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Gene Expression Profiles in Japanese Pine Sawyer, *Monochamus alternatus* Exposed to a Sublethal Dose of Imidacloprid

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Abstract.- The Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is a serious pest management concern in stands of pines. Neonicotinoids are classes of insecticides known to selectively target insect nicotinic acetylcholine receptors. Imidacloprid was introduced as the first commercially available product from the neonicotinoid class of insecticides. In order to understand how *M. alternatus* reacts toxicologically to imidacloprid, transcriptional profiling of *M. alternatus* larvae exposed to a sublethal dose of imidacloprid were monitored using a specific 60-mer oligonucleotide microarray derived from a cDNA library. Treatment of *M. alternatus* with imidacloprid resulted in a large number of differentially expressed genes, principally associated with metabolic pathways and stress responses. A total of 359 unique genes were found to vary at least twofold in terms of accumulation. Of these 359 genes, 41 were upregulated, and 318 were downregulated. A gene ontology enrichment analysis reflected a broad spectrum of the analyzed transcriptome. Our study will assist in a further understanding of networks linking imidacloprid and its potential targets. Identifying the molecular mechanisms of toxicity by imidacloprid is necessary for its future use for controlling *M. alternatus* and other Lepidoptera insects.

Key words: Gene expression profile, *Monochamus alternatus*, imidacloprid.

INTRODUCTION

The Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), a serious pest management concern in stands of pines, occurs widely in China, Korea, Japan, Laos and Vietnam(Sato *et al*., 1999; Da *et al*., 2010; Fan *et al*., 2010). It is the key vector of the exotic pine wood nematode *Bursaphelenchus xylophilus* throughout Asia (Kobayashi *et al*., 1984). This nematode is the causal agent of pine wilt disease, which has caused widespread pine *(Pinus* spp.) mortality in Asia. One method to prevent this nematode from damaging forests is to block its transport by controlling *M. alternatus*.

Neonicotinoids are the fastest-growing class of insecticides in modern crop protection (Jeschke *et al*., 2011). Additional benefits of neonicotinoids include their relatively low risk for non-target organisms and the environment, high target specificity, and versatility in application methods (Cheng *et al*., 2012). Since their introduction onto the market in 1991, they are increasingly used worldwide and sometimes replace the organophosphate and carbamate insecticides (Yassine *et al*., 2011). Imidacloprid was introduced in the early 1990s as the first major commercial product from the neonicotinoid class of insecticides (Elbert *et al*., 1990; Qadir *et al*.,2014) and has since become the most successful proprietary insecticide worldwide. As a systemic seed, soil or foliar treatment, imidacloprid is active against numerous sucking and biting pest insects, including aphids, whiteflies, thrips, leaf miners and beetles (Elbert *et al*., 1998).

Nicotinic acetylcholine receptors are ligand-gated ion channel receptor complexes that mediate fast cholinergic synaptic transmission. They are among the most thoroughly studied molecules in nervous systems. Neonicotinoids are insecticide classes known to selectively target insect nicotinic acetylcholine receptors, and each class acts via a different mechanism (Eugênio *et al*., 2011). Studies performed on dorsal unpaired median and thoracic neurons demonstrated that imidacloprid was a partial agonist of insect nicotinic acetylcholine receptors (Salgado and Saar, 2004; Thany, 2009). Similar electrophysiological data were also found using mushroom body neurons called Kenyon cells in which imidacloprid was also a partial agonist (Deglise *et al*., 2002). Moreover, the insecticidal

