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Gene expression changes in honey bees induced by sublethal imidacloprid exposure during the larval stage

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ABSTRACT

Honey bee larvae exposed to sublethal doses of imidacloprid show behavioural abnormalities as adult insects. Previous studies have demonstrated that this phenomenon originates from abnormal neural development in response to imidacloprid exposure. Here, we further investigated the global gene expression changes in the heads of newly emerged adults and observed that 578 genes showed more than 2-fold changes in gene expression after imidacloprid exposure. This information might aid in understanding the effects of pesticides on the health of pollinators. For example, the genes encoding major royal jelly proteins (MRJPs), a group of multifunctional proteins with significant roles in the sustainable development of bee colonies, were strongly downregulated. These downregulation patterns were further confirmed through analyses using quantitative reverse transcription-polymerase chain reaction on the heads of 6-day-old nurse bees. To our knowledge, this study is the first to demonstrate that sublethal doses of imidacloprid affect *mrjp* expression and likely weaken bee colonies.

1. Introduction

The global decline in honey bee populations is gaining increasing attention. Approximately 20–30% of honey bee colonies were lost over the winters of 2008–2010 in Canada, Europe, and the United States (van der Zee et al., 2012). In 2014–2015, the annual loss of honey bee colonies in the United States increased to 42%, with higher losses during the summer (Seitz et al., 2016). Multiple factors have been associated with colony losses, including the parasitic mite *Varroa destructor* (Rosenkranz et al., 2010), the fungal parasites *Nosema apis* and *N. ceranae* (Antunez et al., 2009), viruses (McMenamin and Genersch, 2015), and widespread pesticide application (Budge et al., 2015; Sanchez-Bayo et al., 2016).

Although the putative causes of honey bee loss have not yet been determined, the large-scale use of systemic pesticides, such as clothianidin, imidacloprid, and thiamethoxam, and other pesticide types has been implicated as a major contributing factor (Henry et al., 2012; Farooqui, 2013; Woodcock et al., 2016). Accumulating evidence indicates that at sublethal doses, neonicotinoids cause honey bee brain dysfunction and reduce immunocompetence, leading to impaired navigation and olfactory learning and memory, and susceptibility to pathogens (Desneux et al., 2007; Yang et al., 2008; Di Prisco et al., 2013; Matsumoto, 2013; Palmer et al., 2013; Williamson and Wright, 2013; Brandt et al., 2016). Moreover, such pesticides disturb the reproductive system of queen bees, i.e., the number of eggs and viability of sperm stored in the spermatheca of queen bees, thereby reducing the numbers of adult bees and broods (Sandrock et al., 2014; Williams et al., 2015; Chaimanee et al., 2016).

The sublethal effects of pesticides on honey bees have also been investigated in the larval development stages. Pesticide exposure during the larval development stages prolongs larval development and shortens adult longevity (Wu et al., 2011). In addition, the density of synaptic units in the calyces of mushroom bodies in the heads decreases; this effect has been further associated with the abnormal olfactory learning ability of adult honey bees exposed to sublethal doses of imidacloprid during the larval stage (Yang et al., 2012; Peng and Yang, 2016). Therefore, numerous physiological aspects of honey bees might be altered after exposure to pesticides at sublethal doses, regardless of the developmental stage.

In adult honey bees, the head is the information center, processing visual, olfactory, and gustatory information through an intricate nervous system. In addition, the head contains several glands, including the hypopharyngeal gland (crucial for larval

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nutrition and colony survival), mandibular gland (producing pheromones to guide bee behaviour), and salivary gland (aiding food processing). We previously demonstrated that larval exposure to sublethal doses of imidacloprid might lead to adult neuronal dysfunction (Yang et al., 2012; Peng and Yang, 2016). Renzi et al. (2016) also found that development of hypopharyngeal gland was repressed when honey bees fed with pollen contained sub-lethal concentrations of thiamethoxam (Renzi et al., 2016). In the present study, we used a high-throughput RNA-sequencing (RNA-seq) platform to detect the dynamic range of the expression levels of all transcribed genes in the heads of adult honey bees after the larvae were exposed to a 2 ng dose of imidacloprid. A differentially expressed gene (DEG) library was constructed after comparing gene expression levels in the heads of the newly emerged workers from imidacloprid-treated and nontreated larvae. This transcriptome library might provide useful information for studying the physiological responses to imidacloprid in the heads of workers. Among these imidacloprid-affected genes, the genes encoding major royal jelly proteins (MRJPs), queen bee determinators and primary nutrient sources for larval development were selected for validation using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

2. Material and methods

2.1. Honey bees

Honey bees (*A. mellifera*) were purchased from a local supplier and maintained at the apiaries in National Taiwan University, Taipei and National Chung Hsing University, Taichung. Each test colony, containing a young normal egg-laying queen, had a working population of 9 frames of comb with larvae, pupae, honey, and pollen; the colonies were maintained according to standard beekeeping practices.

2.2. In vitro artificial larval-rearing system

An artificial larval-rearing system for studying the sublethal effect of imidacloprid on larvae was modified from the previous studies (Aupinel et al., 2005; Kaftanoglu et al., 2011). One-day-old larvae on a comb were grafted into the larva diet (53% royal jelly (produced in the apiary of National Chung Hsing University), 4% glucose, 8% fructose and 1% yeast extract) on a 100 × 15 mm Petri dish. The next day, the larvae on the Petri dish were transferred to a 96-well plate (one larva/well) supplemented with 20 μL of larva diet per well. Subsequently, food was administered every day in different amounts, i.e., 20 μL on day 3, 30 μL on day 4, 45 μL on day 5 and 55 μL on day 6. The total amount of diet administered to each larva reached 170 μL. On day 7, uric acid crystals were observed, and subsequently, the larvae were moved from the 96-well plate to a 100 × 15 mm Petri dish lined with filter paper. The next day the larvae were gently transferred onto clean filter paper in a Petri dish. Prior to eclosion, the pupae were transferred into plastic boxes (14 cm × 13 cm × 9 cm) with metallic mesh on one side. A wax frame (5 cm × 5 cm × 0.1 cm) was provided in each box. After emergence, the workers were supplied with 10 mL of 50% (w/v) sucrose solution and a 1 cm^3 pollen cake (30% pollen (collected by pollen traps in the apiary of National Chung Hsing University), 50% sugar, 10% soybean powder, and 10% yeast extract). The food was replaced every 2 days, and dead bees were removed as required. All stages of the larvae, pupae and adults were maintained in an incubator at 33 °C with 70% relative humidity.

