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Original Article

CHANGES IN ALTERNATIVE SPLICING IN *APIS MELLIFERA* BEES FED *APIS CERANA* ROYAL JELLY

Yuan Yuan Shi[1]
Zachary Y. Huang[2]
Xiao Bo Wu [1]
Zi Long Wang[1]
Wei Yu Yan[1],
Zhi Jiang Zeng[1]*

[1]Honeybee Research Institute, Jiangxi Agricultural University, Nanchang 330045, P.R. China
[2]Department of Entomology, Michigan State University, East Lansing, MI 48824 USA

*corresponding author: bees1965@sina.com
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Abstract

The Western honey bee (*Apis mellifera*) is a social insect characterized by caste differentiation in which the queen bee and worker bees display marked differences in morphology, behavior, reproduction, and longevity despite their identical genomes. The main causative factor in caste differentiation is the food fed to queen larvae, termed royal jelly (RJ). Alternative splicing (AS) is an important RNA-mediated post-transcriptional process in eukaryotes. Here we report AS changes in *A. mellifera* after being fed either *A. mellifera* RJ or *A. cerana* RJ. The results demonstrated that the RJ type affected 4 types of AS in adult *A. mellifera*: exon skipping, intron retention, alternative 5' splice sites, and alternative 3'splice sites. After feeding with *A. cerana* RJ, AS occurred in many genes in adult *A. mellifera* that encode proteins involved in development, growth, the tricarboxylic acid cycle, and substance metabolism. This study provides the first evidence that heterospecific RJ can influence the AS of many genes related to honey bee development and growth.

Keywords: alternative splicing, honey bee, royal jelly, tricarboxylic acid cycle.

INTRODUCTION

The Western honey bee (*Apis mellifera*) is an important model insect because of its precise division of labor, its communication via dance to make effective use of natural resources, and its high levels of social cohesion (Seeley, 1989). In addition, *A. mellifera* is one of the most important insects from an economic standpoint because of its crucial role in pollination (Calderone, 2012).

A honey bee colony usually contains a fertile queen, hundreds of haploid drones, and thousands of nearly sterile workers (Winston, 1987). Despite having identical genomes, the queen and her workers display vast differences in morphology, behavior, physiology, reproduction, and longevity (Weaver, 1957; Weaver, 1966; Guan et al., 2013). The main causative factor is the food fed to queen larvae, termed royal jelly (RJ), which is a yellow milky substance from "nurse bees" that have special hypopharyngeal and mandibular glands (Winston, 1987). RJ has high levels of proteins, sugars, fatty acids, minerals, vitamins, and bioactive substances, especially antibacterial proteins and immunological peptides (Drapeau et al., 2006; Viuda-Martos et al., 2008; Fang et al., 2010). The important components of RJ are the major royal jelly proteins (MRJPs), which play important roles in reproductive maturation and brain development (Drapeau et al., 2006). Royalactin is the monomeric form of MRJP1 and helps increase body size, promote ovary development, and shorten developmental time, thus inducing queen characteristics (Kamakura, 2011).

A. mellifera and *A. cerana* (the Eastern honey bee) are two important honey bee species that are widely bred and studied, and it is likely that they diverged from a common ancestor around three million years ago (Oldroyd and Wongsiri, 2006). Compared with *A. mellifera*, *A. cerana* has stronger resistance to the *Varroa destructor* (Peng et al., 1987). These two species not only display different behavior but also have differences in their RJ (RJM from *A. mellifera* and RJC from *A. cerana*). The two types of RJ differ not only in levels of some MRJPs (Qu et al., 2008; Fang et al., 2010) but also in their DNA content (Zeng et al., 2006). Notably, there are 23 microRNAs (miRNAs) specific to RJM, 2 miRNAs specific to RJC, and 46 miRNAs found in both types of RJ (Shi et al., 2012).

