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Transcriptome changes of *Apis mellifera* female embryos with *fem* gene knockout by CRISPR/Cas9

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ABSTRACT

The sex of honey bees is decided by a regulatory cascade comprising of *csd*, *fem* and *Amdsx*. In order to further identify other genes involved in sex determination and differentiation of honey bees in the early stages of embryo development, the CRISPR/Cas9 method was used to knock out *fem* gene in the embryonic stage of diploid western honey bees, and RNA-seq was used to analyze gene expression changes in the embryo after *fem* knockout. Finally, we found that the bees had undergone gender changes due to *fem* knockout. A total of 155 differentially expressed genes (DEGs) were obtained, with 48 up-regulated and 107 down-regulated DEGs in the mutant group compared to the control group. Of them, many genes are related to sex development or differentiation. In addition, 1502 differentially expressed alternative splicing events (DEASEs) related to 1011 genes, including the main honey bee sex-determining genes *csd*, *tra2*, *fem*, and *Amdsx*, were identified between the mutant group and control group, indicating that *fem* regulates alternative splicing of a large number of downstream genes. Our results provide valuable clues for further investigating the molecular mechanism of sex determination and differentiation in honey bees.

1. Introduction

As important social-economic insects, honey bees not only provide us with abundant high-nutritional bee products, but also play an important role in the maintenance and stability of the entire ecosystem. The sex of honey bee is determined by a haploid-diploid pattern in which queens and workers are diploid females, developing from fertilized eggs, while drones are haploid males, developing from unfertilized eggs [1]. Through controlled mating of the queen and drone of the western honey bee, it was found that the sex of honey bee is actually determined by the composition of alleles at a single locus named complementary sex determiner (CSD): an individual is ultimately female if alleles are heterozygous at the *csd* locus, otherwise hemizygous or homozygous male [2–5]. Honey bee *csd* gene has high polymorphism in *A. mellifera*, *A. cerana*, *A. dorsata*, *A. florea*, and *A. laboriosa* [6–11] due to balance selection, and the polymorphism of the *csd* gene is 7 times higher than that of the neutral region of the honey bee genome [9]. Moreover, it was speculated that there may be 116 to 145 *csd* alleles in nature [12].

The *csd* gene controls the female-specific splicing of *feminizer* (*fem*), and the female-specific FEM protein controls the female-specific splicing of *Amdoublessex* (*Amdsx*) gene [1,13–16], which is a highly conserved sex-determining gene located at the end of sex determination cascades in most insects [17]. These three genes form a regulatory cascade *csd→fem→Amdsx*. *Fem* is the original gene of *csd* and is located 12 kb upstream of *csd* [7]. It encodes a protein with an Arg/Ser-rich region and a Pro-rich domain that is highly homologous to the CSD protein. Besides, FEM has an additional Arg/Ser domain at its amino terminus, but does not have the hypervariable region compared with CSD [6]. Apparently, these two genes are homologous and both encode SR-type proteins, which are generally considered to be involved in the regulation of RNA splicing [18]. Sex-specific transcripts of the *fem* gene share the same 5′ untranslated region (UTR), but differ in their downstream exon composition: compared to the male-specific transcript, part of exon 3 and whole exon 4 and 5 are lacking in the female-specific transcript. The exon 3 of male-specific transcript contains a stop codon, resulting in a truncated FEM protein [7]. Besides regulating female-specific splicing of

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Amdsx, *fem* also maintains and stabilizes embryonic female development. Knocking down the expression of *fem* in female bees through RNAi leads to gonads of 74 % bees change from ovaries to testis, and the heads of these bees became similar to drone heads [19]. Knocking out *fem* in female bees using CRISPR/Cas9 also leads to a transition of honey bee sex from female to male, and the *fem* gene is considered a "switch" that mediates nutritional regulation of ovarian development.

So far, although several key genes for honey bee sex determination have been identified, we do not know whether there are other genes involved in honey bee sex determination. In this study, the CRISPR/Cas9 method was used to knock out the *fem* gene at the embryonic stage of diploid western honey bees, and a lot of DEGs and DEASEs between the mutant group and the control group were identified by RNA-Seq.

2. Material and methods

2.1. Insects

The honey bees (*A. mellifera ligustica*) were reared under natural conditions at the Honeybee Research Institute of Jiangxi Agricultural University (28°46′N, 115°49′E), Nanchang, Jiangxi, China.

2.2. Synthesis of fem sgRNA

To ensure successful editing of the *fem* (LOC724970) gene, this study directly used the *fem*-sgRNA2 designed by Annika Roth et al. [20] as sgRNA sequence.

The template DNA of *fem*-sgRNA2 was synthesized by the company (Nanjing Yaoshunyu Biotechnology Co., Ltd.) and was subjected to PCR amplification and purification, and then the purified DNA template was transcribed into sgRNA using the RNA In Vitro Transcription Kit (MAXIscript T7, Ambion, USA). After transcription, the quality of sgRNA was detected by agarose gel electrophoresis and then was stored at −80 °C.