2.3. Sample preparation and collection

Three honey bee colonies were selected for the experiments. Two frames with eggs were marked in each colony, placed inside the hives, and inspected daily. After the larvae hatched from the eggs, an area with more than 100 cells was encircled with a wire. The next day, the second instar larvae in the colony cells were treated with 1 μL of 0.5 ng imidacloprid (95% TG; Bayer Cropscience AG, Monheim am Rhein, Germany) prepared in a 50% sucrose solution containing 0.05% dimethyl sulfoxide (DMSO). The pesticide was applied once per day for 4 consecutive days (from 2- to 5-day-old), and the total dose of imidacloprid added to the nest cells was 2 ng per larva. For the nontreated groups, the larvae were treated with 1 μL of a 50% sucrose solution containing 0.05% DMSO. Regarding the *in vitro* artificial larval-rearing system, the second instar larvae in the 96-well plate were treated with 1 μL of 0.5 ng imidacloprid prepared in a 50% sucrose solution containing 0.05% DMSO or 1 μL of a 50% sucrose solution containing 0.05% DMSO once per day for 4 consecutive days (from 2- to 5- day-old). Before eclosion, the frames with capped worker broods were removed from the colony, and approximately 60–80 pupae from each frame (the *in vitro* rearing method: 50 pupae from a Petri dish) were carefully selected and confined in plastic boxes (14 cm × 13 cm × 9 cm) with metallic mesh on one side. A wax frame (5 cm × 5 cm × 0.1 cm) was provided in each box, which was subsequently placed in an incubator at 33 °C and 70% relative humidity. After emergence, the workers were supplied with 10 mL of a 50% sucrose solution and a 1 cm^3 pollen cake. The food was replaced every 2 days, and any dead bees were removed.

Three honey bee colonies at the apiary at the National Taiwan University were selected for the RNA-seq experiment. Five heads of newly emerged workers from each colony were collected, immediately frozen in liquid nitrogen, and submitted to a biotechnology company (Welgene Biotech, Taipei, Taiwan) for RNA extraction and RNA-seq analysis.

Three honey bee colonies at the apiary in National Chung Hsing University were used for qRT-PCR validation. Ten heads of 5/6-day-old workers from the *in vitro* rearing system/*in vivo* hive-reared system of each colony were collected and immediately ground in 2 mL of TRIzol reagent (ThermoFisher Scientific, Waltham, Massachusetts, USA). This experiment was carried out two times in a one-month interval, i.e., two batches of three replicate samples were collected. The samples were subsequently stored at −80 °C. Further RNA purification was performed after three replicate samples were collected.

2.4. RNA extraction

Total RNA was extracted using TRIzol reagent and a PureLink® RNA Mini Kit (ThermoFisher Scientific) to obtain high-quality RNA. For the complete removal of contaminating DNA from the RNA preparations, the samples were processed using a TURBO DNA-free™ Kit (ThermoFisher Scientific), according to the manufacturer's instructions. Next, a Qubit fluorometer (ThermoFisher Scientific) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA) with an RNA 6000 LabChip Kit (Agilent Technologies) were used to determine the RNA quality and RNA quantity, respectively.

2.5. RNA-seq library preparation and transcriptome sequencing

All procedures were performed according to the manufacturer's instructions. Sample libraries were constructed using the Agilent

AG-G9691A SureSelect ssRNA Library Kit (Agilent Technologies). The mRNA was enriched using oligo(dT) magnetic beads and subsequently fragmented into shorter fragments of 80–580 bp. The fragmented mRNA was subjected to first- and second-strand cDNA synthesis, followed by adaptor ligation to both ends. The fragments with adaptors were further purified using AMPure XP beads and enriched through PCR amplification. The sequences of the library products were directly determined through 50 bp single-end sequencing on an Illumina Nextseq 500 system (Illumina, San Diego, California, USA).

2.6. RNA-seq analysis

ConDeTri was used to trim the reads according to the quality value 20 (Smeds and Künstner, 2011). Qualified reads were subsequently mapped to the *A. mellifera* genome (Amel 3.0) and guided with the Ensembl gene annotation using TopHat, followed by gene expression analysis using Cufflinks (Trapnell et al., 2012). The gene expression levels were calculated as FPKM (Mortazavi et al., 2008). In differential expression analyses, cummeRbund was employed to perform statistical analyses of the gene expression profiles.

2.7. Functional analysis of DEGs

The functional analysis of 578 DEGs with 2-fold change were proceeded to the Gene Ontology (GO) analysis and functional annotation, respectively. GO annotation proceeded in Blast2GO with default Blast2GO parameters. Enrichment analysis for statistical significance of GO annotation between two groups of annotated sequences was performed, based on hypergeometric test. Regarding gene functional annotation, DNA was analysed using Vector NTI® software V-12 (ThermoFisher Scientific), and the BLAST and alignment analyses were conducted using the alignment program of the same software.