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potency is influenced not only by the intrinsic actions on the targets but also by their accessibility to nicotinic acetylcholine receptors and metabolism in insects (Yamamoto *et al*., 1998). In the green peach aphid *Myzus persicae* (Sulzer), Yu *et al.* (2010) speculated that the balance of biochemical processes was disrupted and achieved a new balance after imidacloprid application. Therefore, we hypothesized that imidacloprid toxicity is not only the result of nicotinic acetylcholine receptor inhibition but also the result of a number of physiological alterations in nervous and other insect tissues. Furthermore, we propose that the transcriptional profiling of larvae exposed to a sublethal insecticide dose could provide unique insights into the reactions of imidacloprid - susceptible larvae by identifying target genes. Because imidacloprid is a relatively new insecticide, the genetic basis of insecticidal potency in *M. alternatus* has not yet been documented. To identify genes that are involved in the toxicological response of *M. alternatus* to imidacloprid, we monitored the gene expression profile in *M. alternatus* after a 4 h exposure to imidacloprid using a 60-mer oligonucleotide microarray derived from a cDNA library. The ultimate goal of this work is to identify new potential non-nicotinic acetylcholine receptor biomarkers as imidacloprid targets in order to assess the insecticide's secondary effects on *M. alternatus* and to gain a better understanding of the networks linking imidacloprid to its targets. Our study could provide a valuable gene resource for the scientific community to study the molecular toxicology of *M. alternatus*.

MATERIALS AND METHODS

Insects

The *M. alternatus* used in this study were obtained from dead, infested *Pinas massoniana* trees. The larvae were reared on artificial diets (Xu *et al*., 2009) in complete darkness at room temperature with a 55% relative humidity (RH) until they reached the third instar larval stage.

Exposure to imidacloprid

The imidacloprid used in the study was a technical grade formulation (95% purity) provided by Huizhou Sino-quick Chemical Co., Ltd., China. The third instar larvae were contacted with filter paper wetted with 1.25 ml of 30 ppm imidacloprid. The sublethal dose of 30 ppm was designed based on previous studies (Ugine *et al*., 2011; Cheng *et al*., 2012) to ensure that the insecticide remained present in the insect tissue, this dosage resulted in less than 10% mortality of *M. alternatus*. To assess the level of gene induction shortly after the insects were exposed to the insecticide, insects were exposed to imidacloprid for 4 h. The control larvae were placed in contact with water. Each experiment was performed in triplicate with 30 larvae per replicate. At the end of the 4 h exposure, the larvae were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction was performed.

RNA extraction and purification

Total RNA was extracted using TRIzol Reagent from Life Technologies, (www.lifetechnologies.com) following the manufacturer's instructions and checked for RNA integrity by assessing the RNA integrity number with an Agilent Bioanalyzer 2100 (www.agilent.com). Qualified total RNA was further purified by an RNeasy Mini Kit and the RNase-Free DNase Set from QIAGEN (http://www.qiagen.com).

cDNA library construction and BLAST search

Standard cDNA libraries were directionally constructed using SMART cDNA Library Construction Kit from Clontech (www.clontech.com), following the manufacturer's instructions. Briefly, first strand synthesis was performed using PowerScript Reverse Transcriptase and an anchored oligo d(T) primer

5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$-N$_{-1}$N-3'

Double-stranded DNA was synthesized by primer extension of cDNA through three cycles and visualized as a 0.1-4 Kb smear showing a strong band at 900 bp. The amplified double-stranded cDNA was digested using Proteinase K and *Sfi*I, size fractionated by Chroma Spin-400, and ligated to a λTriplEx2 Vector containing the asymmetrical *Sfi*I sites (A and B) in the MCS. The average insert size of cDNA libraries was routinely determined by performing colony polymerase chain reaction (PCR)

on 48 randomly selected colonies from the amplified library. PCR amplicons were resolved on 1% agarose gels and visually compared to DNA size markers in the 1 kb DNA ladder from Gibco/BRL (www.gelcompany.com). The libraries were then excised into *Escherichia coli* cells (DH-5α) on LB-ampicillin agar plates and were grown at 31°C overnight for colony picking. Clones were picked from the cDNA libraries and sequenced using the (-47) M13 forward
(5′-: CGCCAGGGTTTCCCAGTCACGAC -3′)
and the (-48) M13 reverse
(5′- AGCGGATAACAATTTCACACAGGA -3′)
primers. VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to trim the vector sequence from raw sequence data. The clone sequences were submitted to GenBank by NCBI(http://www.ncbi.nlm.nih.gov). The predicted biological function of each cDNA was determined based on the blastx hit in the GenBank database with a described gene function.