2.3. Sample collection and mutation rate detection

In this study, a colony headed by a single-male fertilized queen was used. The day before the experiment, a non-grafting-larvae comb was placed into the colony for the workers to clean up. On the day of the experiment, the queen was controlled to lay eggs on the comb for near 2 h, then the queen was released, the comb was taken out, and the eggs were injected using a microinjection system (Eppendorf FemtoJet, Germany) under a microscope (OLYMPUS IX73, Japan). The time from the beginning of limiting the queen on the comb to finish of the injection was no >2.5 h. Each egg in the mutant group was injected with a mixture of 1.5 μl *fem*-sgRNA2 (235 ng/μl) and 4 μl Cas9 protein (242 ng/μl), while the control group was injected with an equal amount of a mixture of *fem*-sgRNA2 (235 ng/μl) and RNase-free water. The injected eggs were promptly put into a plastic box and moved to an incubator with a constant temperature and humidity for cultivation, where the temperature was 34 °C and the humidity was 85 %. At the same time, a small amount of 16 % diluted sulfuric acid was added to the plastic box to inhibit bacterial infection of the damaged eggs [21]. The eggs were collected at 32 h after injection, and bright air cells were observed on the head of each egg, indicating that the eggs were developing normally. Twenty to thirty eggs were collected for each sample in the mutant group and the control group. The collected samples were added Trizol Up (TransGen Biotech, China) for grinding and stored in liquid nitrogen. Finally, transcriptome sequencing was completed in Shanghai Meiji Biomedical Technology Co., LTD.

At the same time, some eggs were randomly selected to test the editing efficiency. In addition, we keep some eggs for artificial cultivation under laboratory conditions until the bees emerge, and their phenotypic changes were analyzed. Subsequently, genomic DNA from eggs and adult tissues were extracted using the TransDirect Animal Tissue Kit (TransGen Biotech, China) and DNA/RNA/Protein Kit (OMEGA, USA) according to the instructions, respectively, and the target site fragments were amplified by PCR using *fem* specific primers (forward primer: 5′CCAAAAAGCCGAGGTAGAAG3′ and reverse primer: 5′ACCCGTTCTTCTTTTGAGCA3′). PCR amplification conditions were as follows: pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 30s, annealing at 60 °C for 30s, extension at 72 °C for 45 s, 30 cycles, and extension at 72 °C for 10 min. Finally, the amplified product was sent to Hunan Qingke Biotechnology Co., Ltd. (Changsha, China) for cloning and sequencing.

2.4. Molecular detection of the sex of mutant bees

The *Amdsx* gene is located at the end of the honey bee sex determination pathway, which is regulated by the *fem* gene. Here, we further validated our experimental results by detecting the expression of the sex-specific variants of the *Amdsx* gene in three adult individuals. The total RNAs of the three individuals were extracted by the TransZol Up RNA Extraction Kit (TransGen Biotechnology, Beijing, China), and the RNAs were reverse transcribed into cDNA by the MLV Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). Then the target fragments were amplified by the primers of the *Amdsx* gene (*Amdsx*-F: 5′-CTATTGGAGCACAGTAGCAAACTTG-3′, *Amdsx*-R1: 5′-GGCTACG-TATGTTTAGGAGGACC-3′, *Amdsx*-R2: 5′-GAAACAATTTTGTTCAAAA-TAGAATTCC-3′). Primer pair *Amdsx*-F/*Amdsx*-R1 was used to amplify the male- and female-specific products, and *Amdsx*-F/*Amdsx*-R2 was used to amplify the female-specific products. Amplification products were verified by agarose gel electrophoresis.

In addition, to ensure the scientific accuracy of the experiment, we identified alleles at the *csd* locus of the three adult individuals. For the DNA template extracted above, the *csd* gene primers (forward primer: 5′-AATTGGATTTATTAATATAATTTATTATTCAGG-3′ and reverse primer 5′-RTCATCTCATWTTTCATTATTCAAT-3′) and high-fidelity LA Taq DNA polymerase (Takara, Beijing, China) was used to amplify the hypervariable region of the *csd* gene, and then the amplified product was sent to Hunan Qingke Biotechnology Co., Ltd. (Changsha, China) for TA cloning and sequencing analysis.

2.5. cDNA libraries construction and sequencing

The total RNA of each sample was extracted separately, and the RNA quality was measured to ensure it meets the requirements of library construction. For each RNA sample, mRNA molecules from 1 μl total RNA were enriched using Oligo (dT) magnetic beads and were randomly disrupted into mRNA fragments. The first cDNA strands were synthesized using random hexamers and M-MuLV reverse transcriptase, and then the second strands were synthesized using enzyme buffer, dNTPs, RNase H, and DNA polymerase I. The synthesized cDNAs were purified using AMPure XP beads (Beckman Coulter, USA) and then were subjected to end repairing, poly (A) tailing, and the addition of sequencing adaptors. cDNA fragments of 240 bp in length were preferentially selected using AMPure XP beads, and the cDNA libraries were constructed by PCR amplification, enriched, and sequenced using the Illumina Hiseq 4000 platform (Illumina, CA, USA).

