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Corrigendum

Corrigendum to "Towards understanding the molecular basis of cockroach tergal gland morphogenesis. A transcriptomic approach" [Insect Biochem. Mol. Biol. 63 (2015) 104–112]



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The authors regret that the order of the authors that appeared in the published article (Xavier Belles and Guillem Ylla) is reversed with respect to the order stated in the original manuscript. The right order of the authors is Guillem Ylla and Xavier Belles. The authors would like to apologise for any inconvenience caused.

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



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A R T I C L E I N F O

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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 overrepresented 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.

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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).

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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 (Krh1), 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 Krh1 (Belles and Santos, 2014), closes the circle and establishes the 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 nonmetamorphosing 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 μ l of acetone (as control treatment) just after the molt (transcriptome N6D1C); and 1-day-old sixth instar nymph topically treated with 20 μ g of JH III (Sigma) in 2 μ l 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 μ g 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 μ g 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.

length" = 20; "minimum overlap length" = 40; "minimum overlap identity" = 90%). The Newbler software allows removing reads from possible contaminations by including as a parameter a "fasta" file with the genomes of the putative contaminant organisms. We prepared this "fasta" file including: five genomes of *B. germanica* bacteroid endosymbionts of the genus *Blattabacterium* (Ref seq. NC_013454; NC_017924.1; NC_016621.1; NC_013418.2; NC_016146.1), the whole genome of *B. germanica* densovirus (Ref seq. NC_005041.2), *B. germanica* mitochondrial genome (Ref seq. NC_012901.1), *Caenorhabditis elegans* nuclear genome WBcel215 (GCA_000002985.1), and *Escherichia coli* genome (AE014075.1).

2.5. Functional annotation

We carried out two rounds of blasts of the contigs obtained using different databases. The first blast was a Blastn (Altschul et al., 1990) against a dataset of sequences of *B. germanica* that have been the object of previous functional studies in our laboratory and that had been manually curated. With the sequences that did not reach a match with e-value < 0.001, a blastx against the NCBI arthropod sequences was performed. Whenever a sequence from our reference transcriptome was getting a blast match with an evalue < 0.001, the accession code, the descriptor, and also the GOterms (Ashburner et al., 2000) of the matched sequences when available, were transferred to our sequence. To study the PFAM motifs, we first translated our reference transcriptome to the 6 possible open reading frames with the package "Transeq" from the EMBOSS software suite version 6.4.0.0 (Rice et al., 2000). Then with the standalone version of PfamScan software and the PFAM-A database (Finn et al., 2014) and an e-value = 0.05, we predicted the PFAM motifs in the reference transcriptome.

2.6. Preprocessing reads for mapping

The ".sff" files corresponding to the 11 transcriptomes used (Table 1) were first transformed to "fastq" files with homemade scripts. Then, we trimmed the adapters from all reads. Since RSEM (Li and Dewey, 2011) takes into account the quality values of the reads, we did not apply a further reads quality control.

2.7. Mapping

The reference transcriptome, which was in "fasta" format, was transformed into a reference format with the RSEM software (Li and Dewey, 2011) using the command "rsem-prepare-reference". Then, using the command "rsem-calculate-expression", we

obtained the expected counts, and the normalized values in FPKMs in each T7-8 transcriptome. The software RSEM had previously shown good performance in estimating abundances mapping reads in an rna-seq *de novo* assembly (Haas et al., 2013).

2.8. Differential expression analysis

Using R language (Team, 2003) with the files produced during the mapping, we constructed a data frame with all the reference sequences and their abundance in FPKMs (Trapnell et al., 2010) for each T7-8 transcriptome. With the NoiSeq package (Ferrer et al., 2011) for R available in Bioconductor (Gentleman et al., 2004), we performed the differential expression analysis between N6D5-7 and N5D3-5, and between N6D1C and N6D1JH. NoiSeq software is non-parametric and data-adaptative, which allows performing differential expression analyses without the need of replicates thanks to the series of simulations that it executes. It was run with the following parameters: pnr (size of the simulated samples) = 0.2; nss (number of replicates to be simulated) = 5; v (variability) = 0.02; replicates = "no".