2.8. RNA-seq data validation using qRT-PCR

The reverse transcription step was performed using 1 μg of total RNA and the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). Gene-specific primers (Table S1) were designed using the online programmes Primer3Plus and Primer-BLAST to ensure primer specificity. Each qRT-PCR reaction in a 96-well microtitre plate contained 10 μL of 2X iQ™ SYBR® Green Supermix (Bio-Rad), 2.5 μL of 1.6 μM of each gene-specific primer, and 5 μL of diluted cDNA in a final volume of 20 μL. The PCR was performed using an iCycler iQ5 Detection System (Bio-Rad). The cycling programme comprised an initial step at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C (depending on the Tm value of primers) for 30 s. A melting curve analysis of the final amplified product was performed by taking continuous readings over increasing temperatures from 55 °C to 95 °C to ensure amplification specificity. The PCR efficiency of each primer pair was assessed using serially 10^{-1}-to-10^{-5}-fold diluted cDNA samples. The qRT-PCR data were collected using Bio-Rad iQ5 2.0 Standard Edition Optical System Software V2.0 (Bio-Rad) and normalized to the geometric mean of three selected reference genes (*actin*, GB17681; *rpl32*, GB10903; and *ef1a*, GB16844) (Vandesompele et al., 2002; Lourenço et al., 2008; Scharlaken et al., 2008). The relative gene expression data were analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Each qRT-PCR experiment was performed using three independent biological replicates with three technical replicates for each experiment. The student's t-test analysis was conducted using Sigma-Plot 12.0 (Systat Software Inc., San Jose, California, USA) with a *P*-value (<0.05) to determine statistical significance.

3. Results

3.1. Construction of the honey bee head transcriptome after exposing the larvae to sublethal doses of imidacloprid

To obtain the imidacloprid-affected transcriptome of the adult head, two pooled total RNA samples were prepared from the heads of workers newly emerged from imidacloprid-treated and non-treated larvae using single-end cDNA library construction and sequencing. The statistics for both samples are summarized in Table S2. Approximately 10 million clean, single-end mRNA reads with a mean size of 50 bp and a Q20 percentage of 85% were generated and sequentially aligned to the reference genome.

Using uniquely mapped single-end reads, we detected a total of 15,314 assembled unigenes expressed in the heads of the newly emerged workers, of which 578 unigenes had an absolute value of $\log_2$ ratio $\geq$1 (2-fold change), which was the significance threshold for DEGs between the treated and nontreated larvae (Fig. S1). These 578 imidacloprid-affected genes represented 3.8% of the head transcriptome genes, and their expression levels were classified into eight grades, which were further grouped into low (Fragments Per Kilobase of transcript per Million mapped reads (FPKM), <10), moderate (FPKM, 10–500) and high (FPKM, > 500) expression levels (Table S3). The majority of the genes (approximately 90%) were expressed at levels of 0.1–500 FPKM. Pairwise comparisons between the imidacloprid-treated and nontreated samples revealed that imidacloprid changes the gene expression pattern by approximately 3% in low and high gene expression groups (Table S3).

3.2. Screening and functional analysis of DEGs

The 578 DEGs with 2-fold change were first classified by function using the gene ontology (GO) and can be categorized into 365 functional groups in three main categories (molecular function, biological process, and cellular component) (Table S4). GO enrichment indicated that genes involved in monooxygenase activity, oxidoreductase activity and steroid hydroxylase activity in the category of molecular function, fatty acid metabolic process, lipid biosynthetic process, steroid metabolic process, small molecule biosynthetic process and monocarboxylic acid metabolic process in the category of biological process, and extracellular matrix in the category of cellular component were dominant (Fig. 1).

To further investigate the gene functional roles in honey bee physiology, the 578 DEGs were proceeded to gene functional blast in NCBI. Among the 578 transcripts expressed in the heads of adult honey bees, 329 genes with known functions were further classified into 11 groups: detoxification, immunity, sensation, transporters and receptors, neuron development, transcription factors, structural proteins, signalling pathway, metabolism, mitochondrial proteins, and others (i.e., proteins with unknown/multiple physiological pathways and roles; Fig. 2A). These genes presented dramatic expression pattern differences when comparing nontreated and imidacloprid-treated samples (Fig. 2B and Table S5). However, the functionality of the remaining 249 genes requires further characterization.

Among the 329 imidacloprid-affected genes, 129 genes with multifunctional or unique physiological roles were classified into the “others” group, whereas the remaining 200 genes with explicit functions were classified into the 10 remaining groups. Among the 129 genes, the group of genes encoding MRJPs, which play critical and multifunctional roles in the physiology, development, and colonial extension of honey bees (Buttstedt et al., 2014), were initially downregulated approximately 3–32,000-fold (Fig. 3A and Table S5). MRJPs are the major protein moieties present in the royal

