CYP6AS8, a cytochrome P450, is associated with the 10-HDA biosynthesis in honey bee (*Apis mellifera*) workers

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Abstract – Mandibular gland (MG) secretions of honey bee are synthesized through a three-step bifurcation pathway, and the initiate hydroxylation step is carried out by cytochrome P450 enzymes (P450). We have previously revealed that *CYP6AS8* and *CYP6AS11* are possibly participating in this process. In this study, we evaluated the function of *CYP6AS8* in honey bee worker's MG using RNA interference technology, and we investigated the expression pattern of *CYP6AS8* in MGs from workers of different ages. We found that the expression of *CYP6AS8* was detectable in MGs across the whole life cycle of *Apis mellifera* except for newly emerged workers and predominately expressed at day 15. RNAi knockdown revealed that the production of 10-HDA was synchronized with the expression level of *CYP6AS8* in MG of honey bee workers; the downregulation of *CYP6AS8* caused a significant reduction of 10-HDA content, demonstrating that *CYP6AS8* is indispensable for 10-HDA biosynthesis. Our current results confirm that *CYP6AS8* modulates 10-HDA biosynthesis in MGs of western honey bee worker and provided the first evidence to identify the certain P450 participate in the MG secretion biosynthesis of honey bee workers.

cytochrome P450 / mandibular gland / biosynthesis / CYP6AS8

1. INTRODUCTION

Cytochrome P450s (P450s) constitute one of the largest family of genes, with representatives in virtually all living organisms, from bacteria to protists, plants, fungi, and animals (Werck-Reichhart and Feyereisen 2000; Feyereisen 2012). P450s use a variety of small and large molecules as substrates in enzymatic reactions and catalyze a broad diversity of reactions (Feyereisen 2005, 2012). They are involved in an increasing number of physiological processes in insects, including the biosynthesis of

endogenous pheromones and hormones (Gilbert 2004), the detoxification of natural and synthetic xenobiotics in insects (Joussen et al. 2012), as well as the biosynthesis of long chain hydrocarbons (Qiu et al. 2012).

The honey bee genome contains 46 putatively functional P450 genes, belonging to four phylogenetically distinct clades (Claudianos et al. 2006; Berenbaum and Johnson 2015). Our knowledge of the specific functions of these P450s is quite limited. Enzymes involved in the metabolism of endogenous substrates, such as steroid hormones, tend to be highly conserved; thus CYP307B1, CYP314A1, CYP315A1, CYP302A1, and CYP306A1 are considered to be involved in the biosynthesis of ecdysteroid in honey bee (Yamazaki et al. 2011). The function of some members of the CYP6 subfamily is found to be

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involved in the metabolism of quercetin, a flavonoid component of honey and pollen (Mao et al. 2009). Heterologous expression of CYP9Q1, CYP9Q2, and CYP9Q3 confirmed their activity to tau-fluvalinate, an acaricide used for mite control in honey bee colonies (Mao et al. 2011).

The honey bee mandibular gland (MG) is a key exocrine gland that develops in all three castes (drones, queen, and worker) but serves different functions (Snodgrass 1984; Wu et al. 2017). In queens, MGs are responsible for the production of decenoic acids hydroxylated at ω -1-position, including 9-oxo-2-decenoic acid (9-ODA) and 9-hydroxydec-2-enoic acid (9-HDA) (Plettner et al. 1997), which function as important pheromones in honey bee colony (Slessor et al. 2005), while worker MGs mainly produce ω -hydroxylated decenoic acids, including 10-hydroxydec-2-enoic acid (10-HDA) and 10-hydroxydecanoic acid (10-HDAA) (Plettner et al. 1997), which are important nutrition to honey bee larvae (Kinoshita and Shuel 1975). Studies with deuterated substrates have revealed that P450s are likely to catalyze the hydroxylation of fatty acid at ω - and ω -1-position in honey bee MGs (Plettner et al. 1998), which leads to the important structure difference between queen and worker MG products. However, the specific P450s responsible for this key step remain unknown.