Microarray construction

M. alternatus DNA microarrays were customized using the Agilent eArray 5.0 program according to the manufacturer's recommendations (https://earray.chem.agilent.com/earray/). The customized microarray contained 15000 spots in triplicate with gene-specific 60-mer oligonucleotides representing 3786 protein-coding genes in *M. alternatus* based on the sequenced clones from the cDNA library.

RNA amplification and labeling

Total RNA was amplified and labeled by the Low RNA Input Linear Amplification kit from Agilent Technologies (www.agilent.com) using 5-(3-aminoallyl)-UTP (Ambion, Austin, TX, US) and Cy3 NHS ester (GE healthcare Biosciences, Pittsburgh, PA, US) by following the manufacturer's instructions. Labeled cRNA were purified by the RNeasy Mini Kit from QIAGEN (http://www.qiagen.com).

Microarray hybridization

Each slide was hybridized with 600 μg of Cy3-labeled cRNA at 65°C using the Gene Expression Hybridization Kit from Agilent Technologies (www.agilent.com) in a Hybridization Oven from Agilent Technologies (www.agilent.com) following the manufacturer's instructions. After 17 h of hybridization, the slides were washed in staining dishes (/www.thermoscientific.com) using the Gene Expression Wash Buffer Kit from Agilent Technologies (www.agilent.com) and dried using the Stabilization and Drying Solution from Agilent Technologies (www.agilent.com), following the manufacturer's instructions.

Scanning, data normalization and analysis

The slides were scanned at a 5 μm resolution using the Agilent Microarray Scanner (www.agilent.com) and Feature Extraction Software 10.7 (www.agilent.com). Both 10% and 100% photomultiplier tube (PMT) settings were selected, and the combined images were exported. The signal intensities of all spots were quantified for each image. The raw data were normalized using the Quantile algorithm using Gene Spring Software 11.0 (www.agilent.com). The average normalized expression value for the three biological replicates for each probe is reported as the expression value of a predicted gene. Differentially expressed genes were identified using the empirical Bayes test (Daglish and Nayak, 2012; Smyth *et al*., 2005) with the application of the Benjamini and Hochberg (2000) multiple testing correction. Genes were considered to be differentially expressed if they possessed both an adjusted p-value <0.05 and a log-fold expression change (LFC) $>|1.0|$. The regulated genes were functionally classified by gene ontology (http://www.geneontology.org/). We used Fisher's exact test to compare the gene ontology terms associated with significantly regulated genes to those associated with genes with unchanged expression following treatment. The significance of enrichment of gene ontology terms was analyzed separately for upregulated and downregulated genes. The criteria for the significance of a gene ontology term required that it possessed both an adjusted P-value<0.05 and that the number of altered targets for that term was ≥ 2. To determine the relationship among these differentially expressed genes, molecular pathway analysis of the regulated genes

was carried out based on the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/). Fisher's exact test was used to identify the significant pathways, and the threshold of significance was defined by a P-value<0.05.