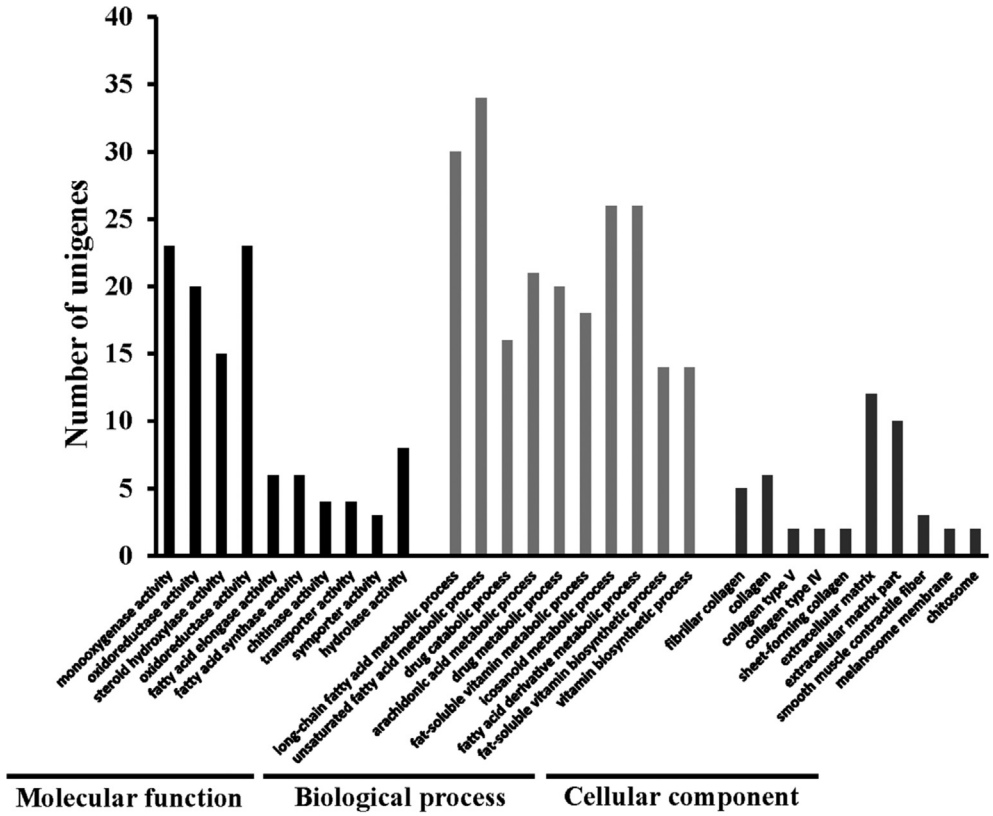

Fig. 1. Gene Ontology classification of DEGs. Ten GO terms with the lowest *P* value from each three main categories: molecular function (black), biological process (grey) and cellular component (light black), were listed. The left y-axis indicates the number of unigenes involved in a GO term. See also Table S4 shown the whole GO enrichment analysis of 578 DEGs.

jelly for the queen bee and serve as rich nutrient resources in larval foods. Due to their important roles in honey bees, MRJP expression levels were further validated using qRT-PCR.

The genes encoding glutathione *S*-transferases (GSTs) and cytochrome P450s (CYP450s) were assigned to the detoxification category, although both groups contained multifunctional proteins essential for detoxification and biosynthesis. The expression levels of two GSTs, namely GSTS1 and a GST partial sequence (GB49614), and nine CYP450s with different isoforms, namely 4aa1, 4g11, 4c3, 6a8, 6a13, 6a14, 6k1, 9e2, and 305a1, were affected by imidacloprid (Fig. 3B and Table S5). GSTS1, responsible for antioxidation(Corona and Robinson, 2006), was downregulated by approximately 4-fold, whereas GB49614 was upregulated 8-fold. The CYP450 family 4 and 305 members are responsible for the metabolism of endogenous substrates, including pheromones and hormones, whereas the CYP450 family 6 and 9 members are involved in xenobiotic detoxification (Claudianos et al., 2006). The transcriptome data revealed the upregulation of only six CYP450s, namely 4c3, 6a8, two 6a14 isomers, one 6k1 isomer, and 305a1; in particular, the expression levels of CYP450 4c3 and 305a1 were significantly increased 16- and 65,000-fold, respectively. The function of CYP450 305a1 has been associated with ecdysone-related physiology, whereas the function of CYP450 4c3 remains unknown (Mello et al., 2014). The remaining 16 CYP450s were downregulated 2.8–64-fold, among which CYP450 4aa1 and 4g11 are involved in pheromone synthesis (Malka et al., 2009) and chemoreception, respectively (Le Conte et al., 2011; Mao et al., 2015).

In the immunity category, 5 genes encoding antimicrobial peptides, namely hymenoptaecin (GB51223), abaecin (GB47318), apidaecin (GB47546), apisimin (GB53576), and lysozyme (GB40164), were significantly downregulated 4–256-fold after imidacloprid treatment (Fig. 3C and Table S5).

The genes encoding 10 odorant-binding proteins, 3 chemosensory proteins, 2 opsin proteins, and nephrin-, chaoptin-, and retinaldehyde-binding proteins were grouped into the sensory processing category (Fig. 3D and Table S5). Most of these genes were downregulated 4–16-fold after imidacloprid exposure. Only 4 genes, one odorant binding protein-encoding gene (GB50936), two chemosensory protein-encoding genes (GB55547 and GB52326), and one retinaldehyde-binding protein-encoding gene (GB45850), were upregulated approximately 8-fold. Consequently, pesticide exposure might affect the vision and olfaction functions of the honey bee.

After imidacloprid exposure during the larval stage, 46 and 23 pesticide-affected genes were grouped into a transporter and receptor category and a neuron development category, respectively. Among the 46 genes in the transporter and receptor category, 10 genes were upregulated, whereas 36 genes were downregulated (Table S5). For example, a dopamine transporter-encoding gene (GB40867) and a glutamate receptor-encoding gene (GB54881) were downregulated 16-fold, whereas a GABA neurotransmitter-encoding gene (GB54918) was upregulated 4-fold. These effects can disrupt the honey bee nervous system. Furthermore, the facilitated trehalose transporter-encoding gene (GB47931) was downregulated 4-fold in a manner consistent with the transcriptome analysis of the honey bee response to pesticides (Aufauvre et al., 2014). Notably, prostaglandin E2 receptor-encoding gene (GB49727) was upregulated 32,000-fold, indicating that the lipid-mediator receptor-associated physiology is strongly affected; however, the precise physiological interactions

