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Towards understanding the molecular basis of cockroach tergal gland morphogenesis. A transcriptomic approach

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ABSTRACT

The tergal gland is a structure exclusive of adult male cockroaches that produces substances attractive to the female and facilitates mating. It is formed *de novo* in tergites 7 and 8 during the transition from the last nymphal instar to the adult. Thus, the tergal gland can afford a suitable case study to investigate the molecular basis of a morphogenetic process occurring during metamorphosis. Using *Blattella germanica* as model, we constructed transcriptomes from male tergites 7–8 in non-metamorphosing specimens, and from the same tergites in metamorphosing specimens. We performed a *de novo* assembly all available transcriptomes to construct a reference transcriptome and we identified transcripts by homology. Finally we mapped all reads into the reference transcriptome in order to perform analysis of differentially expressed genes and a GO-enrichment test. A total of 5622 contigs appeared to be over-represented in the transcriptome of metamorphosing specimens with respect to those specimens that did not metamorphose. Among these genes, there were six GO-terms with a p-value lower than 0.05 and among them GO: 0003676 ("nucleic acid binding") was especially interesting since it included transcription factors (TFs). Examination of TF-Pfam-motifs revealed that the transcriptome of metamorphosing specimens contains the highest diversity of these motifs, with 29 different types (seven of them exclusively expressed in this stage) compared with that of non-metamorphosing specimens, which contained 24 motif types. Transcriptome comparisons suggest that TFs are important drivers of the process of tergal gland formation during metamorphosis.

1. Introduction

Insect metamorphosis is essentially regulated by two hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH). 20E triggers the successive molts throughout the life cycle, whereas JH prevents metamorphosis from taking place (Hiruma and Kaneko, 2013; Riddiford, 2012; Truman and Riddiford, 2002). A great deal of information about the 20E signaling pathway and the corresponding transcription factors, many of which belong to the nuclear receptor superfamily, has already been obtained (King-Jones and Thummel, 2005; Nakagawa and Henrich, 2009). In contrast, the molecular mechanisms underlying the action of JH have remained elusive until recently, when the transcription factor Methoprene-tolerant (Met) has been reported to be the JH receptor (Charles et al., 2011), and a number of components of the JH signaling pathway have been identified (Belles and Santos, 2014; Jindra et al., 2013).

Regarding the molecular action of JH and 20E, most of the information has been obtained in holometabolan species, especially in the model par excellence, *Drosophila melanogaster*. Conversely, data available in hemimetabolan species are more limited, which is a serious drawback if one aims at comparing the two modes of metamorphosis and at drawing conclusions in evolutionary terms. Concerning the hemimetabolan mode, the best known species is the cockroach *Blattella germanica*, where the factors operating in the 20E signaling pathway have been extensively studied over the last ten years (Cruz et al., 2006, 2007, 2008; Maestro et al., 2005; Mane-Padros et al., 2012, 2008; Martin et al., 2006). More recently, the components of the JH receptor complex, Methoprene tolerant (Met) and Taiman (Tai), as well as Krüppel homolog 1 (Kr-h1), the master transducer of the JH signal, have been reported in *B. germanica* in the context of metamorphosis regulation (Lozano and Belles, 2011, 2014; Lozano et al., 2014). Finally, the discovery of the transcription factor E93, which triggers adult morphogenesis (Belles and Santos, 2014; Ureña et al., 2014) and is repressed by Kr-h1 (Belles and Santos, 2014), closes the circle and establishes the

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basic MEKRE93 (Met-Kr-h1-E93) pathway that switches adult morphogenesis off and on (Belles and Santos, 2014).

In the present work, we have focused our attention to the formation of male tergal glands of *B. germanica*, a complex structure that is formed during the imaginal molt. The tergal gland is a specific structure of male cockroaches that produce substances that are attractive to the female, and serve to arrest her movement while she licks the gland secretion long enough for the male to clasp her genitalia and consummate mating (Roth, 1969). In *B. germanica*, the tergal gland has been studied morphologically (Sreng and Quennedey, 1976), as well as in terms of the chemical composition of its secretions, which contain pheromonal compounds (Sreng, 2006), phagostimulants (oligosaccharides and phospholipids) (Nojima et al., 2002) and enzymes (Saltzmann et al., 2006).

Our interest has been motivated because the gland is formed *de novo* in tergites 7 and 8 during the transition from the last nymphal instar, where these tergites have a flat morphology, to the adult, where they contain the complex glandular structure (Fig. 1). Thus, the tergal gland can afford a suitable case study to investigate the molecular basis of a morphogenetic process occurring during metamorphosis because it is well bounded in space (circumscribed to tergites 7 and 8) and in time (it is formed during the 3 days for which the peak of circulating 20E occurs in the absence of JH, in the last nymphal stage). The approach followed has been to compare transcriptomes of tergites 7 and 8 in metamorphosing and non-metamorphosing specimens as a first step to uncover the main genes involved in the formation of the tergal gland. Despite that the tergal gland is a structure specific of adult male cockroaches, we presume that the molecular players uncovered with our study would not only describe the formation of the gland but also contribute to the knowledge of the whole morphogenetic processes occurring during metamorphosis.

