female flies exposed to insecticides.

Troponin C isoform 4 and flightin were also underexpressed in all six insecticide treatments. Troponin C plays an important role in regulating insect behavior including surviving, feeding and breeding via controlling muscle contraction and relaxation activity (Weber et al. 1973). Flightin encodes an indirect flight muscle specific protein but not in other muscle types (Vigoreaux et al. 1993). In salmon lice *Lepeophtheirus salmonis*, an insecticide-resistant strain showed significantly less expressed troponins compared to a susceptible strain (Carmichael et al. 2013). When *D. melanogaster* and *Neophotettix cincticeps* were challenged by Nora virus and a rice dwarf virus-encoded nonstructural protein 10, the expression of troponin and flightin, and troponin, were suppressed, respectively (Cordes et al. 2013; Lan et al. 2018). Taken together, it can be speculated that down-regulation of troponin C isoform 4 and flightin expression reduce the muscle excitability, thereby increasing resource and energy conservation. With this in mind, down regulation of the gene groups involved muscle contraction like results in a similar physiological outcome as the underexpression of genes related with mitochondrial energy generation.

Additionally, a long non-coding RNA (lncRNA) CR43459 was under-transcribed in all insecticide treatments, lncRNA was known to have regulator function, to affect the expression level of other genes such

as genes related with detoxification metabolism and metamorphosis whereas lncRNA itself could be regulated by xenobiotic stress (Liu et al. 2017; Lawrie et al. 2020). The connection between this specific lncRNA and tolerance induction need to be further studied.

4.5 Comparison of DEG profiles among *D. melanogaster*, *F. occidentalis* and *P. xylostella*

In previous studies, I identified the differentially expressed genes from *P. xylostella* and *F. occidentalis* transcriptome data following exposure to sublethal concentrations of insecticides (Gao et al. 2018; Gao et al. 2020). When comparing with *D. melanogaster* transcriptome data, overall DEG profiles shared a limited similarity, suggesting that xenobiotic tolerance induction mechanism at the early stage of intoxication might be common in some species, but most mechanisms looked like species-specific. Other possible explanations for such a lack of commonly responding genes to insecticides across different species include the differences in insect developmental stage, insect physiology and treatment method between the experimental conditions with different species (Gao et al. 2018; Gao et al. 2020).

One of main species-specific feature of gene expression patterns was that up-regulation of cuticular protein genes was only observed in *P.*

xylostella larvae, which was suggested to be associated with desiccation resistance. No cuticular protein genes were overexpressed in either *F. occidentalis* or *D. melanogaster.* This difference can be speculated to be due to that fact that water preservation may be more crucial in the larval stage of *P. xylostella* larvae than the adult stage with well-developed cuticle of *F. occidentalis* and *D. melanogaster.*

The second main difference/similarity was found in the mitochondrial respiratory chain system. Several components were commonly underexpressed in all insecticide treatments in *P. xylostella* and *D. melanogaster*, where reduced energy generation was proposed to be beneficial for acquiring tolerance. However, no similar tendency of down-regulation was found in *F. occidentalis,* suggesting that the role of energy metabolism in the tolerance induction process in *F. occidentalis* differs from those of *P. xylostella* and *D. melanogaster*.

The third main difference was observed in the expression pattern of AMP genes. Various AMP genes were overexpressed in *D. melanogaster* following exposure to sublethal concentrations of insecticides whereas few AMP genes were differentially expressed in all insecticide treatment either in *P. xylostella* or in *F. occidentalis*. It is unclear yet how insecticide exposure induced a such strong humoral immune response in *D. melanogaster.* Nonetheless, it is worthy to note that the insecticide

treatment methods were different among all three studies. *P. xylostella* and *F. occidentalis* were treated either using air dried leaf disc or glass vial without using any solvent but *D. melanogaster* were directly treated with insecticides dissolved in acetone. Direct contact of acetone to the cuticle might lead to a unique immune response in *D. melanogaster*. Further study is needed to identify the similarities and discrepancies of tolerance induction in different species of insects.

A total of 12 genes were identified in dinotefuran treatments in all three species (Table 2). Among the 12 common DEGs in dinotefuran treatments, only two of them showed consistent over/underexpression. Three genes belonged to CYPs (i.e. Cyp4d21, Cyp6a18 and Cyp6w1) showed a common differential expression patter in all three species treated with dinotefuran, but their expression patterns were distinct each other. All the three CYPs were all underexpressed in *P. xylostella*, whereas they were overexpressed in *F. occidentalis*. In *D. melanogaster*, however, one was overexpressed but other two were underexpressed. Although the expression level of three CYPs have been reported to be elevated in *D. melanogaster* and *Palaemon elegans* by different insecticide treatments (Le Goff et al. 2006; Olsvik et al. 2017). The obvious discrepancies on expression regulation may indicated they are not the specific detoxification factors for dinotefuran. Five genes, which