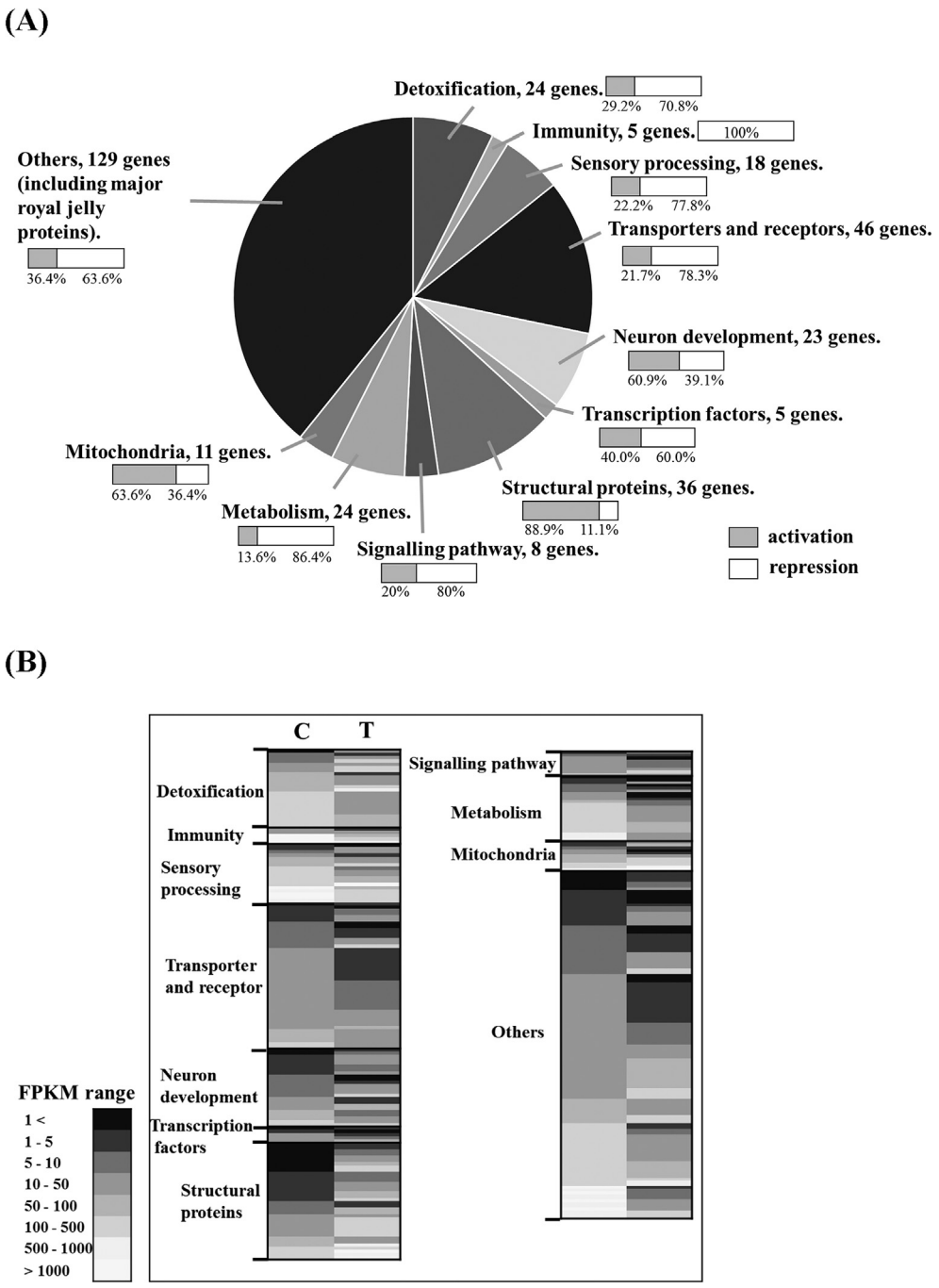

Fig. 2. Expression patterns and functionality of the 329 genes with known functions among the 578 differentially expressed transcripts with greater than 2-fold changes. (A) According to gene functionality, 329 genes with known functions were classified into 11 groups. (B) Pairwise comparison of the expression of 329 genes with known functions. Right column represents the treatment (imidacloprid-treated) group (larvae were treated with a total of 2 ng of imidacloprid) compared with the control (nontreated) group (larvae received 4 μL of 0.05% DMSO) in the left column. Each row represents a gene, and the gene expression level (Fragments Per Kilobase of transcript per Million mapped reads (FPKM)) is shown using different colours: black to white represent the gradation of gene expression abundance from low to high.

remain unknown. Of the 23 genes associated with neuron development, 19 genes were downregulated (Table S5). Considering the genes showing significant changes in expression, a synaptic vesicle glycoprotein-encoding gene (GB49708) and a circadian clock-controlled protein-encoding gene (GB42798) were downregulated and upregulated 16-fold, respectively. This fluctuating gene expression might impair the honey bee brain function.

Mitochondria are the powerhouses of the cell because they generate the majority of cellular energy. A constant mitochondrial membrane potential is critical for generation of ATP for high energetically demanding cells, i.e. neurons. In bumble bees (*Bombus terrestris*), chronic exposure to neonicotinoids causes mitochondrial depolarization in neurons, preventing mitochondria from generating energy (Moffat et al., 2015). The aforementioned effects might be associated with brain dysfunction (Tome et al., 2012). In the imidacloprid-affected transcriptome library constructed in the present study, 11 genes were involved in mitochondrial functions (Table S5). Modifications to mitochondrial gene