Previously, we have investigated the global gene expression patterns of MGs from queens, queenright workers, and queenless workers using high-throughput RNA-sequencing technology, and we analyzed the gene expression pattern of six P450s gene across different castes, ages, tasks and tissues (Wu et al. 2017). We found that *CYP6AS11* and *CYP6AS8* are the most abundantly expressed P450 genes in worker and queen MGs, respectively, and are selectively expressed in the tissue of MGs compared to other tissues. These indicated that *CYP6AS8* and *CYP6AS11* are involved in the process of MG secretion biosynthesis, but functional assays are still required to confirm their functions.

RNA interference (RNAi) is a valuable research tool for probing gene function and has already been widely applied to P450 function analysis and honey bee biology research (Li-Byarlay et al. 2013; Li et al. 2016). In the work

described here, we investigated the potential involvement of *CYP6AS8* in the biosynthesis pathway in honey bee worker's MG using RNAi technology and analyzed the expression profile of *CYP6AS8* in the MGs of workers in different ages. We found that silencing *CYP6AS8* lead to the downregulation of honey bee 10-HDA biosynthesis.

2. MATERIALS AND METHODS

2.1. Honey bee rearing and collection

Honey bee (*Apis mellifera ligustica*) colonies were reared at the Honey Bee Research Laboratory in the College of Animal Sciences, Zhejiang University, Hangzhou, China. To obtain the workers of specific age, we removed sealed brood combs containing emerging adult workers from natural colonies and placed them in an incubator overnight at $34.5 \pm 1^\circ\text{C}$ and $70\% \pm 5\%$ relative humidity (RH). The following day, the newly emerged workers were collected for expression pattern analysis and RNAi experiment.

2.2. Analysis of *CYP6AS8* expression pattern in worker MGs at different ages

Thirty newly emerged workers were sampled as day 0 workers. Then the rest of the workers were marked and transferred into health host colonies. Workers were sampled on days 5, 10, 15, 20, and 25. On each day, 10 workers were sampled and pooled together for further process and analysis. MG dissection, RNA extraction, and qPCR analysis were carried out as described by Wu et al. (2017). In brief, 10 pairs of mandibular glands were under a stereo microscope (SZ-61, Olympus Life Science) in RNase-free water and placed in chilled vials. Total RNA was extracted using RNeasy Pure Total RNA Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China) according to the manufacturer's protocol. The RT-PCR reaction was performed using 0.5 μg total RNA with ReverTra Ace qPCR RT Kit (Toyobo life science, Shanghai, China). *Apis mellifera* actin-related protein 1 (*Arp1*) was used as an internal reference gene. Primers for qPCR analysis were

Table I. The primers used in this study. T7 promoter sequences for dsRNA are in lower case and underlined

	Gene symbol and accession No.		Sequence (5'-3')
qPCR	<i>CYP6AS8</i> (XM_006565013.2)	F	ACCGCCAATAAACTCAGAGGAATGT
		R	ACTTCTCGCACATTGACAGGTTCTC
	<i>Arp1</i> (NM_001185145.1)	F	TGCCAACACTGTCCTTTCTG
		R	AGAATTGACCCACCAATCCA
RNAi	<i>CYP6AS8</i> (XM_006565013.2)	F	GGATCC <u>taatacgactcactataggg</u> TTTCGGCGTAGAAATGAACTC
		R	GGATCC <u>taatacgactcactataggg</u> CATTTGTTACGTCAAGGCCA

designed using Primer Premier 6.0 and listed in Table I. Reaction mixtures were setup with 1 µl cDNA (10X diluted), 0.5 µl of forward and reverse primers (10 µM), 5 µl Thunderbird SYBR Green qPCR Mix (Toyobo life science, Shanghai, China), and 3 µl distilled water. Transcription levels were quantified using the StepOne Plus Real-Time PCR System. The relative expression levels of the selected genes were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The experiment was carried out in triplicate using 3 different colonies.