2. Materials and methods

2.1. Insects

B. germanica specimens were obtained from a colony reared in the dark at 29 ± 1 °C and 60–70% r.h. (Belles et al., 1987). All dissections and tissue sampling were carried out on carbon dioxide-anesthetized specimens. Tissues were frozen on liquid nitrogen and stored at −80 °C until use.

2.2. mRNA transcriptome construction and sequencing

Tergites 7 and 8 (T7-8) from male nymphs in four different stages and experimental conditions were used to build four T7-8 transcriptomes: 3 to 5-day-old fifth instar nymphs (transcriptome N5D3-5); 5 to 7-day-old sixth instar nymphs (transcriptome N6D5-7); 1-day-old sixth instar nymph topically treated with 2 μl of acetone (as control treatment) just after the molt (transcriptome N6D1C); and 1-day-old sixth instar nymph topically treated with 20 μg of JH III (Sigma) in 2 μl of acetone just after the molt (transcriptome N6D1JH). JH III is the native JH of *B. germanica* (Camps et al., 1987), and the commercial source used is a mixture of isomers containing ca. 50% of the biologically active (10R)-JH III, thus the active dose applied was around 10 μg per specimen. This dose produces 100% inhibition of metamorphosis (Lozano and Belles, 2011).

For the RNA extractions, we started with pools of T7-8 from 5 individuals, and then we pooled these 5-individuals pools until obtaining a minimum of 10 μg of total RNA. For the N5D3-5 and N6D5-7 transcriptomes, we pooled two 5-individuals pools per each one of the three days encompassing the ecdysone peak, which represents six 5-individuals pools (T7-8 from 30 individuals). For the N6D1C and N6D1JH transcriptomes we followed the same approach but collecting six 5-individuals pools for each transcriptome, which represents also 30 individuals. Total RNA was extracted using the GenElute Mammalian Total RNA kit (Sigma) following the manufacturer's protocol. Up to 10 μg of total RNA from pooled samples were used to prepare the T7-8 transcriptomes. The mRNAs were isolated by magnetic beads using the Dynabeads® Oligo (dT)25 (Invitrogen, Life Technologies) and following the manufacturer's protocol, and the quality and quantity of the mRNAs were validated by a Bioanalyzer (Aligent Bioanalyzer® 2100).

The purified RNA was sent to the UPF Genomics Core Facility (PRBB, Barcelona. Spain), where it was sequenced with Roche's pyrosequencing technology (454 GS Junior System), a method of choice for generating transcriptome data from those species without genome annotated (Kumar and Blaxter, 2010; Mukherjee et al., 2004), as it provides large reads that facilitates the *de novo* assembly. Data from the four T7-8 transcriptomes are accessible at the GEO database (accession code GSE63993).

2.3. Other B. germanica transcriptomes used in the analysis

For assembling purposes, we used the following seven transcriptomes obtained for previous studies (the number represent the accession code in GEO or SRA): GSM1560373 (adult ovaries), GSM1560374 (sixth nymphal instar epidermis), GSM1560375 (adult female fat body), SRX796238 (adult ovaries), SRX796239 (adult ovaries sunder hydric stress), SRX796244 (sixth nymphal instar ovaries), SRX790658 (fifth nymphal instar wing primordia).

2.4. De novo assembly

De novo assembly was carried out with the software Newbler 2.5p1 (Roche) using the standard parameters ("minimum read

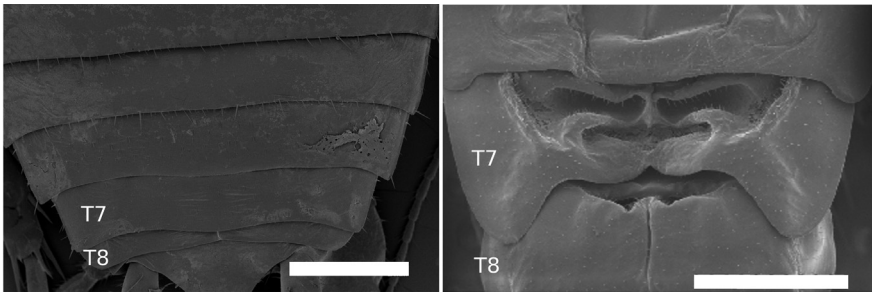

Fig. 1. Tergites 7 and 8 (T7 and T8) of male *Blattella germanica*. Left: from a sixth instar nymph, showing a flat morphology. Right: from an adult, showing the structure of the tergal gland. The scale bar represents 1 mm.