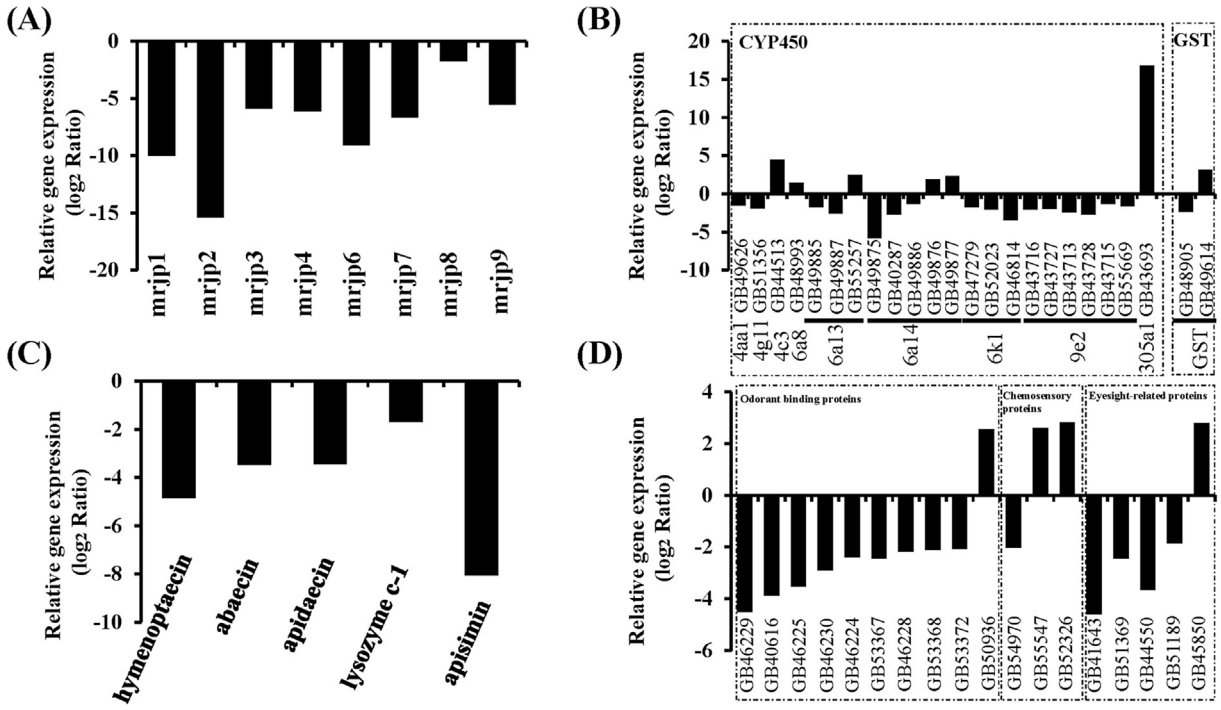

Fig. 3. The relative gene expression of 4 functional gene groups from the constructed transcriptome library. (A) MRJP-encoding gene group, (B) detoxification gene group, (C) immunity gene group, and (D) sensory processing gene group.

expression might be associated with mitochondrial dysfunction and might lead to brain dysfunction in response to pesticide exposure.

In upstream cellular processes, 5 transcription factor-encoding genes were identified, of which 3 genes were downregulated after imidacloprid exposure (Table S5). Moreover, 8 cellular signalling genes and 24 glycolytic- and carbohydrate metabolism-related genes were affected, with approximately 70% downregulated genes in both groups (Table S5). The abnormal expression of the aforementioned 37 genes involved in cellular processes accounted for the downstream physiological disorders of honey bees. These results are consistent with those of previous studies, reporting that pesticides downregulate the expression of genes encoding several transcription factors and suppress glycolytic metabolism (Derecka et al., 2013; Aufauvre et al., 2014).

The significant overexpression of genes encoding structural proteins, including cuticular proteins, chitin synthases, actins, and cellular skeleton-associated proteins, were present in the transcriptome library constructed herein (Table S5).

3.3. Validation of reference genes for the quantification of gene expression in honey bee heads

To acquire biologically meaningful data, the use of validated stable reference genes is crucial for quantifying gene expression (Vandesompele et al., 2002; Guo et al., 2013). Otherwise, false results, which show incorrect significant or nonsignificant differences between experimental samples, might be obtained (Vandesompele et al., 2002; Cameron et al., 2013). Furthermore, Vandesompele et al. (2002) demonstrated that the conventional use of a single gene for normalization could lead to erroneous normalization in gene expression up to 3–6-fold, even 20-fold in a specific case (Vandesompele et al., 2002). These authors also revealed that the minimal use of the three most stable reference genes is required for gene expression normalization.

Thus far, three studies have investigated stable reference genes for gene expression analysis in honey bees (Lourenço et al., 2008; Scharlaken et al., 2008; Cameron et al., 2013). However, none of these studies have investigated suitable reference genes for quantifying adult gene expression after pesticide treatment during the larval stage. In the present study, 7 candidate genes, namely *actin*, *α-tubulin*, *rpL13A*, *rpL32*, *rpS18*, *rpL27A*, and *ef1a*, which have been used as housekeeping genes in previous studies of *A. mellifera*, were selected and evaluated for the stability of reference gene expression in the heads of 6-day-old workers after treating the larvae with 2 ng of imidacloprid. None of the 7 reference genes showed any significant changes in expression levels in response to pesticide exposure ($P > 0.1$; Fig. S2). According to the P values, the reference genes *actin*, *rpL32*, and *ef1a* had the lowest variation in Ct values; hence, these genes were selected for gene normalization.

3.4. In vitro artificial larval-rearing system reveals that imidacloprid minimally affects the expression of mrjps

The main advantage of *in vitro* larval-rearing methods is the lack of hive-related interfering factors in pesticide-treated samples. Therefore, the *in vitro* larval-rearing method was used, obtaining approximately an 80% successful emergence rate (Fig. S3). Samples from the *in vitro* rearing method after pesticide treatment were further prepared to investigate the downregulated expression of *mrjps* in the transcriptome library. As MRJP expression reaches the maximal level in 5/6-day-old workers (Feng et al., 2009), the heads of 5-day-old workers from nontreated and imidacloprid-treated larvae were collected for RNA extraction and *mrjp* expression analysis. Following normalization with the reference genes *actin*, *rpL32*, and *ef1a*, the ΔCt values of the *mrjp* genes, i.e., *mrjp1*, *mrjp2*, *mrjp4*, *mrjp5*, and *mrjp8*, were significantly different between nontreated and imidacloprid-treated workers. However, the relative gene expression analysis showed that imidacloprid repressed the expression of *mrjps* genes, i.e., *mrjp1*, *mrjp2*, *mrjp4*, *mrjp5*, and