Verification of expression with RT-qPCR

We verified microarray results with RT-qPCR using the ΔΔCT method. RNA was extracted following the same procedure used in the microarray experiments, and three replicates were performed. Cytoplasmic actin of *M. alternatus* was used as the endogenous control and normalized to be in equal quantity in every cDNA treatment. Primers for RT-qPCR target genes were designed online by GenScript Real-time PCR Primer Design (https:// www.genscript.com/ssl-bin/app/primer). Melting curves for each template/primer pair were examined for non-specific amplification. The RT-qPCR data were acquired on a LightCycler Real Time PCR using SYBR Premix Ex Taq™ (www.takara-bio.com) and universal thermocycler conditions according to the manufacturer's protocol from Roche Diagnostics (www.roche.com). Briefly, The PCR was conducted in a 20 µl final volume reaction with a PCR mix containing 2 µl of cDNA at 4 ng/µl (*i.e.*, a quantity of cDNA corresponding to 20 ng of total RNA), 10 µl of SYBR Premix Ex Taq™, 0.4 µl of PCR Forward Primer, 0.4 µl PCR Reverse Primer, and 7.2 µl of dH_2O. The amplification scheme was: 95°C for 30 sec, 95°C for 5 sec, and 60°C for 20 sec. The dissociation curve method was applied according to the manufacturer's protocol (60°C to 95°C) to ensure the presence of a single specific PCR product. The annealing temperature was 60°C and the RT-qPCRs were performed using 40 cycles. For each cDNA, three RT-qPCR reactions were performed and standard curves were generated. To minimize the positional effect on PCR reactions because of variations of block temperature, the PCR reactions for target gene and reference gene were placed on the cycler in a randomized block design. The threshold cycle (CT) and the relative expression levels were calculated using the LightCycler480 1.5 software from Roche Diagnostics (www.roche.com).

RESULTS

Microarray overall results

In this study, global changes in *M. alternatus* gene expression were assessed by microarray analyses 4 h after imidacloprid treatment. Some genes were represented with more than one probe set. Therefore, the number of responsive probe sets is greater than the number of responsive genes. A total of 1012 probe sets (GenBank accession number: JZ143720-JZ144731) corresponding to 359 genes were found to vary in accumulation at least two-fold. Of these 359 unique genes, 41 were upregulated, and 318 were downregulated, which suggests that the gene repression was more sensitive to imidacloprid than enhanced expression. More than 10 nicotinic acetylcholine receptor genes have been cloned in insects, and post-translational modifications have been observed on subunit mRNAs (Grauso *et al*., 2002; Lansdell and Millar, 2002; Matsuda *et al*., 2005; Millar and Gotti, 2009; Sattelle *et al*., 2005), suggesting the existence of many nicotinic acetylcholine receptor subtypes (Ihara *et al*., 2006). Five nicotinic acetylcholine receptor transcripts were identified in this study, all of which were downregulated by the imidacloprid treatment.

The unique genes showing altered transcriptional patterns were primarily related to metabolic pathways, oxidative phosphorylation and various enzymes (*e.g.,* cytochrome P450 reductase, juvenile hormone esterase and serine protease) involved in the general stress response. Imidacloprid serves a more complex function in insect toxicology than previously believed.

Gene ontology enrichment analysis

To infer the relationship between the gene expression pattern and the functional categories, gene ontology enrichment analysis was performed to identify the overrepresented categories for both induced and repressed genes (Figs.1-2). The differentially expressed genes were widely distributed among the molecular function, biological processes, and cellular component categories, reflecting a broad spectrum of the analyzed transcriptome.