have serine-type endopeptidase activity, were identified in dinotefuran-treated samples, among which only the hypodermin-B was consistently underexpressed. In *P. xylostella* and *F. occidentalis*, four and three out of five proteolysis-related genes were overexpressed, respectively. However, these five genes were all underexpressed in *D. melanogaster*. The distinct expression pattern about these genes may indicate the expression regulation of proteolysis-related genes are more closely related with the different physiology of these species rather than common tolerance factors induced by dinotefuran. Two structural components of muscle (bent and unc-89) were also identified in the three species treated with dinotefuran. Both of them were up-regulated in *F. occidentalis* but down-regulated in *P. xylostella* and *D. melanogaster*. In bumblebee *Bombus terrestris*, imidacloprid treatment increased the expression of unc-89 (Erban et al. 2019); whereas dual stressors of emamectin benzoate treatment and microsporidian *Facilispora margolisi* increased the expression level of unc-89 as well as the components of energy generation system (Poley et al. 2017).

A total of 22 genes were identified in spinosad-treated three species, among which four genes were CYPs (Cyp4d1, Cyp4e3, Cyp6a2 and Cyp6a8) (Table 3). All the four CYPs were commonly overexpressed in the three species when treated them with sublethal concentrations of

spinosad. As introduced previously, CYPs were widely involved in the xenobiotic metabolism in insects, and many members from CYP6 groups were found to be responsible for the insecticide resistance in various insects (Feyereisen 2006). Here in my study, although we used distinct insect species and treatment methods, the CYPs were commonly overexpressed by spinosad treatments, indicating these four CYPs were specifically involved in the spinosad detoxification. Nine serine-type endopeptidase genes were commonly identified in spinosad treatments to the three insect species, among which most were consistently overexpressed. Similar overexpression of the chymotrypsin-like and trypsin-like genes were found in various insecticide treated insect species (Zhang et al. 2013; Lv et al. 2016; Silva et al. 2010). In addition, three genes related with lipid metabolism were commonly overexpressed in all three spinosad-treated species. Lipid-metabolizing enzymes were reported to confer to pyrethroid insecticides in *C. pipiens pallens* and *S. zeamais*, but how these genes involved in the detoxification of macrocyclic lactones still remained unclear (Hu et al. 2020; Araujo et al. 2008). The olfactory response was upregulated when *S. littoralis* is exposed to sublethal doses of deltamethrin. When *An. gambie* is exposed to permethrin, two transcripts of antennal carrier protein TOL-2 were overexpressed. Since antennal protein 10 was commonly overexpressed in different species it is likely to be involved in general antennal

detoxification against spinosad or antennal stress response to spinosad treatment (Lalouette et al. 2016; Vontas et al. 2005).

When comparing the common DEG lists between dinotefuran and spinosad treatments, the DEGs induced by dinotefuran treatments in three species were rather randomly regulated, whereas spinosad treatments induced consistent over/under expression DEGs in the three insect species. Further studies are needed to verify the potential roles of the putative tolerance factors induced by dinotefuran- and spinosad-treatments.

5. CONCLUSION

Comparative analysis of sublethal insecticide-treated transcriptomes of *D. melanogaster* suggested that several genes with differential expression may contribute to the insecticide tolerance directly or indirectly. The common responsiveness to six insecticides with distinct structures and modes of action indicates their potential roles in the general defense system. Cross-comparison with other species treated with the same insecticides under the similar conditions revealed that genes commonly responding to insecticide exposure are scarce across different species. The species-specific DEG set indicated that the strategy for general defense may be diverse from species to species.

Therefore, it would be difficult to deduce a generalized model for tolerance induction in various species of insects. Identification of such species-specific DEGs would help our comprehensive understanding of both universal and specific insecticide tolerance factors, which further benefits the proactive management of resistance.

GENERAL DISCUSSION

In these three chapters, the comparative transcriptome analysis of different insecticides treatments in three insect species were conducted. Chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb and spinosad were applied to diamondback moth larvae. Chlorfenapyr, dinotefuran and spinosad were applied to western flower thrips females. Chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad were applied to female common fruit flies.