2.3. Production of dsRNA

Primers for double-strain RNA (dsRNA) were designed from the sequence of the *A. mellifera* *CYP6AS8* gene (XM_006565013.2) using the E-RNAi web service (Horn and Boutros 2010). Primer sequences were fused with the T7 promoter sequence; extra bases (GGATCC) upstream of the minimal T7 RNA polymerase promoter sequences were added to increase dsRNA yield (Table I).

For producing the template of dsRNA synthesis, KOD FX (Toyobo life science, Shanghai, China) was used for PCR amplification, and the reaction mixture was prepared as follows: 50 µl 2XPCR buffer, 20 µl dNTPs, 3 µl forward primer, 3 µl reverse primer, 2 µl cDNA, 2 µl KOD FX, and 20 µl H₂O. The PCR program was 94 °C for 2 min, followed by 40 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 1 min. Then the PCR product was purified by Axyprep DNA Gel Extraction Kit and used as the template for dsRNA synthesis.

The production of dsRNA was carried out by using the T7 RiboMAX™ Express RNAi System (Promega, Madison, Wisconsin, USA). The transcription reaction mixture was assembled according to the manufacturer's instruction. The dsRNA was further purified following precipitation using a G25 micro spin column (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturer's instructions and quantified by absorbance at 260 nm using a nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The pGEM® Express Positive Control Template (Promega, Madison, Wisconsin, USA, Catalog Number: P2561) provided in T7 RiboMAX™ Express RNAi System was used as the template for negative control dsRNA (NCdsRNA)

2.4. Application of RNAi

The newly emerged workers were randomly transferred into bee-rearing cages. In each cage, 50% sucrose solution was supplied using 2 ml syringe, and pollen was supplied in the bottom of the cage for 50 worker bees. All the sucrose solution and pollen were changed daily. Workers were assigned to three experiment groups: control group (C) which was fed with normal sucrose syrup, *CYP6AS8* interference group (6AS8i), and negative control group (NC), which were provided sucrose solution containing dsRNA. dsRNA for *CYP6AS8* and NCdsRNA was diluted with sucrose solution to final concentrations of 60 µg/ml and applied to corresponding cages.

All cages were incubated at 31 °C ± 1 °C and 75% ± 5% RH; the number of dead bees was

Figure 1. The overview of dsRNA application in our experiment.

recorded daily and removed; at day 10, all the survived bees were collected and used for qPCR to evaluate the gene knockdown efficiency or for GC analysis to measure the 10-HDA content (Figure 1). The experiment was replicated three times..

2.4.1. Evaluation of the gene knockdown efficiency

A total of 30 workers from each group were used to test the efficiency of *CYP6AS8* gene knockdown (10 workers per replicate and pooled together for further process). MG dissection, RNA extraction, and qPCR analysis were carried out as described above.

2.5. Measurement of 10-HDA content

In order to evaluate the 10-HDA synthesis ability, 10 workers from each cage were collected, and the 10-HDA content in their heads was measured. Heads were dissected and extracted with 1 ml ethanol with 0.075 mg/ml methyl 4-hydroxybenzoate added as internal standard for 15 min. Next the ethanol solvent was removed; 1 ml ether was added and filtered through 0.22 μ m filter membrane. After evaporation of the ether, 400 μ l pyridine and 80 μ l BSTFA (Sigma, Kawasaki, Japan) were added, sealed, and heated at 60 $^{\circ}$ C for 60 min to derivatize samples.