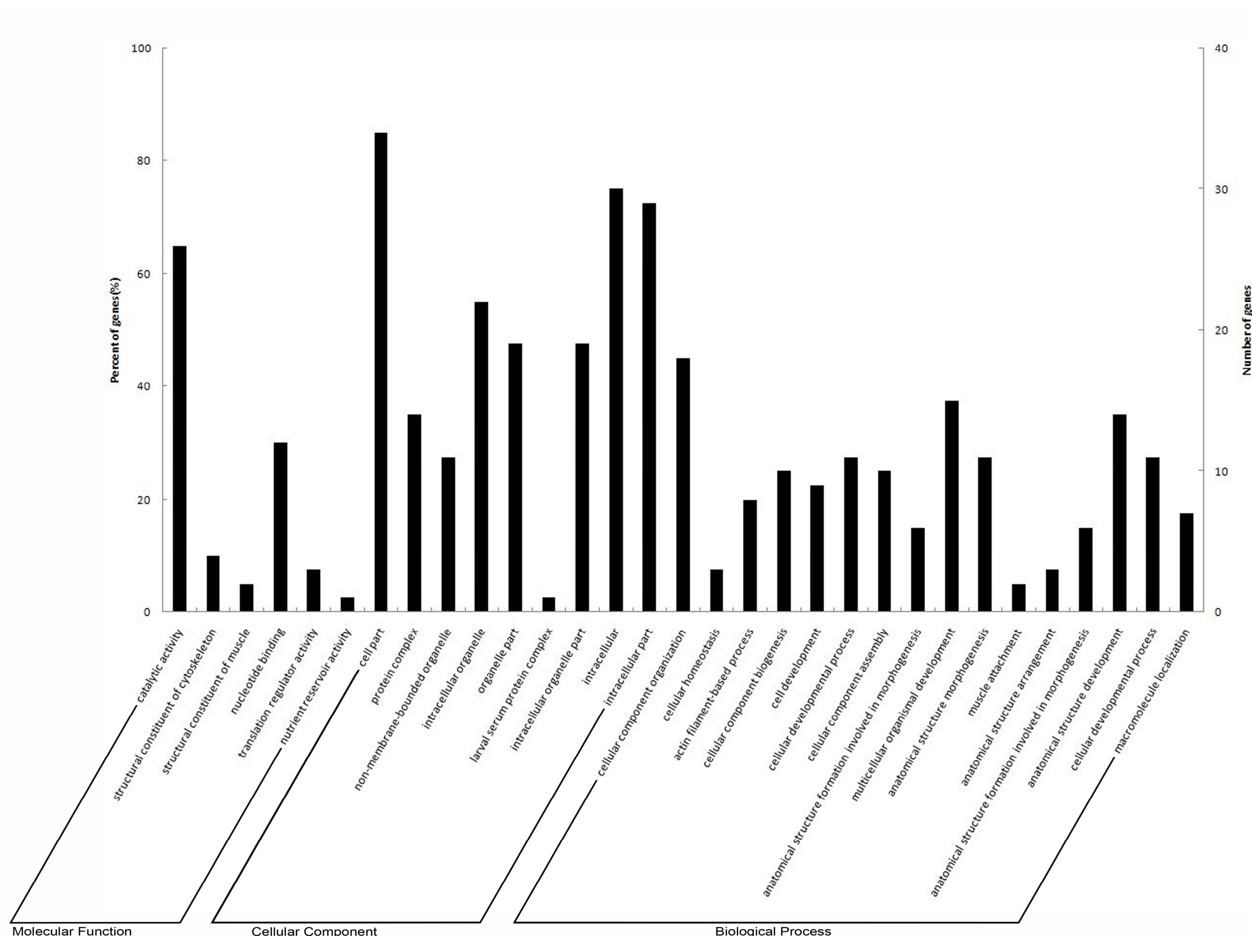

Fig. 1 Gene ontology enrichment analysis of induced genes

Among the various molecular functions, catalytic activity was the most highly represented term in upregulated genes, followed by nucleotide binding, structural constituent of cytoskeleton and translation regulator activity. The oxidoreductase atcivity and substrate-specific transporter activity were the most highly represented in downregulated genes. The subcategories associated with the oxidoreductase activity, structural constituent of ribosome, and substrate-specific transporter activity encompassed the majority of the downregulated genes.

Within the cellular component category, the upregulated genes comprised 9 categories, among which cell part, intracellular, and intracellular part each represented more than 29 genes. Intracellular part and protein complex were the most represented Gene ontology terms in the enriched categories from downregulated genes. Other sub-categories such as membrane part, synapse part, larval serum protein complex, and organelle envelope were also identified.

In the biological process category, the cellular component organization term was represented most frequently in upregulated genes, followed by multicellular organismal development, anatomical structure development, cellular developmental process, and anatomical structure morphogenesis. Among downregulated genes, the transport, regulation of biological quality, and oxidation reduction terms were enriched most frequently.