In *P. xylostella*, the main commonly overexpressed DEGs included cuticular protein genes, cuticle modification related protein genes and cytochrome P450s, whereas the commonly underexpressed DEGs included the components from mitochondrial respiratory chain and a ryanodine receptor 44F. In the Asian honey bee *Apis cerana*, Cyp9e2, one of the two commonly overexpressed Cyp450s, was overexpressed in the flumethrin-treated worker bees, suggesting this gene is likely involved in the xenobiotic metabolism and detoxification (Wujun 2016). Cuticle is the first barrier of insect against xenobiotics penetration and water conservation, since there were multiple cuticular protein genes were commonly overexpressed along with a glucose dehydrogenase, a chitinase and a laccase, all of which are closely related with cuticle

modification, there was a clear tendency of increasing and changing the protein components of cuticles. In several mosquito species, the resistant strains had significant thicker cuticles and overexpressed more cuticle proteins than susceptible strains, thereby decreasing insecticide penetration (Fang et al. 2015; Lilly et al. 2016; Vannini et al.2014). Considering of the different modes of action and chemical structures, the common overexpression of these cuticle protein genes may confer to the direct tolerance to insecticides and/or dessication resulted from intoxication. In contrast, an apparent underexpression of mitochondrial respiratory chain was observed. The reduced energy generation maybe beneficial to *P. xylostella* larvae for increasing the survival rate, or the mitochondria dysfunction caused by xenobiotics.

In F. occidentalis, commonly overexpressed DEGs included genes related with basic biological process, such as proteolysis, glycogenolysis and lipid metabolism, detoxification genes such as cytochrome P450s, UGTs, esterases, a GST and a ABCT. Compared with the commonly overexpressed DEGs, only a few DEGs were commonly underexpressed, including transcription factors and an antibacterial peptide diptericin A. Thirty-four proteolysis related genes were commonly overexpressed in all three insecticide treatments. Among them, chymotrypsins have been found to be overexpressed in insecticide-treated thrips and involved in

deltamethrin metabolism in *C. pipiens pallens*, knockdown of chymotrypsin-like genes significantly enhanced *Bacillus thuringiensis* insecticidal efficiency in *Ostrinia furnacalis* (Zhang et al. 2013; Lv et al. 2016; Guan et al. 2017). These findings suggest that chymotrypsin-like genes likely responds to intoxication by neurotoxic insecticides and bacterial endotoxins. A total of 18 glycosidase genes were overexpressed by sublethal treatment with three insecticides, similar overexpression or activity up-regulation were also reported in DDT exposed *D. melanogaster* and cyfluthrin fed *S. littoralis* (Seong et al. 2017; Bernard et al. 1993). Taken together, these findings indicate that xenobiotic insecticide stress impaired the homeostasis of energy metabolism, thus inducing glycogenolysis to meet the energy demands. There were 14 detoxification genes that were also overexpressed in all three insecticide treatments. Among them, four genes belonged to CYP6 group, which is well known to be a major part of xenobiotics metabolism, thus conferring tolerance/resistance (Feyereisen 2006). Four of these detoxifications were UGTs, involved in phase II detoxification, catalyzed the conjugation of small lipophilic molecules with UDP sugars, thus increasing their water solubility for efficient elimination.

In *D. melanogaster*, commonly overexpressed DEGs included olfactory related genes and immune response related genes such as

antimicrobial peptides, on the other side, commonly underexpressed DEGs included the components of mitochondrial respiratory chain and chorion protein genes. OBPs are responsible for providing the chemical signals to ORs which originated from initial molecular interactions (Zhou et al. 2009). Several studies have reported that the expression of OBPs can be overexpressed when exposed insects to either imidacloprid or a mixture of pesticides and herbicides (Nkya et al. 2014; Liu et al. 2020). Moreover, OBPs have distinct binding affinity to various chemicals including insecticides, thus we conclude that the commonly overexpressed OBPs have a broad binding spectrum to chemicals and function as non-specific bioscavengers (Zhang et al. 2020). A total of seven immune related genes were commonly overexpressed in all six insecticide treatments, and most of these genes were AMPs. In two honey bee species, the expression level of *defensin1/2* was significantly up-regulated when treated with imidacloprid and clothiaidin (Li et al. 2017). When exposed the *D. melanogaster* to acetic acid, ethanol and 2-phenylethanol, several AMPs regulated by Toll and IMD pathway were upregulated (Seong et al. 2020). Theses overexpressed AMPs may just be the response after identifying the non-self-object. Besides the overexpressed DEGs, some genes were commonly underexpressed following all six insecticide treatments. Similar to my previous study of *P. xylostella*, seven components of mitochondrial respiratory chain were

commonly overexpressed in *D melanogaster*. Such decreased total energy generation and consumption may be necessary for the flies to conserve energy source, increase survival rate and gain tolerance, or the function of mitochondria was impaired (Martelli et al. 2020). Six chorion protein genes were underexpressed in all six insecticide treatments. It is not clear yet how and why these egg-protecting factors were underexpressed since they seem to be not related with survival rate or tolerance, however, it can be speculated that underexpression of chorion formation might be a front-row option for resource trade-off in intoxicated flies. The overall DEGs that identified in three different insect species shared a limited similarity, suggesting that xenobiotic tolerance induction mechanism at the early stage of intoxication might be common in some species, but most mechanisms looked like species-specific. Other possible explanations for such a lack of commonly responding genes to insecticides across different species include the differences in insect developmental stage, insect physiology and treatment method between the experimental conditions with different species.