2.6. Alignment of sequencing data with the reference genome

FastQC software was used to perform quality control on the raw data, filter out low-quality reads (adapter sequences and reads with no inserts due to self-ligation of the adaptor, etc.), trim out the bases with low quality (quality value <20) at the 3′ end of the sequence, and remove reads containing N (modular bases), adapters, and sequences <30 bp in length after trimming. The raw data were submitted to the NCBI SRA database under accession number PRJNA878648.

HISAT2 [22] software was used to align the filtered data with the *A. mellifera* reference genome version Amel_HAv3.1 (https://www.ncbi.

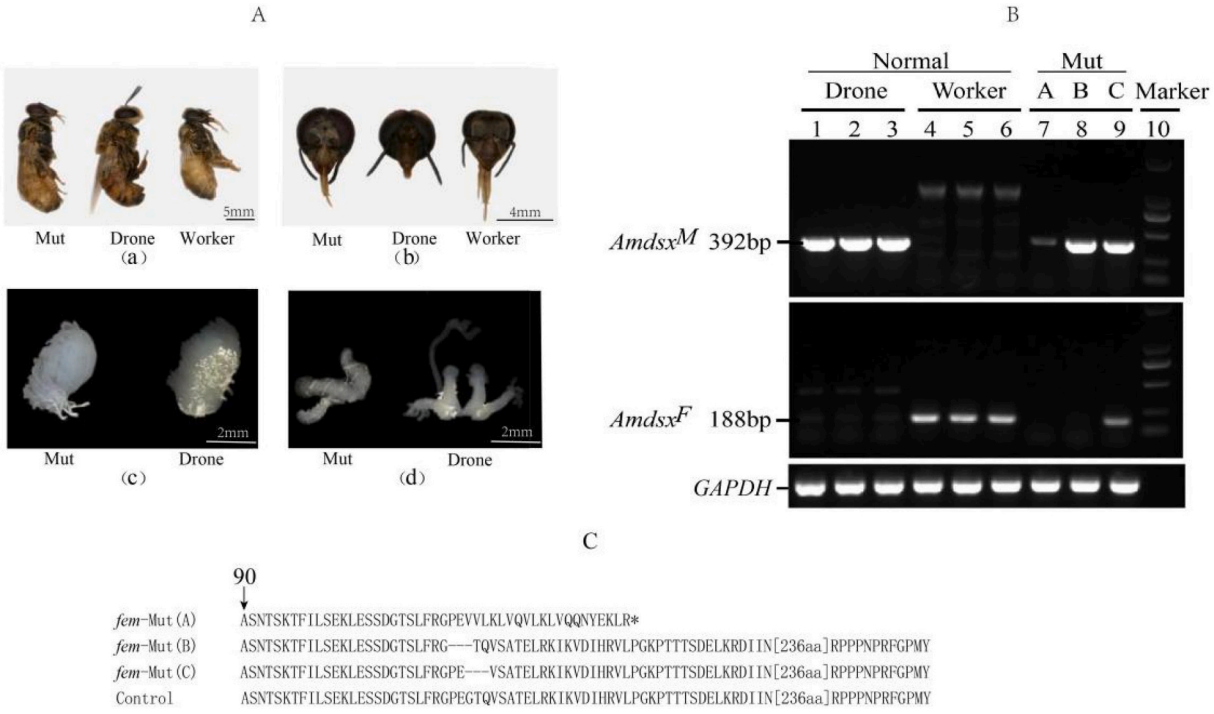

Fig. 1. Sex change of individuals with mutation of fem gene. A: (a) Comparison of phenotypes between mutant individual and normal drone and worker; (b) Comparison of the heads between mutant individual and normal drone and worker; (c) Comparison of testis between mutant individual and normal drone; (d) Comparison of mucous glands of mutant and normal drone. B: Identification of *Amdsx* gene expression in mutant individuals. C: Amino acid sequence analysis of mutant individuals.

nlm.nih.gov/assembly/GCA_003254395.2).

2.7. Screening of DEGs and DEASEs

Using the Cuffquant and Cuffnorm components of Cufflinks software, the expression levels of transcripts and genes were quantified by the positional information of the matched reads on the genes. TPM (transcripts per million reads) was used as an indicator to measure the expression level of transcripts or genes [23].

DESeq was used to analyze differentially expressed genes between sample groups [24]. The relative expression difference fold ≥ 2 or ≤ 0.5 and *P*-value <0.05 were used as screening criteria. All DEGs were mapped to the GO database for GO enrichment analysis and the KEGG database for pathway enrichment analysis.

Differential expressed alternative splicing events were analyzed using rMATS software with FDR < 0.05 and $|\Delta\psi| > 0.0001$ as the screening criteria [25].