All derivatized extracts were analyzed on GC-FID (GC-2010, Shimadzu, Kyoto, Japan) using InertCap-5 column (30 m $\times$ 0.25 mm I.D. $\times$ 0.25 μ m film). 1 μ l sample was injected with the help of AOC-20i auto-injector (Shimadzu, Kyoto, Japan). Nitrogen was used as a carrier gas, and flow was kept constant at 1 ml/min. The injector was held at 280 $^{\circ}$ C and worked on split mode (split ratio 10:1). The initial column temperature was 60 $^{\circ}$ C and held for 5 min and then raised to 300 $^{\circ}$ C at 5 $^{\circ}$ C/min and held for 5 min. The detector was performed at 325 $^{\circ}$ C.

2.6. Data analysis

The Kaplan–Meier survival regression analysis, expression levels of *CYP6AS8* gene in MGs, and the 10-HDA content in the heads of workers were analyzed using SPSS software version 22.0. Statistical comparison of the data was performed by one-way ANOVA; *p* values < 0.05 were considered as statistically significant.

3. RESULTS

3.1. Characterization and sequence analysis of *A. mellifera* CYP6AS8

The full length of *CYP6AS8* gene was 3574 bp spliced from 5 exons and 4 introns,

Figure 2. Sequence analysis of *Apis mellifera* CYP6AS8. **(A)** Exon-intron organization of CYP6AS8. **(B)** Schematic diagram of RNAi against CYP6AS8 mRNA. **(C)** Multiple alignment of *A. mellifera* (XP_006565076.1), *A. cerana* (XP_016922092.1), *A. dorsata* (XP_006614287.1), and *A. florea* (XP_003698349.1), CYP6AS8 protein sequences. Deep blue (100%) and pink (75%) shading indicate high degree of conservation of amino acids.

including a 549 bp 3'-UTR region and a 359 bp 5'-UTR region as shown in Figure 2a. The target site for dsRNA was designed in the fourth exon (Figure 2b). The identified *A. mellifera* CYP6AS8 gene encodes a protein of 500 amino acids; the conserved site of the P450 monooxygenase was identified at 436 AA to 445 AA using InterProScan tool (Finn et al. 2017). Sequence alignment searching through BLAST and multiple alignment analysis using DNAMAN demonstrated that this P450 is relatively conserved in 10-HDA producing *Apis* species, with sequence similarity ranging from 81.00% (*Apis cerana*) to 69.43% (*Apis florea*) (Figure 2c). This finding is consistent with findings of Plettner et al. (1997) wherein they found that these same species of *Apis* all produce 10-HDA.

3.2. The survival rate of honey bee workers after RNAi treatment

We tested whether our dsRNA have caused any harm to the caged honey bee workers. As shown by Kaplan–Meier survival regression analysis (Figure 3), cumulative survival of bees treated with different dsRNA showed no significant variation ($F_{(2, 450)} = 1.306, p > 0.05$), and the mortality is no more than 10%, demonstrating that our dsRNA have no significant impact on the health of workers and can be used in further experiments.

3.3. The silencing of CYP6AS8 with dsRNA

As shown in Figure 4, after 10 days of dsRNA treatment, a marked decline of CYP6AS8 level

Figure 3. Effect of *CYP6AS8* gene silencing on the life span of caged honey bee workers.

was detected. The expression level of *CYP6AS8* in the interference group is significantly knocked down by 57.1% when compared to control group ($F_{(1, 6)} = 94.476, p < 0.05$), while between control group and negative control group, no significant difference was found ($F_{(1, 6)} = 0.019, p > 0.05$). Thus, our RNAi treatment can successfully downregulate the expression of *CYP6AS8* in workers' MGs.

3.4. Effect of *CYP6AS8* knockdown on the level of 10-HDA

The 10-HDA content in worker's head was evaluated by GC-FID, and compared to the control group, the ds*CYP6AS8* treatment triggered a 30% decrease in 10-HDA content ($F_{(60, 1)} = 4.955, p < 0.05$) (Figure 5). In contrast, feeding of NCdsRNA did not quantitatively alter the content of 10-HDA.