Alternative splicing (AS) is an important RNA-mediated post-transcriptional process in eukaryotes that creates functionally different proteins. AS plays important roles in development, cell differentiation, and cancer (Modrek and Lee, 2002; Black, 2003; Wang et al., 2012). Recent studies have demonstrated that AS is widespread in *A. mellifera* (Jarosch et al., 2011; Foret et al., 2012; Li et al., 2012), and AS was recently found to be the mechanism that controls social parasitism in Cape honey bees (*A. mellifera capensis*) (Jorasch et al., 2011). In this study, we compare AS in genes in *A. mellifera* that were fed either RJM or RJC. These bees were termed mRJM and mRJC, respectively.

MATERIAL AND METHODS

Experimental bee colonies

Honey bee colonies (*A. mellifera* and *A. cerana*) were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46ºN, 115.49ºE) using standard beekeeping techniques.

Harvesting RJM and RJC

RJM and RJC were produced according to standard practices in China (Zeng et al., 2009). Briefly, the queen was confined in a queen-excluding cage. Queen cups with one-day-old larvae were introduced into the colony and fed by workers for 2 days. First we carefully removed 3-day-old larvae using either a grafting tool or a pair of forceps and then we removed the RJ with a spatula.

mRJM and mRJC

A. mellifera larvae were reared in 24-cell tissue culture plates (Costar, NY, USA) in an incubator (35°C, 75 ± 3% relative humidity (RH)). Each cell was primed with 200 µL of freshly collected RJ from RJM or from RJC and then a 1-day-old larva was transferred into it. Every 8 h, larvae were transferred to another plate with new food. For pupation, 6-day-old larvae were transferred to 6-cell tissue culture plates lined with pieces of Kimwipe and kept in an incubator (35°C, 75 ± 3% RH). We only used samples with fully developed queens (larger body size, notches in their mandibles, and 16 days of development); we did not use other individuals, such as workers or intercastes. A total of 1,400 larvae were reared for this experiment (Shi et al., 2012). After adult queen emergence, we obtained one sample per treatment, i.e. one mRJM sample and one mRJC sample. Each sample consisted of 10 adult queens (5 from each of the two colonies), and their heads were used for global transcriptome analysis. These samples were kept at -80°C until use.

Transcriptome and alternative splicing analysis

Global transcriptome analysis of mRJM and mRJC was performed by the Beijing Genomics Institute (Shenzhen, China) as described previously (Shi et al., 2012). To construct a cDNA library, total RNA was extracted with TRIzol regent (Invitrogen, USA). The library (200-bp inserts) was sequenced with an Illumina HiSeqTM 2000 (Illumina Inc., CA, USA). A total of 48,971,186 reads from the mRJM library and 49,358,642 reads from the mRJC library were obtained after discarding the empty adapters (Shi et al., 2012). After mapping to the *A. mellifera* reference genome (*Amel* 4.5), we obtained 38,461,269 (78.54%) mapped reads from the mRJM library and 38,759,459 (78.54%) mapped reads from the mRJC library (Shi et al., 2012). AS was identified using the Short Oligonucleotide Analysis Package (SOAP) splice software (Huang et al., 2011). The reported AS gene numbers are those identified by SOAP splice software and analyzed by the National Center for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov/ gene/?term=gene numbers + in + apis). Data were analyzed using Contingency Table Analysis with StatView (v 5.01, SAS Institute, Gary, NC, USA).

RESULTS

We identified 4 types of AS in mRJM and mRJC: exon skipping, intron retention, alternative 5' splice sites, and alternative 3'splice sites (Fig. 1). The distribution of the number of genes with different types of AS were not significantly different in mRJM versus