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ABSTRACT IN KOREAN

현대 농업에서는 여러 종류의 해충방제를 위해 다양한 살충제가 도입되었으며, 이러한 화학 물질의 범지구적 사용으로 살충제 내성/저항성이 등장하였고 이는 시급하게 해결해야 될 문제이다. 따라서 살충제 스트레스에 의해 유도될 수 있는 유전자를 찾아내고 특징을 확인하기 위해 *Plutella xylostella* (배추좀나방, DBM), *Frankliniella occidentalis* (꽃노랑총채벌레, WFT) 및 *Drosophila melanogaster* (노랑 초파리, CFF)를 모델 시험곤충으로 선정하여 다양한 살충제들의 아치사량으로 처리한 후 전사체 데이터를 분석하였다. 1장에서는 잎침지법으로 *P. xylostella* 의 3령 유충에 chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb 및 Spinosad 약제를 아치사농도(LC_{10})로 전처리 한 후 반수치사농도 (LC_{50})에 노출 시켰을 때 살충제에 대한 내성이 크게 향상됨을 확인하였다. 전사체 데이터를 통해 chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb 및 spinosad 처리군에서 과발현된 전사체는 각각 125, 143, 182, 215 및 149개인 반면, 저발현된 전사체는 67, 45, 60, 60 및 38 개임을 확인하였다. 차별발현 유전자(DEG) 중 가장 전사량 차이가 컸던 유전자는 두 개의 사이토크롬 P450 유전자(Cyp301a1 및 Cyp9e2)와 9 개의 표피 단백질 유전자였다. 반대로, 미토콘드리아 에너지 생성 시스템을 구성하는 몇몇 유전자는 모든 처리군에서 적게 전사되었다. 이 결과는 P. xylostella의 경우, 대부분의 DEG가 살충제의 구조와 작용 기작에 관계없이 중독 초기 단계에서 일반적인 화학적 방어에 관여함을 시사한다.

2장에서는 잔류접촉법(RCVpW)으로 *F. occidentalis* 의 암컷 성충에 chlorfenapyr, dinotefuran 및 spinosad 약제의 아치사농도 (LC_{10})를 전처리한 후 반수치사농도(LC_{50})에 노출시켜 살충제

내성이 크게 향상됨을 확인했다. 전사체 분석 결과 chlorfenapyr, dinotefuran 및 spinosad 처리시 404, 386, 756 개의 유전자의 발현량이 증가했고, 124, 107, 169개의 유전자의 발현량이 감소했다. 이 중 199개의 전사체는 세 가지 약제 처리 시 공통적으로 상향조절 되었으며, 31개가 하향조절 되었다.

대부분의 상향조절 된 전사체는 단백질 분해와 지질 대사와 같은 기본적인 생물학적 과정으로 분류되었다. 해독 유전자에 속하는 glutathione S transferase 1개, UDP- glucuronosyltransferases 3개, CYP450 4개, ABC transporter 1개는 세 살충제 처리군에서 공통적으로 상향조절되었다. 공통적으로 과발현 된 유전자 중 5개의 RNAi를 시행했을 때, 3개의 살충제 모두에서 사망률이 증가하였는데, 세 살충제의 구조와 작용 기작이 서로 다르기 때문에 공통과발현 유전자는 살충제 내성에 관여한다고 보았다.

3장에서는 *D. melanogaster* 암컷 성충을 국소처리법으로 chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad 아치사농도 (LC_{10})에 노출시킨 후 반수치사농도 (LC_{50})로 처리하였고, 이전 실험들과 마찬가지로 살충제 내성이 크게 증가하였다. 전사체분석을 통해 chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, ivermectin and spinosad에서 각각123, 173, 75, 245, 368, 145개의 과발현된 유전자를 확인하였고, 137, 108, 202, 83, 59, 126개의 저발현된 유전자를 확인하였다. 이러한 DEG 중 26개와 30개의 유전자가 6개 살충제 모두에서 공통적으로 상향, 하향조절 되는 것으로 나타났으며, 공통적으로 상향조절된 유전자는 attacin-A/C, dptericin A/B, drosocin, immune induced molecule 18 등과 같은 항균성 펩타이드를 만드는 면역관련 유전자가 대부분이었다. 미토콘드리아 호흡계를 구성하는 유전자들이 공통적으로 하향조절되었으며, 이러한 유전자들이 일반적으로 내성에 어떻게 관여하는지에 대해 기술

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