Molecular pathway analysis

To determine the function and relationship of

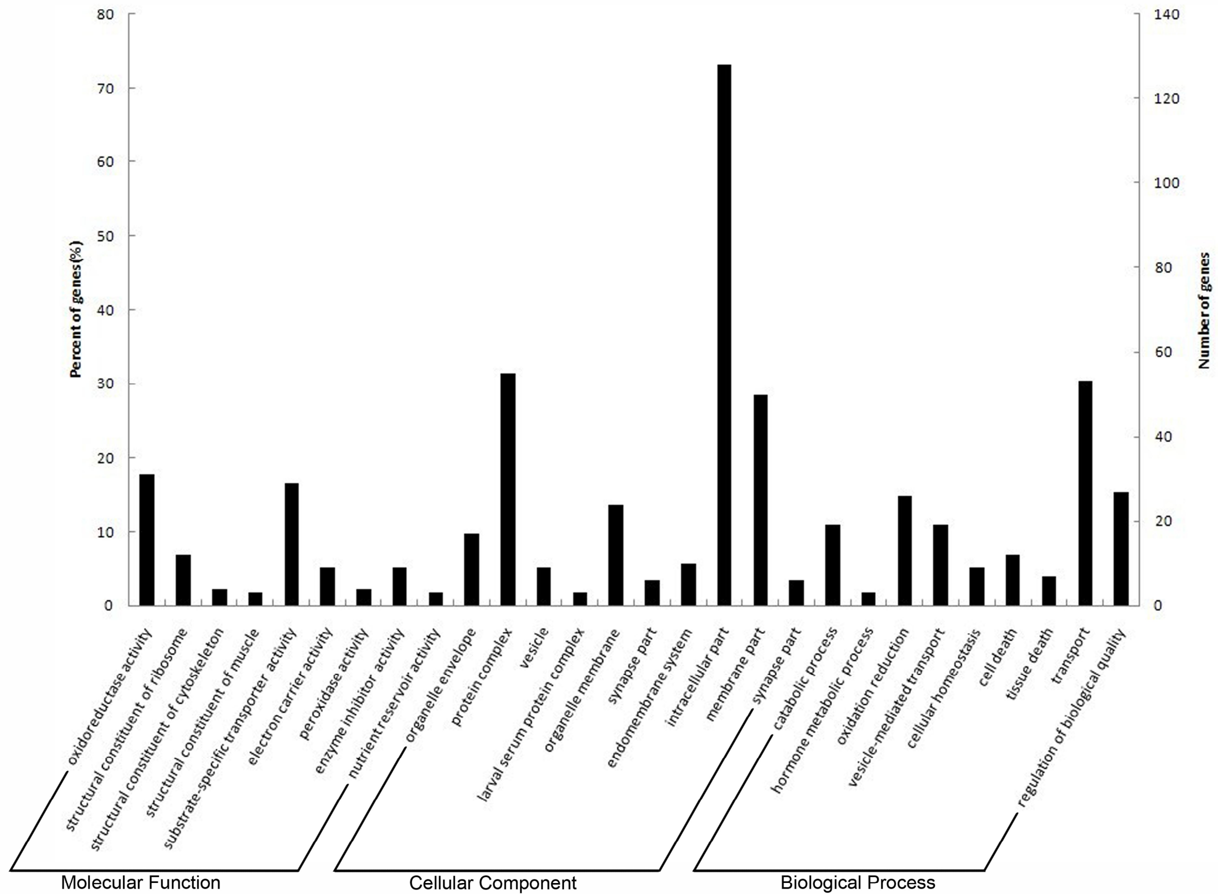

Fig. 2. Gene ontology enrichment analysis of repressed genes

these differentially expressed genes, molecular pathway analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes database.

Molecular pathway analysis showed that a large portion of the differentially expressed genes was associated with metabolic pathways (Table I). For example, the genes *Ahcy13*, *ATPsyn-b*, *ATPsyn-d*, *Cyp9f2*, *fab1*, *GlcT-1*, *kdn*, *ND75*, *UGP*, *ND75* and *Odc2* were in metabolic pathways, and most were downregulated.