2.8. Quantitative real-time PCR (qRT-PCR)

The above RNA samples remaining from transcriptome sequencing were reverse transcribed into cDNA using the MLV Reverse Transcriptase Kit (Invitrogen, CA, USA). A total of 10 differentially expressed genes were selected for quantitative PCR verification, and the western honey bee *Gapdh* gene was used as the internal reference gene. The primer sequences (Table S1) were designed based on the mRNA sequence of each gene from the GenBank database using the Prime Primer 5.0 software. The qRT-PCR reaction system consists of 5 μl of SYBR®Premix Ex Taq™II, 0.2 μl of ROX calibration solution, 0.4 μl of forward primer, 0.4 μl of reverse primer, 1 μl of cDNA, and 3 μl of H_2O. Reaction process: 95 °C, 5 min; 94 °C, 2 min; 40 cycles of (95 °C, 30s, Tm, 30s, 72 °C, 45 s); 72 °C, 10 min. The data were analyzed using $2^{-\triangle\triangle CT}$ [26]. Using SPSS17.0 software, significant differences were analyzed by *t*-test, and *P*-value <0.05 was regarded as significant.

3. Results and discussion

3.1. Editing efficiency detection

A total of eight embryos were randomly selected for testing editing efficiency. The results showed that for each embryo all the sequenced *fem* genome sequences are mutated at the sgRNA region and caused amino acid sequence changes (\s. S1, S2), which means double knockout of the two *fem* alleles in these embryos. It suggests that the double knockout rate of the *fem* gene of the embryo samples for transcriptome sequencing reached to 100 %. In addition, by culturing some embryos until the later stage, a total of three individuals developed into adults. Through phenotypic and anatomical structure analysis, it showed that these three individuals have undergone sex transition, and the heads of the individuals are biased towards the round shape of the drone head. The drone testis and mucous glands were dissected from these three individuals (Fig. 1A). Through detection genomic mutation of *fem* gene, we found that all the three individuals had mutated in the sgRNA region of *fem* (Fig. S3), and also caused amino acid sequence changes (Fig. 1C), suggesting that knockout of *fem* lead to sex change of these honey bees.

The expression of the *Amdsx* gene in the three individuals was detected. It can be seen from the results of agarose gel electrophoresis that the female-specific splicing variant of the *Amdsx* in the three mutant individuals was very weak or even disappeared, while the male-specific splicing variant of the *Amdsx* was very obvious, which further confirmed that the individual gender has shifted (Fig. 1B).

In addition, through analysis of TA cloning results of the three adult individuals, it was found that these three individuals had two allele forms at the *csd* locus (Fig. S4). That is, they were heterozygous at this locus, indicating that these three individuals were genetically female, which also ruled out the possibility of haploid and diploid males, and confirmed the reliability of the results.

Table 1
Statistics of transcriptome sequencing results.

Sample	Total raw reads	Total clean reads	Total clean bases	Clean reads Q30 (%)	GC content(%)	Uniquely mapping(%)
Mut1	44,175,388	43,698,792	6,350,109,241	93.29	36.84	93.41
Mut2	44,308,328	43,907,388	6,352,665,839	93.43	37.28	93.89
Mut3	44,278,750	43,872,682	6,409,713,230	93.17	37	93.87
Mut4	45,009,854	44,583,142	6,424,583,596	93.41	36.58	93.61
Con1	45,178,724	44,836,622	6,518,160,665	93.55	37.92	93.89
Con2	47,993,744	47,641,330	6,908,175,665	93.35	37.85	94.06
Con3	44,618,780	44,195,958	6,431,920,376	93.22	36.73	93.72
Con4	50,587,960	50,239,672	7,270,538,975	93.52	37.9	94.06

3.2. Statistics of transcriptome sequencing results

A total of eight RNA-seq libraries were obtained from four biological replicates of the mutant and control group (Table 1). The number of filtered reads from the eight samples in this experiment ranged from 43,698,792 to 50,239,672. The cumulative sequence length of each sample was between 6.35 Gb and 7.27 Gb. The percentages of Q30 bases were not <93.17 %, indicating that the sequencing accuracy of base calling was very high. Compared with the western honey bee genome sequences, the number of reads aligned to the unique position of the reference genome for each sample ranged from 40,820,634 to 4,725,386, and the ratio was between 93.41 % and 94.06 %.

The Pearson correlation coefficient r was used as an evaluation index for biological replicate correlation [27]. The values within each group are higher than 0.9 (Table S2), indicating the high reliability of biological replicates.

3.3. Co-expressed and specifically expressed genes

Co-expressed and specifically expressed genes between samples or groups were obtained through Venn analysis. The number of co-expressed genes between the two groups was 9,255, and the number of specifically expressed genes between the control group and the mutant group was not significantly different, with 174 and 170, respectively (Fig. S5).

3.4. DEGs between the mutant and control groups

Through analysis, a total of 155 genes with significant differences ($P < 0.05$, Table S3) were identified between the mutant and control groups. The volcano map of these DEGs is shown in Fig. 2A. There are 48 up-regulated genes and 107 down-regulated genes in the mutant group compared to the control group (Fig. 2B). Among these DEGs, the *vitellogenin* (*vg*) gene was significantly down-regulated in the mutant group.