3.5. The expression pattern of *CYP6AS8* in MGs from workers of different ages

The expression pattern of *CYP6AS8* in MGs at different ages is presented in Figure 6. We sampled workers at different time points, and the expression of *CYP6AS8* in MGs varied at different ages. On day 0, the expression of *CYP6AS8* was barely detectable in the MGs of newly emerged workers. Then its expression level was significantly upregulated and eventually peaked on day 15, which is higher than any other time point. Later the expression of *CYP6AS8* was decreased; however, no significant difference was found among day 5, day 10, day 20, and day 25.

4. DISCUSSION

The function of MG is a crucial difference between honey bee queens and workers. The

Figure 4. The expression level of *CYP6AS8* in the MGs after dsRNA treatment. The data represents mean expression level $\pm$ standard errors. Asterisk represents significant statistical difference ($p < 0.05$).

Figure 5. The 10-HDA content in the heads of worker. X represents the mean value; asterisk represents significant statistical difference between control group and 6AS8 interference group ($p < 0.05$); dots indicate outliers.

Figure 6. The expression pattern of *CYP6AS8* in the MGs from workers of different ages. Data represents mean expression level $\pm$ standard errors. Different letters denote statistical difference at $p < 0.05$.

MG of queens produce fatty acids hydroxylated at the ω -1-position. In the contrast, fatty acids from worker MGs are hydroxylated at the ω -position; Plettner et al. (1998) found that the ω/ω -1 hydroxylation does not involve a terminal double bond but occurs by a 1-center reaction, which suggests that this process may be catalyzed by cytochrome P450 fatty acid hydroxylases.

P450-catalyzed fatty acid ω and ω -type hydroxylation have been found in variety of living organisms (Miura 2013). Unfortunately, our knowledge about the P450-mediated fatty acid ω -hydroxylation in insect is quite limited. In mammals, six CYP4 gene subfamilies have been identified CYP4A, CYP4B, CYP4F, CYP4V, CYP4X, and CYP4Z. Three of these subfamily members (CYP4A, CYP4B, and CYP4F) have been shown to ω hydroxylate saturated, branched, and unsaturated fatty acids and eicosanoids (Hardwick 2008). However, in *A. mellifera*, the CYP4 gene family is reduced to only 4 genes (Claudianos et al. 2006; Berenbaum and Johnson 2015), and no insect P450 gene belonging to the CYP4 family has been confirmed to function in terminal fatty acid hydroxylation. Interestingly,

CYP6A8 of *Drosophila* was shown to catalyze laurate ω -1 hydroxylation (Helvig et al. 2004), indicating that fatty acid hydroxylase activity is not restricted to P450s from the CYP4 clade in insect.

CYP18A1 and CYP4AA1 were once considered as the P450s regulating this step, because they were found differentially expressed in the MGs of honey bee queens, queenright workers, and queenless workers (Malka et al. 2009). However, further transcriptome analysis on honey bee MGs has refuted this conjecture (Malka et al. 2014; Wu et al. 2017). In our previous study (Wu et al. 2017), the expression level of *CYP6AS8* in MGs of worker was over 100-fold higher compared to the MGs of queens and was selectively expressed in MG compared to other tissues. Furthermore, proteomic research on honey bee exocrine glands has confirmed our findings at the protein level (Fujita et al. 2018), suggesting that CYP6AS8 are involved in the hydroxylation of MG secretions.

In this study, a functional assay using RNAi knockdown was carried out to verify the function of *CYP6AS8*. We treated caged worker

with dsRNA via feeding. The qPCR results revealed that *CYP6AS8* transcript levels were significantly downregulated in MGs of workers from 6AS8i compared to both the control and negative control, demonstrating a successful and accurate gene knock down. Then we found that the dsRNA-mediated RNAi of *CYP6AS8* resulted in a significant reduction of 10-HDA content. Therefore, we conclude that *CYP6AS8* is closely associated with the biosynthesis of 10-HDA in honey bee worker MG and *CYP6AS8* may be responsible for the hydroxylation of stearic acid.