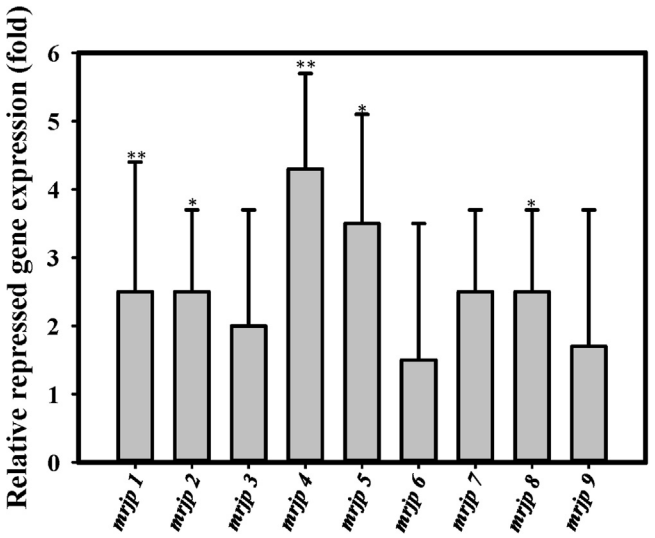

Fig. 4. qRT-PCR analysis of the expression of *mrjps* in the heads of honey bees developed from an *in vitro* artificial larval-rearing system. RNA samples from the heads of 5-day-old workers treated with 2 ng imidacloprid or DMSO per bee during the larval stage were prepared for qRT-PCR. The ΔCt value was calculated as the Ct value of *mrjp* normalized to the geometric mean of three selected reference genes (*actin*, *rpl32*, and *ef1a*). The relative gene expression was analysed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean of three repeats, with error bars indicating standard deviation. The relative transcriptional expression was analysed by the student's t-test. Genes with significantly different expression between control and treatment are indicated using asterisks: $^{*}P < 0.05$ and $^{**}P < 0.01$.

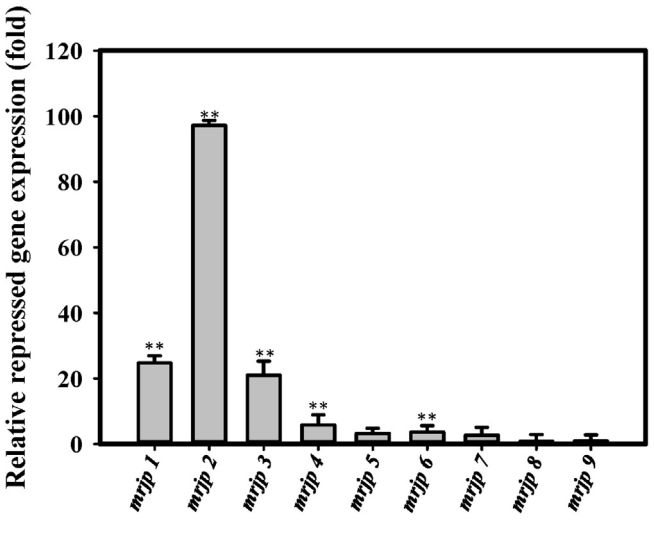

Fig. 5. qRT-PCR analysis of the expression of *mrjps* in the heads of honey bees developed from the hive. RNA samples from the heads of 6-day-old workers treated with 2 ng imidacloprid or DMSO per bee during the larval stage were prepared for qRT-PCR. The ΔCt value was calculated as the Ct value of *mrjp* normalized to the geometric mean of three selected reference genes (*actin*, *rpl32*, and *ef1a*). The relative gene expression was analysed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean of three repeats, with error bars indicating standard deviation. The relative transcriptional expression was analysed by the student's t-test. Genes with significantly different expression between control and treatment are indicated using asterisks: $^{*}P < 0.05$ and $^{**}P < 0.01$.

mrjp8, only 1- to 4-fold (Fig. 4), revealing that a sublethal dose of imidacloprid did not significantly affect *mrjps* expression. This result is not consistent with the repressed pattern of *mrjps* in the RNAseq library. However we indeed found the big difference of *mrjps* expression level between control and imidacloprid treatment groups, i.e., the raw expression data of *mrjps* in RNAseq library presented that imidacloprid would repress the expression of *mrjps* to a very low level comparing to that of control group (Table S5). Inspecting the difference between hive rearing and *in vitro* rearing methods, we considered the time of exposure to the pesticide residue would be a critical point. For the *in vitro* rearing method, the pupae were transferred from a 96-well plate to a clean Petri dish for further development and were not subjected to continuous exposure to the pesticide residue. Therefore, further pesticide treatment was performed in the comb, and the frames in the hive were maintained to investigate *mrjps* expression in the workers.

3.5. Pesticide-exposed hive-reared honey bees show higher repression of mrjps expression

Next, we collected the heads of 6-day-old workers from non-treated and imidacloprid-treated larvae reared in the hive for *mrjps* expression analysis. Following normalization with the reference genes *actin*, *rpL32*, and *ef1a*, the ΔCt values of the *mrjp* genes, i.e., *mrjp1*, *mrjp2*, *mrjp3*, *mrjp4*, and *mrjp6*, were significantly different between nontreated and imidacloprid-treated workers. The relative gene expression analysis further showed that *mrjp1*, *mrjp2*, *mrjp3*, *mrjp4*, and *mrjp6* were downregulated 25-, 97-, 21-, 5.8-, and 4-fold, respectively (Fig. 5). However, the expression levels of *mrjp5*, *mrjp7*, *mrjp8*, and *mrjp9* were not significantly affected after imidacloprid exposure. These qPCR results were mostly consistent with the RNA-seq data, indicating that pesticide treatment in the comb might prolong pesticide exposure time, resulting in higher repression of *mrjps* expression in nurse bees.