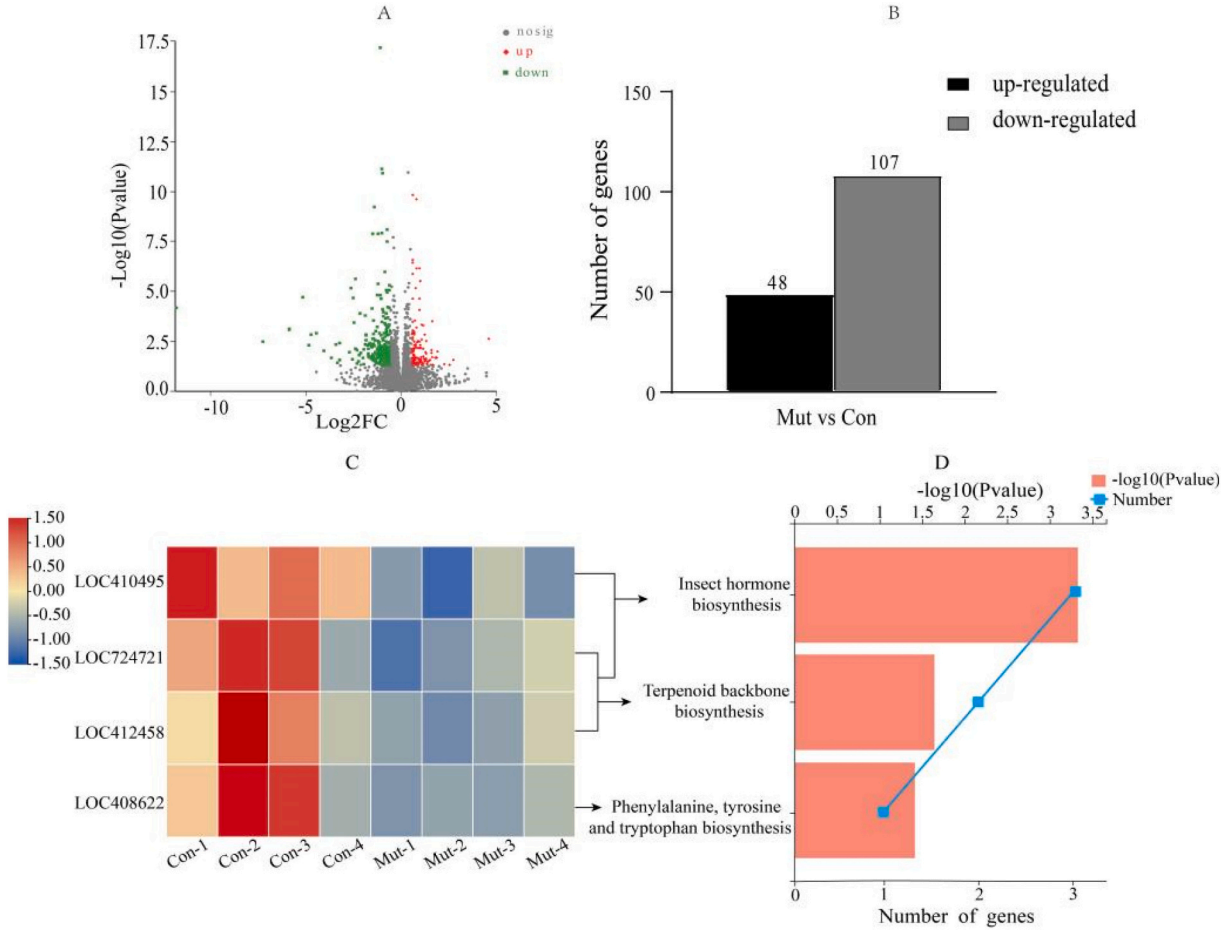

Fig. 2. DEGs between mutant group and control group. (A) Volcano plot of DEGs; (B) Numbers of up-regulated and down-regulated DEGs in mutant group compared with the control group; (C) Heatmap of DEGs in the KEGG pathways; (D) KEGG significantly enriched pathways.