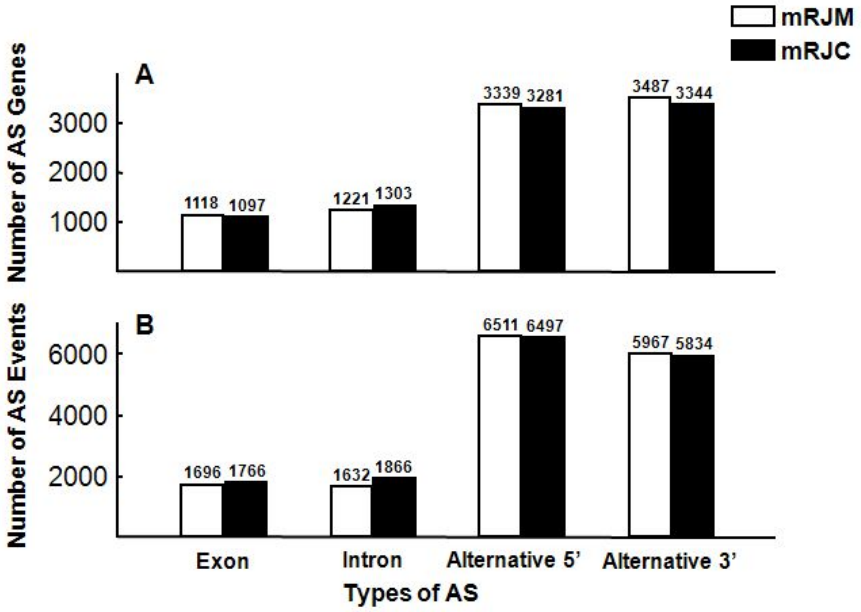

Fig. 1. The number of alternatively spliced (AS) genes (A) and the number of AS events (B) in genes expressed in the heads of adult *Apis mellifera* fed royal jelly from *A. mellifera* (mRJM; open bars) or from *A. cerana* (mRJC; solid bars). The distribution of the number of genes showing different types of AS in mRJM and mRJC were not significantly different ($P>0.05$). The distribution of the number of different types of AS events in mRJM and mRJC were significantly different ($P<0.01$).

mRJC (Contingency Table Analysis, $\chi^2 = 5.29$, df = 3, $P>0.05$). However, the distribution of the number of different types of AS events in mRJM and mRJC were significantly different (Contingency Table Analysis, $\chi^2 = 17.81$, df = 3, $P<0.01$). The two supplementary tables show all genes with AS in the mRJM and mRJC samples.

AS affected genes involved in arginine, histidine, and proline metabolism only in the mRJC sample (Tab. 1). Three AS genes (408862, 551507, and 410638) were found to be involved in arginine, histidine, and proline metabolism; notably, α-ketoglutarate biosynthesis is based on these amino acids. We detected AS in the mRJC sample in the genes for ketohexokinase, sorbitol dehydrogenase, hydroxyglutarate oxidase, and fumarase. These AS events are therefore predicted to affect fructose and mannose metabolism and the tricarboxylic acid (TCA) cycle. Two genes, 410999 (encoding hydroxyglutarate oxidase) and 724321 (encoding fumarase), showed AS in the mRJC sample. AS in the mRJM sample occurred in the genes for tetrahydrofolate dehydrogenase and for the NADH dehydrogenase Fe-S protein complex, which is predicted to affect the synthesis of ATP. In addition, three AS genes (726522, 408871, and 551785) were found that encoded proteins that are involved in regulation of fructose and mannose metabolism and in the pentose phosphate pathway; these AS events may affect glucose metabolism. The TCA cycle and glucose metabolism are critical for generating energy, which is stored as ATP, for cell-to-cell signaling, for synthesizing new structures, and for the movements of motile cells.

AS in the mRJM and mRJC samples influenced genes encoding the following types of proteins: proteins involved in important signaling pathways (circadian rhythm, the Wnt signaling pathway, cellular detoxification, the Jak-STAT signaling pathway, and the notch signaling pathway); signaling molecules (calcium signal modulators, the major facilitator superfamily, PDZ signaling molecules, and WD40); and other proteins that are important for cellular function (mitochondrial carrier protein, odorant receptor, cold-shock protein, dynactin p62 family, juvenile hormone binding protein, and secretory proteins) (Tab. 2). AS in the mRJM sample, but not in the mRJC sample, affected some specific genes encoding proteins involved in the circadian rhythm and in the Wnt signaling pathway, as well as some leucine-rich repeat proteins. The circadian rhythm pathway (406112) may affect hormone release, body temperature, sleep cycles, and other processes. The leucine-rich repeat (409027, 413332, 413310, 725782, and 100577587) is a structural motif involved in molecular recognition processes, such as signal transduction, cell

Table 1.