CYP6AS8 belongs to the *CYP6AS* subfamily, which is unique to species in the order Hymenoptera and is the largest CYP subfamily in the *A. mellifera* genome, comprising 15 genes and accounting for approximately 54% of 28 genes in all of clade 3 and 75% of 20 genes in the CYP6 family (Claudianos et al. 2006). Four more P450s belong to *A. mellifera* CYP6AS subfamily: *CYP6AS1*, *CYP6AS3*, *CYP6AS4*, and *CYP6AS10* were capable of metabolizing quercetin, the main component of the flavonoids contained in honey and pollen (Mao et al. 2009). Therefore, the CYP6AS subfamily is considered to be responsible for xenobiotic detoxification. However, our findings demonstrated that members of *A. mellifera* CYP6AS subfamily have broader and more varied functions.

Honey bee workers perform a complex age-dependent division of labor (Huang and Robinson 1996) with related function changes in glands, such as the hypopharyngeal gland (Kubo et al. 1996), which mainly produces royal jelly protein when workers are performing nursing activities and switch to produce digestive enzymes when workers start to forage (Kubo et al. 1996). Interestingly, as the gland produces royal jelly acids, the amount of 10-HDA contained in MGs increases as the workers became older and remain to be high even in the MGs of foragers (Boch and Shearer 1966). However, according to their ages, workers are performing different task, and the 10-HDA secretion demand varied (Huang and Robinson 1996); nurse workers are likely to secreting more 10-HDA than foragers. That is to say, the 10-HDA contained in MG can only

demonstrate the balance between 10-HDA biosynthesis and secretion but not the biosynthesis ability of worker MGs at different ages.

In this study, we analyzed the expression pattern of *CYP6AS8* in the MGs at workers of different ages. Based on the fact that *CYP6AS8* is involved in the 10-HDA biosynthesis, the expression of *CYP6AS8* gene and the changes of 10-HDA content (Boch and Shearer 1966; Yang et al. 2017) in the MGs of worker bees at different ages. We hypothesized that the synthesize ability of the worker MG is upregulated with age growth and reaches peak at around 15 days old, and then the 10-HDA biosynthesis in MG is reduced as the workers shift from nurse to forager. This suggest that the secretory cells of MGs remained functioning throughout the worker bee life and the most active period appeared to be around day 15. Furthermore, unlike the hypopharyngeal gland, the forager's MGs still have a high 10-HDA synthesis ability beyond 15 days.

In conclusion, we have successfully performed RNAi against *CYP6AS8* in the honey bee worker's MG and noticed a significant influence on the 10-HDA biosynthesis in MG; in addition, the expression of *CYP6AS8* revealed the changes of 10-HDA synthesis in MGs from workers of different ages. Our findings confirmed that *CYP6AS8* is closely related to the biosynthesis of mandibular acids in honey bee worker and possibly responsible for the hydroxylation of steric acid. These not only shed light on the molecular architecture underlying the mandibular gland secretion biosynthesis but also broaden our knowledges about fatty acid ω hydroxylation in insect.

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AUTHORS' CONTRIBUTION

YQW, QZH, and FLH conceived this research and designed experiments; YQW and YFZ performed experiments and analysis; YZS and SW participated in the experiments; YQW, YFZ, HLB, HQZ, and FLH wrote the paper and participated in the revisions of it; All authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Competing interests The authors declare that they have no competing interests.

Le CYP6AS8, un cytochrome P450, est associé à la biosynthèse 10-HDA chez les ouvrières de l'abeille (*Apis mellifera*).

cytochrome P450 / glande mandibulaire / biosynthèse / CYP6AS8.

CYP6AS8, ein Cytochrome P450, ist verbunden mit der 10-HDA-Biosynthese bei Honigbienen-Arbeiterinnen (*Apis mellifera*).

cytochrome P450 / Mandibeldrüse / Biosynthese/ CYP6AS8.

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