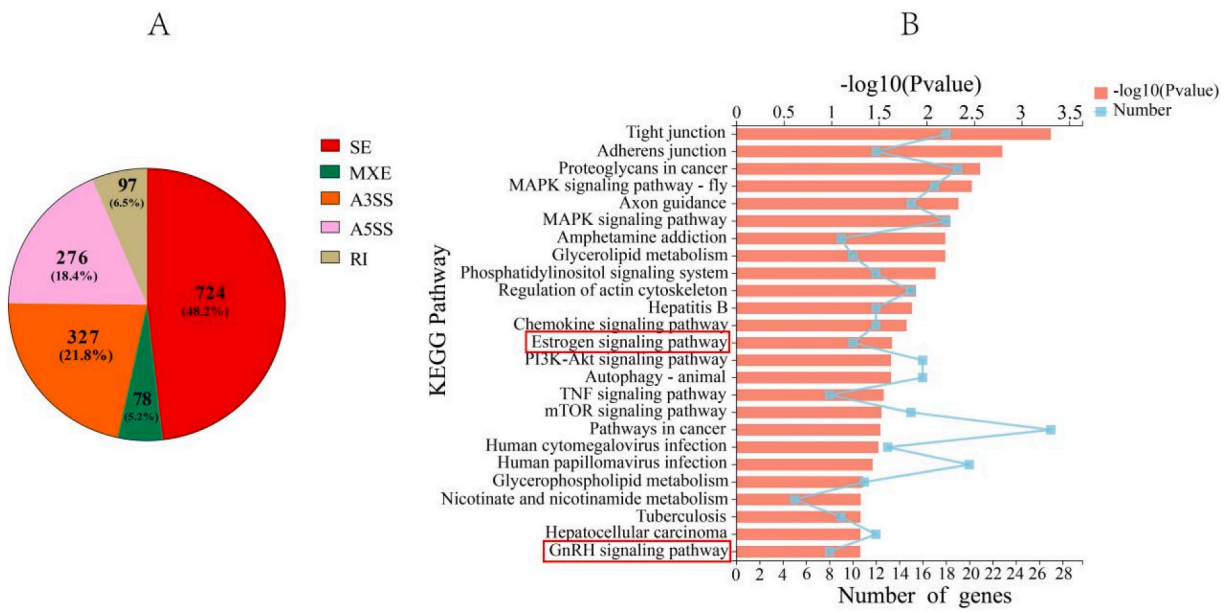

Fig. 3. DEASEs between mutant group and control group. A: Statistics on the number of DEASEs; B: KEGG enrichment analysis of DEASE associated genes.

The VG protein was synthesized in the fat body and ovary of most insects [28] and then was taken up by the developing oocyte [29]. During embryonic development, VG acts as a repository for amino acids in protein synthesis [29]. The down-regulation of *vg* after *fem* knockout during the embryonic stage suggests that *vg* is involved in female development. Two DEGs, *LOC724721* and *LOC412458*, both encode a synthetic farnesol dehydrogenase, which oxidizes farnesol in the pharyngeal body of insects to farnesal, a precursor of insect juvenile hormone (JH) [30], are down-regulated in the mutant group. A recent study showed that the JH level in drones was lower than that in queen larvae [31]. The down-regulation of these two genes may be caused by the sex transition after *fem* gene knockout, and the expression of these two genes may be differ between males and females in early embryonic stage. Another important DEG is *krüppel homolog 1* (*kr-h1*), which encodes a C_2H_2 zinc finger transcription factor and is the direct target of JH. The growth, development and morphogenesis of insects are regulated by JH through initiating transcription of the specific gene *kr-h1* in the cell [32]. Studies have shown that the increase of JH content can cause up-regulation of *kr-h1* expression in honey bees, and the two showed a positive correlation [33]. In our results, the *kr-h1* gene was down-regulated in the mutant group, indicating that the transcription of this gene was decreased due to the decreased synthesis of JH. This is consistent with previous results [33]. In addition, *cyp307a1* (LOC410495), which acts as a regulator of ecdysone in insects [34] and plays a role in the early stages of ecdysone (20-hydroxyecdysone, 20E) synthesis [34–36], is also an important DEG. The ecdysone is required for the growth and development of individual insects and the maturation of female adult oocytes [37,38]. The expression of *cyp307a1* was down-regulated in the mutant group, indicating that the ecdysone level in *fem* mutant embryos might be decreased in the early stages of embryonic development.

GO enrichment analysis was performed on the DEGs, and the results

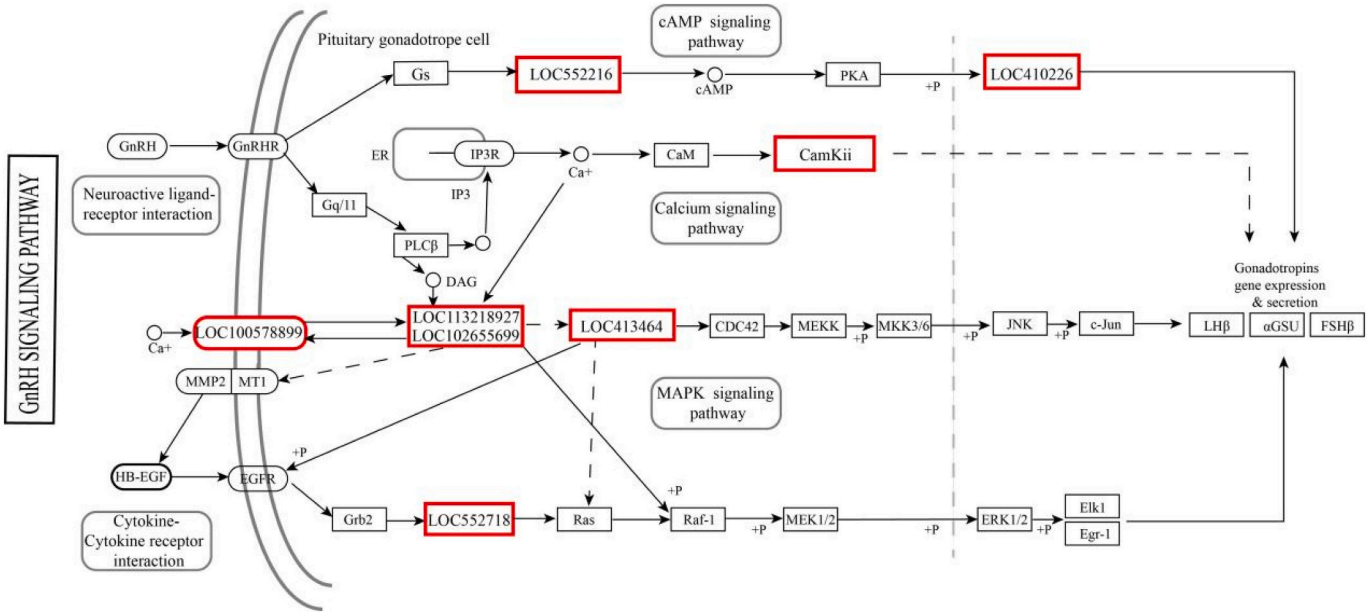

Fig. 4. GnRH signaling pathway. Genes boxed with red color are associated with DEASEs.