Genes related to the tricarboxylic acid cycle (TCA) and substance metabolism that show alternative splicing in adult *A. mellifera* queens that were fed royal jelly from *A. mellifera* (mRJM) or royal jelly from *A. cerana* (mRJC)

Pathways	Alternatively spliced genes in mRJM	Alternatively spliced genes in mRJC
Alanine, aspartate and glutamate metabolism	408955, 412923, 551670	408955, 412923, 551670
Arginine and proline metabolism		**408862, 551507**
Cysteine and methionine metabolism	410798	410798**, 551341**
Glycine, serine and threonine metabolism	410550, **413885**, 552523	**410432,** 410550, 552523, **725967**
Histidine metabolism		**410638**
Phenylalanine, tyrosine metabolism	725400	**551858**, 725400
Valine, leucine and isoleucine degradation	408288, **408716**, 410554, **412027**, **100578450**	408288, 410554
Fructose and mannose metabolism, Ketohexokinase		**726522**
Fructose and mannose metabolism, Sorbitol dehydrogenase		**408871**
Pentose phosphate pathway		**551785**
TCA cycle, Glycolysis / Gluconeogenesis	408654, 409624, **410999**, **411697**	408654, 409624, **410122**, **727153**
TCA cycle, hydroxyglutarate oxidase		410999
TCA cycle, pyruvate dehydrogenase	100578821	100578821
TCA cycle, isocitrate dehydrogenase	410396	410396
TCA cycle, succinate dehydrogenase	**409549**, 550667	550667
TCA cycle, fumarase_classII		**724321**
TCA cycle, dihydrolipoyl acyltransferase	409155	409155
Oxidative phosphorylation	**409103**, 409148, 409930, 411183, 552682	409148, 409930, 411183, **550667**, 552682, **726120**
Coenzyme metabolism	**408766**, 409766, **411447**, **725105**, **727189**, **100578821**	**409017**, 409766, **411759**, **550745**, **552104**, **552364**
Cytochrome	411057, 552679	**410936**, 411057, **413083**, **551223**, 552679, **100576555**
NAD; tetrahydrofolate dehydrogenase	**412276**, **55166**	
NADH dehydrogenase Fe-S protein complex	**408367**, **411411**	

The gene numbers in bold were specific (not shared) between the two types of queens, while plain font indicates that these genes were shared in the two types of queens.

development, cell adhesion, DNA repair, and RNA processing. The Wnt signaling pathway (551258 and 552177) is a conserved signaling pathway that plays very important roles in physiological processes. AS in the mRJC sample occurred in genes encoding proteins that are important in cellular detoxification (411045, 408551, 100578051, and 412436) and in genes encoding a cold-shock protein (409854), the dynactin p62 family of proteins (725207), hemolymph juvenile hormone-binding protein (551572, 408320), proteins in the Jak-STAT signaling pathway (550857, 725976, 408557), proteins in the notch signaling pathway (551225, 100576446, 551599, 410819), and vigilin (413416, 552845, 725982). Notably, these proteins help regulate development and growth.

DISCUSSION

A. mellifera and *A. cerana* share a common ancestor but display marked differences in morphology, physiology, and disease resistance. Feeding *A. mellifera* queens *A. cerana* RJ was demonstrated previously to cause morphological changes. Specifically, the RJ type affects proboscis length, anterior wing area, the length of the third and fourth dorsal plate of the abdomen, the length of the fourth dorsal plate of the tuberculum, the area of the sixth abdominal segment, and the area of wax mirrors (Xie et al., 2008). The same study also demonstrated that RJ type affects mite resistance. Another study showed that *A. mellifera* fed *A. cerana* RJ display many differentially expressed genes

Table 2.

Genes related to bee development and growth show alternative splicing in adult *Apis mellifera* that were fed royal jelly from *A. mellifera* (mRJM) or royal jelly from *A. cerana* (mRJC)