A differential transcription was observed in genes involved in the endocytosis pathway. Six endocytosis pathway-associated genes were enriched in the downregulated gene group, including *Hsc70-4* and *stam*, coding for heat shock protein cognate 4 and signal transducing adaptor molecule, respectively. Additionally, the *Rab11* gene encoding Rab-protein 11 and *Vps4* (CG6842 gene product) were in the upregulated gene group.

It was noted that a single regulated gene may belong to more than one pathway. For instance, the *Ahcy* gene encoding adenosylhomocysteinase was in the cysteine and methionine metabolism, selenoamino acid metabolism and metabolic pathways. The gene *ATPsyn-b* encoding ATP synthase, subunit b, was in both the oxidative phosphorylation and metabolic pathways.

Confirmation of the gene expression by RT-qPCR

To verify the reliability of the microarray data,

Table I.- Molecular pathway analysis of 20 genes that associated with metabolic pathways.

Name	Hits	Total	Percent (%)	Enrichment test *P*-value
Arachidonic acid metabolism	2	15	13	0.0284
beta-Alanine metabolism	2	17	12	0.035
Endocytosis	8	86	9	1.00E-04
Fatty acid metabolism	4	34	12	0.0208
Glutathione metabolism	4	63	6	0.0208
Glyoxylate and dicarboxylate metabolism	2	16	13	0.0316
Limonene and pinene degradation	5	82	6	0.0118
Lysosome	6	89	7	0.0038
Metabolic pathways	42	996	4	0
Oxidative phosphorylation	16	274	6	0
Pentose and glucuronate interconversions	3	39	8	0.0278
Pentose phosphate pathway	3	23	13	0.0075
Phenylalanine metabolism	2	20	10	0.0458
Proteasome	3	50	6	0.05
Regulation of autophagy	2	14	14	0.0253
Ribosome	9	89	10	0
Selenoamino acid metabolism	2	16	13	0.0316
Tryptophan metabolism	2	18	11	0.0385
Valine, leucine and isoleucine degradation	4	31	13	0.034
Wnt signaling pathway	4	74	5	0.034

Table II.- Expression pattern of 9 genes with known functions in the RT-qPCR and the microarray experiments.

GenBank Accn.	Drosophila homologue	RT-qPCR	Microarray
77933472	l(2)efl	0.2549	0.132
JZ143892	Tal	1.56	4.17
JZ144071	Antp	0.7301	0.44
JZ144277	COX2	1.236	2.1148
JZ144285	Ef2b	0.3051	0.201
JZ144463	CG5023	1.32	9.647
JZ144492	Cpr49Aa	1.769	2.24
JZ144519	eRF1	0.6495	0.429
JZ144687	ND4	1.184	2.392

nine genes with known functions that exhibited differential expression in the microarray were randomly analyzed by RT-qPCR. Table II demonstrates that most genes followed the same expression pattern in the RT-qPCR and the microarray experiments. In the genes for JZ143892 and JZ144463, fold-change values were noticeably different between the RT-qPCR and microarray results, likely due to signal amplification in the microarray experiments. However, the gene expression patterns observed were identical. Therefore, the microarray experiments give an accurate depiction of the gene expression patterns elicited.

DISCUSSION

This study exploited a DNA microarray platform to identify differentially expressed genes in *M. alternatus* exposed to imidacloprid.A total of 359 genes were differentially expressed by at least two-fold, most of which were downregulated, and were related to metabolic detoxification, oxidative phosphorylation, stress responses.

Differentially expressed genes involved in metabolic detoxification

The overexpression of the cytochrome c oxidase subunit I gene (*COX1*) has been linked to pyrethroid resistance in the *Blattella germanica* (Pridgeon and Liu, 2003) and has been reported to be involved in the resistance of *Schistosoma mansoni* to praziquantel, a drug used to control schistosomiasis (Pereira *et al*.,1998). Our result infers that high imidacloprid-induced *COX1* overexpression plays a specific role in metabolic detoxification. Further study of the relationship between *COX1* and imidacloprid detoxification will shed light on the importance of *COX1* in insecticide detoxification or resistance.