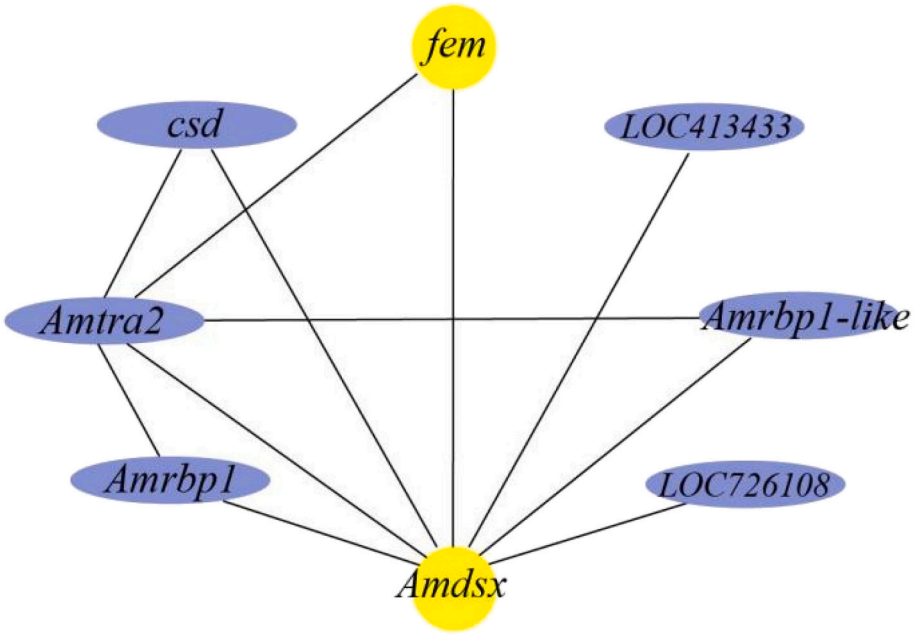

Fig. 5. Mapping of gene interactions associated with *fem* and *Amdsx*.

showed that a total of 84 GO entries were significantly enriched (*P*-value <0.05, Table S4), among which, "sperm chromatin condensation (GO: 0035092)" is related to spermatogenesis in mammalian [39], it may has similar function in honey bees. KEGG pathway enrichment analysis of the DEGs indicated that three KEGG pathways were significantly enriched (*P*-value <0.05, Fig. 2D), including a total of four DEGs (Fig. 2C). Three DEGs are in the insect hormone biosynthesis (map00981) pathway: LOC410495, LOC724721 and LOC412458, of which two genes, LOC724721 and LOC412458, are shared by the terpenoid backbone biosynthesis (map00900) pathway. The Phenylalanine, tyrosine and tryptophan biosynthesis (map00400) pathway contains one DEG.

3.5. DEASEs between the mutant and control group

Alternative splicing is very common in eukaryotes and is involved in many biological processes. For example, in *Drosophila*, an X:A → *sxl*→*tra*→*dsx* regulatory cascade formed by alternative splicing controls *Drosophila* sex development [40]. In this study, five alternative splicing forms between the mutant group and the control group were analyzed, and a total of 92,211 and 92,916 splicing forms were detected in the four biological replicates of the mutant group and the control group, respectively. Skipped exon (SE) is the most selective type of alternative splicing. In addition, there were 1,502 DEASEs between these two groups (Fig. 3A), which are related to 1,011 genes, including the main sex-determining genes: *csd*, *tra2*, *fem*, and *Amdsx* of the honey bee.

The GO and KEGG enrichment analysis of genes related to DEASEs showed that a total of 75 GO terms were significantly enriched (*P*-value <0.05, Table S5), and most of these terms belonged to molecular functions. The KEGG pathway enrichment analysis showed that a total of 25 KEGG pathways were significantly enriched (*P*-value <0.05, Fig. 3B). Among these pathways, the Estrogen signaling pathway (map04915) and the GnRH signaling pathway (map04912) were related to male and female development. Of them, estrogen signaling pathway maintains the secondary sexual characteristics of females [41] and enhances the "self-priming" effect of GnRH on the pituitary [42], thereby affecting the release of gonadotropins. This pathway may also play a role in sex differentiation of honey bees. Another signaling pathway is the GnRH