4. Discussion

The DEG library accounts for the multifaceted impacts of imidacloprid on honey bee physiology. Notably, the toxicity index LD_{50} towards imidacloprid for the honey bee in the present study was 71.5 ng/bee (Fig. S4). Different honey bee genotypes show different insecticide susceptibilities (Schmuck et al., 2001; Laurino et al., 2013; Rinkevich et al., 2015). For example, Schmuck et al. (2001) reported that the oral LD_{50} of imidacloprid to honey bees is 3.7–40.9 ng/bee (Schmuck et al., 2001). Rinkevich et al. (2015) further demonstrated that honey bees with different genetic backgrounds have different sensitivities towards neonicotinoid pesticides, i.e., Italian bees are sensitive to imidacloprid with a lower LD_{50} of 2.5 ng/bee; however, Carniolan and Russian bees are 33- and 16-fold less sensitive, respectively, to imidacloprid than Italian bees (Rinkevich et al., 2015). Laurino et al. (2013) also observed a similar phenomenon, reporting that different honey bee genotypes have different sensitivities towards neonicotinoid pesticides (Laurino et al., 2013). As the mortality rate of larvae and adult honey bees treated with 2 ng of imidacloprid was not significantly different from that of the control bees, this dose could be considered sublethal. We also speculated that the honey bees with higher sensitivities towards imidacloprid, such as Italian bees (Rinkevich et al., 2015), might have higher DEG patterns than observed in the present study with larvae exposed to 2 ng imidacloprid.

Previous studies have shown that the expression of *mrjp1–9* can be detected in the heads of honey bees at different levels (Buttstedt et al., 2013). In the present study, the expression of *mrjps* in the heads of honey bees was analysed in two independent experiments using RNA-Seq and qRT-PCR. Both experiments showed that imidacloprid exposure during the larval stage represses the expression of *mrjps* in the adult head, particularly *mrjp1–4*. Regarding their functionality, MRJP1, MRJP2, and MRJP7 might be involved in brain function because these proteins are expressed in the mushroom

body of the head (Hojo et al., 2010). Moreover, because MRJP1 and MRJP3 induce queen larval growth, these proteins are considered queen bee determinators (Kamakura, 2011; Huang et al., 2012). In addition, MRJP1 exhibits antimicrobial properties after its C-terminal region is hydrolysed to form an 8-amino-acid peptide, jelleine, which effectively kills Gram-positive and Gram-negative bacteria (Fontana et al., 2004). Nevertheless, the functions of MRJP4, MRJP5, MRJP6, MRJP8, and MRJP9 require further elucidation. To our knowledge, this study is the first to demonstrate the effect of imidacloprid on *mrjp* expression. The downregulated expression of MRJPs might lead to nutritional deficiencies in larvae, likely reflecting the phenomenon by which pesticides prolong larval development and reduce the number of *A. mellifera* (Wu et al., 2011). We have treated the health colony (8 frames with about 20,000 adult bees) by feeding 400 mL of 50% sucrose solution containing 800 ng imidacloprid, i.e. 2 pg/μL imidacloprid. The pesticide was applied once per day for 15 consecutive days to make sure all the larvae would receive the pesticide. In this condition, we found the sign of colony collapse in 2 months, i.e. the 8-frames population of honey bee dropped to 5-frames population (Fig. S5A). In 4 months, the 8-frames population dropped to 2-frames population with the queen only laying unfertilized eggs (Fig. S5B). The control group was treated with DMSO (0.05%) developed normally in 4 months, even we needed to move the extra-population to other hive for maintaining 8-frames population (Figs. S5C and D). By this result we strongly assume that the royal jelly production (including the major royal jelly protein content) in imidacloprid-treated colony would largely reduce. A previous study investigated pyriproxyfen effect on honey bee also found that low dose of pyriproxyfen can reduce yield of royal jelly in queen cells (Chen et al., 2016). Moreover, they also found that queen cell acceptance rate would reduce due to pyriproxyfen treatment (Chen et al., 2016).

In the transcriptome library constructed in the present study, the up- and downregulation of several genes involved in detoxification, sensory processing, metabolism, neuron development, transporter and receptor, mitochondria and cellular processes, e.g., transcription and cell signalling pathways, was observed after larval stage exposure to a sublethal dose of imidacloprid (Fig. S6). This fluctuating gene expression reflected that multifaceted physiologies of honey bee might be affected, leading to threaten the survival of honey bees. For example, in detoxification, the downregulated expression of CYP450 family members 6 and 9 renders honey bees more sensitive to environmental xenobiotics. Additionally, the up- and downregulation of CYP450 family 4 and 305 genes might induce chaotic behaviour among honey bees, as CYP450 4aa1 and 4g11 are involved in pheromone synthesis (Malka et al., 2009) and chemoreception, respectively (Le Conte et al., 2011; Mao et al., 2015), and CYP450 305a1 is associated with hormone-related physiology (Mello et al., 2014). CYP450 305a1, showing higher imidacloprid-induced expression levels, is an interesting target and has been implicated in the synthesis of JH according to sequence analysis. Such phenomenon also was supported by GO enrichment analysis which shown the genes involved in lipid biosynthetic process and steroid metabolic process were dominant in imidacloprid-affected honey bee physiology. Related studies of CYP450 305a1, including age-specific gene expression profiles, relevant juvenile hormone levels and imidacloprid effects are under investigation.

With respect to immunity, these results confirm those of previous studies, which showed that pesticides downregulate immunity-related genes, such as lysozyme- and hymenoptaecin-encoding genes (Aufauvre et al., 2014; Brandt et al., 2016). However, the proteins involved in pathogen recognition and signalling proteins in upstream immunity pathways were not significantly altered after imidacloprid treatment. Thus, as a consequence of immune dysfunction, honey bees might undergo disease pathogenesis.

Interestingly, we also observed that genes encoding cytoskeleton proteins were upregulated in response to pesticide treatments, suggesting that barrier defence was initiated upon pesticide exposure. These results are consistent with those of a previous study reporting that cuticular proteins are upregulated when honey bees are exposed to parasite and insecticide treatments (Aufauvre et al., 2014).

In the end, it is worth mentioning that the repressed *mrjps* expression level between hive rearing and *in vitro* rearing methods are significantly different. This might be due to the length of pesticide exposure time. The data from imidacloprid-treated hive reared honey bees also truly reflect what the honey bees in fields would face. This is a good reference for beekeepers and farmers to know the effects of pesticides on honey bee and farmers to accurately use pesticides in agriculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2017.06.016.

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