Pathways	Alternatively spliced genes in mRJM	Alternatively spliced genes in mRJC
Circadian rhythm	**406112**	
Leucine-rich repeats	**409027, 413332, 413310, 725782, 100577587**	
Wnt signaling pathway	**551258, 552177**	
Calcium sensors and calcium signal modulators	408562, 409474, 551291	**408528,** 408562, 409474, 551291, **1005784334, 100577456**
Major Facilitator Superfamily (MFS) is secondary transporters that includes symporters, antiporters)	408478, 408828, **409924, 410067**, 410278, 410448, **410705**, 412399, **412620**, 552708, **724578, 725015**, 725192, 725462, **725470, 727496**	408478, 408828, **409933**, 410278, 410448, **410613, 411052**, 412399, **412809**, 552708, 725192, 726412, 725462
Mitochondrial carrier protein	409508, 409122, **410791**, 413551	409508, 409122, 413551, **460791, 552595**
Odorant receptor	**724760,** 725483, 726459, **100578045**	**406109, 409159, 410068, 552478, 677677, 677678, 724237,** 725483, 726459, **100579048**
PDZ signaling (signaling molecules)	**409249, 410204,** 724411, 726121, 726548, 726668	**408333, 408780, 408945, 409193, 409571, 410128, 410133, 552101,** 724411, 726121, 726548, 726668, **100577530**
WD40 (regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly)	**411748, 412155, 412406, 413399,** 413404, **551531, 552021, 724567, 724658, 724809, 724976, 725546, 726566 726631, 100576958**	**410497, 410771, 411068,** 413404, **413957**
Cellular detoxification		**411045, 408551, 100578051, 412436**
Cold-Shock Protein		**409854**
Dynactin p62 family		**725207**
Haemolymph juvenile hormone binding protein		**551572, 408320**
Jak-STAT signaling pathway		**550857, 725976, 408557**
Notch signaling pathway		**551225, 100576446, 551599, 410819**
Secretory proteins		**100576855, 408596, 551703, 552489, 408361, 552408**
Vigilin		413416, 552845, 725982

The gene numbers in bold fonts indicate that they were specific (not shared) between the two types of queens, while plain font indicates these genes were shared in the two types of queens.

encoding proteins that are mainly involved in endocytosis, metabolic pathways, regulation of the actin cytoskeleton, and signaling pathways (Shi et al., 2012). The present study extends this knowledge by revealing that mRJC show AS in many genes that are important for bee development and growth and in the TCA cycle and substance metabolism.

There are 7 types of AS: exon skipping, intron retention, alternative 5′ splicing sites, alternative 3′ splicing sites, alternative first exon, alternative last exon, and mutually exclusive exons (Black, 2003). We identified 4 types of AS in mRJM and mRJC, namely exon skipping, intron retention, alternative 5′ splice sites, and alternative 3′ splice sites. More AS (in numbers of both genes and events) occurred at alternative 5′ and alternative 3′ sites. About 60% of the approximately 25,000 human genes undergo AS (Black, 2003). An AS rate with 60% of human genes displays evidence of at least one alternative splice form (Modrek and Lee, 2002). Because one gene can

produce many different transcripts using different types of AS (Black, 2003), one cannot simply sum up the number of AS genes in Fig. 1 to obtain the total number of AS events. The total number of AS genes was 5358 and 5300, in the mRJM and mRJC samples, respectively.

The TCA cycle generates ATP, which is needed for glucose, amino acid, and lipid metabolism. There is a clear difference between the data in Table 1 (<50 differentially spliced genes between the experimental groups) and Fig. 1 (>1000 such genes). Some breakdown products of the TCA cycle are involved in insulin signaling in the brains of adult worker bees engaged in different activities (Foret et al., 2012), so changes in proteins involved in the TCA cycle would further affect the development and growth of honey bees. After feeding the *A. mellifera* larvae RJC, we found that several genes that displayed AS in the *A. mellifera* queen were related to the TCA cycle.

The development of an individual larva is an ongoing dynamic process that is controlled by genetic factors, nutritional status, hormones (especially juvenile hormone and ecdysteroids), and other factors. The queen bee and her workers express different genes (Barchuk et al., 2007) and have different protein expression patterns (Wu and Li, 2010) and DNA methylome profiles (Foret et al., 2012). The increased DNA methylation seen in worker bees appears to specifically affect splicing (Lyko et al., 2010). Our study suggests that AS in the honey bee might also play a role in caste differentiation during feeding of the *A. mellifera* larvae with different types of RJ.

CONCLUSIONS

This study provides the first evidence that heterospecific RJ influences many genes related to honey bee development and growth through AS.

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