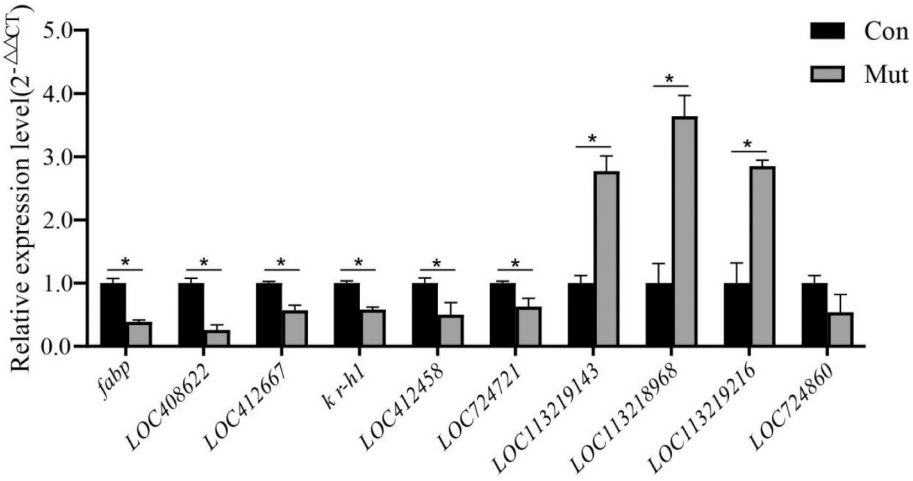

Fig. 6. Quantitative RT-PCR verification of ten DEGs between the mutant (Mut) group and control (Con) group. The X-axis indicates the gene names. The Y-axis shows the relative expression levels of genes.*represents significant difference.

signaling pathway. As shown in Fig. 4, in this pathway, after GnRH signaling binds to the GnRH receptor on the surface of pituitary gonadotrope cells, it goes through three intracellular signaling pathways in the cell, and finally regulates gonadotropin gene expression and secretion to generate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) necessary for female development [43–45].

Taken together, DEASEs play an integral role in male and female growth and development.

3.6. Genes interacting with fem and Amdsx

To identify genes interacting with *fem* and *Amdsx*, all the DEASEs related genes were analyzed by the string online software (Fig. 5). The genes interacting with *fem* are *Amtra2* and *Amdsx*. In addition to *fem*, there are other six genes interacting with *Amdsx*, including *csd*, *Amtra2*, *Amrbp1*(*LOC727349*), *LOC726108*, *Amrbp1-like* (*LOC413835*) and *LOC413433*. Among these genes, *Amtra2* is an RNA-binding cofactor essential for regulating alternative splicing of *fem* and *Amdsx* [46]. In *Drosophila*, *rbp1*, *rbp1-like* and *tra2* regulate alternative splicing of *dsx* by recognizing the *rbp1* target sequence in the pre-mRNA of the *dsx* gene [47,48]. We speculate that these three genes play similar roles in honey bees and are involved in regulating alternative splicing of *Amdsx*. The *LOC726108* gene encodes the synthetic E3 ubiquitin-protein ligase MSL-2, which is a RING-finger protein required for X chromosome dose compensation in male *Drosophila* [49]. *Msl-2* is involved in the formation of the MSL complex, which up-regulates the transcription level of most genes on the X chromosome in male individuals to compensate for the difference in X chromosome dose between males and females [49]. We speculate that dose-compensation mechanism may also exist in honey bees and *msl-2* plays a key role in this process.

3.7. Validation of DEGs by qRT-PCR analysis

Ten DEGs between the mutant group and control group were selected for verifying the reliability of the RNA-seq results using qRT-PCR. The results indicated that nine of the ten genes were significantly differentially expressed between the two groups with three of them up-regulated and six down-regulated in the mutant group compared to the control group, which are consistent with the RNA-seq results, and the remaining one showed a similar expression trend as the transcriptome result although the expression difference between the two groups is not significant in statistical tests (Fig. 6). These results indicated that the transcriptome results are highly reliable.

4. Conclusions

In this study, CRISPR/Cas9 technology was used to knock out *fem* gene at the diploid embryonic stage, which led to change of individual sex from female to male, which further confirmed importance of *fem* to the sex development of honey bees. The transcriptome comparison of diploid fem mutant embryos and blank controls from the same queen identified a lot of DEGs and a much larger number of DEASEs, which indicated that the fem gene might affect alternative splicing of many downstream genes that are involved in regulating sex differentiation of honey bees. These results provide very valuable information for exploring the molecular mechanism of honey bee sex determination and differentiations at embryo stage.

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CRediT authorship contribution statement

Wang Zilong: Conceived and designed the experiments, Writing-reviewing and editing.

Cheng Fuping: Performed the experiments, Analyzed the data, Writing- Original draft preparation.

Hu Xiaofen: Performed the experiments, Analyzed the data.

Gong Zhixian: Performed the experiments.

Pan Luxia: Analyzed the data.

Qin Kaixin: Analyzed the data.

Li Zhen: Analyzed the data, Review.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zi Long Wang reports financial support was provided by National Natural Science Foundation (No. 32160134). Zi Long Wang reports financial support was provided by Natural Science Foundation of Jiangxi Province (No. 2018ACB21028).

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