Reduced cuticle permeability is one of the modes of insect resistance to insecticides (Ahmad *et al*.,2006). The putative cuticular protein genes in *Leptinotarsa decemlineata* were highly induced by the insecticide azinphos-methyl (organophosphorus) 2 to 3 weeks after adult molting. Several cuticular protein genes whose products are associated with the structural constituent of a chitin-based cuticle were identified in our study, including *Cpr49Aa*, *Cpr67B* and *Cpr11A*. However, three cuticular protein genes were significantly downregulated, which indicated they might not reduce cuticular

penetration and contribute to resistance or tolerance to imidacloprid.

Differentially expressed genes involved in oxidative phosphorylation

Protein phosphorylation is a critical factor in signal transduction. Many physiological processes in the cell are regulated by protein phosphorylation (Greengard, 1978). Protein phosphorylation studies have provided solid evidence to elucidate the mechanisms of many molecular events (Miyazawa and Matsumura, 1990; Enan and Matsumura, 1991; Schuh *et al*., 2002). In our study, the oxidative phosphorylation pathway genes *ATPsyn-d*, *mtacp1* and *ND75* (encoding ATP synthase subunit d, mitochondrial acyl carrier protein 1, and NADH: ubiquinone and reductase 75 kD subunit precursor, respectively) were down-regulated, suggesting toxicological changes caused by oxidative phosphorylation. We infers that the insecticidal effects on protein phosphorylation may be one imidacloprid toxicity mechanism in *M. alternatus* and possibly in other insects, and proteins that change phosphorylation levels in the insect may provide non-classical targets for insecticides.

Differentially expressed genes involved in stress responses

Several genes displayed decreased transcriptional activity after imidacloprid exposure, including those encoding serine protease, cytochrome P450 reductase, juvenile hormone esterase, cysteine proteinase and thioredoxin peroxidase.

Serine proteases are reportedly involved in the immune response (Jiang and Kanost, 2000; Jiang *et al*., 2003a,b; Kanost *et al*., 2004), food protein digestion (Mazumdar and Broadway, 2001; Herrero *et al*., 2005; Broehan *et al*., 2008), and the molting process (Samuels and Reynolds, 1993; Chamankhah *et al*., 2003; Zhao *et al*., 2007; He *et al*., 2009) in insects. Our data shows that imidacloprid repressed the transcription of serine protease. Studies have shown that reduced levels of gut serine proteases may play a role in resistance to Cry toxins (Oppert *et al*., 1997). In our opinion, imidacloprid may induce the defense system in *M. alternatus* (*e.g.*, melanization) by modulating serine protease gene transcription. Such information will help elucidate the relationship between imidacloprid exposure and serine protease -related immune responses in *M. alternatus*.

Insect cytochrome P450 reductases have been placed in a critical pathways involving metabolism-based insecticide resistance and were considered to be novel targets for the development of synergists (Lian *et al*., 2011). The inactivation of all cytochrome P450 reductases has been proposed to cause multiple developmental defects and embryonic lethality in mice (Shen *et al*., 2002). In this study, imidacloprid exposure in *M. alternatus* downregulated the cytochrome P450 reductase transcript level and thus suggested that imidacloprid may delay insecticide resistance and impede *M. alternatus* development.

It will be interesting to determine whether *M. alternatus* proteins are involved in the downregulation of juvenile hormone esterase mRNAs.It has been shown that juvenile hormone esterase inhibition results in supernumerary larval molts (İçen *et al*., 2005). We found the level of juvenile hormone esterase transcripts in *M. alternatus* larvae was reduced, suggesting that either reduced gene transcription or reduced juvenile hormone esterase RNA stability is responsible for the decrease in juvenile hormone esterase activity, and malformation would be formed during the development of *M. alternates*.

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