2.9. GO enrichment analysis

With the differentially expressed selected contigs and the GOterm obtained in the functional annotation step, we performed a GO-enrichment analysis for molecular function with the R package also available in Bioconductor "TopGO" (Alexa and Rahnenfuhrer, 2010).

2.10. Quantitative analysis of transcription factors

In order to retrieve only the sequences corresponding to transcription factors (TFs) from our reference transcriptome, we selected the sequences with a PFAM-domain contained in the list of PFAM-domains directly related to TF activity published by de Mendoza et al. (2013). We added the number of "expected counts" (from the RSEM output in each T7-8 transcriptome) for each TF-pfam-motif. Finally, a chi-square with Yates continuity correction was used to compare the proportions of expected counts corresponding to TFs in N6D5-7 vs. N5D3-5, and N6D1C vs. N6D1JH.

2.11. Qualitative analysis of transcription factors

Before examining qualitative differences of TF-families in the four T7-8 transcriptomes and in order to avoid the bias due to different transcriptome sizes, we selected 82,279 random reads,

Table 1

Number of reads and bases, before and after trimming, for each one of the 11 specific transcriptomes of *Blattella germanica* used for tergal gland studies and to obtain a reference transcriptome.

Transcriptome	Number of raw reads	Number of reads after trimming	Number of raw bases	Number of bases after trimming	
^a N5D3-5	102,019	100,268	40,697,795	40,087,037	
^a N6D5-7	131,329	128,976	50,601,265	49,759,967	
^a N6D1C	100,140	97,844	38,263,300	37,416,662	
^a N6D1JH	82,279	80,604	34,388,508	33,739,945	
^b GSM1560373	157,564	156,227	40,762,859	40,203,489	
^b GSM1560374	212,195	210,973	52,334,374	51,796,827	
^b GSM1560375	154,932	144,829	40,304,096	37,244,372	
^b SRX796238	139,895	135,144	60,435,716	58,413,046	
^b SRX796239	88,487	85,430	37,167,556	35,841,093	
^b SRX796244	61,219	60,856	18,491,286	18,350,726	
^b SRX790658	106,552	104,858	43,783,056	43,168,565	
TOTAL	1,336,631	1,306,009	457,229,811	446,021,729	

^a Transcriptomes used for tergal gland studies. Data of these four T7-8 transcriptomes are accessible from the GEO database (accession code GSE63993).

^b Transcriptomes coming from 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 under hydric stress), SRX796244 (sixth nymphal instar ovaries), SRX790658 (fifth nymphal instar wing primordia).

which is the size of the smallest T7-8 transcriptome (N6D1JH, see Table 1) from each one of the four T7-8 transcriptomes. We mapped again these reads in to the reference transcriptome using the software RSEM, and we obtained a new table of counts for each sequence. Finally, a list of different TF-pfam-motif detected in each T7-8 transcriptome was obtained. This information was plotted in a Venn diagram with the R package "VennDiagram" (Chen and Boutros, 2011).

2.12. Validation of transcriptomic data by qRT-PCR

A selection of factors that resulted promising from transcriptomic comparisons (Table S1) were validated using qRT-PCR. For the N5D3-5 and N6D5-7 transcriptomes, we used male nymphs of N5D4 and N6D6, respectively. For the N6D1C and N6D1JH transcriptomes, we carried out the JH treatment on N6 male nymphs as when preparing the transcriptomes. RNA extraction was performed from T7-8 of male nymphs of chosen ages using the miRNeasy extraction kit (QIAGEN). A sample of 500-ng from each RNA extraction was treated with DNase (Promega) and reverse transcribed with first Strand cDNA Synthesis Kit (Roche) and random hexamers primers (Roche). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000[®] (NanoDrop Technologies). Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR[®]Green (Power SYBR[®] Green PCR Master Mix: Applied Biosystems). A template-free control was included in all batches. The primers used to detect mRNA levels are detailed in Table S1. The efficiency of each set of primers was first validated by constructing a standard curve through four serial dilutions. Levels of mRNA were calculated relative to BgActin-5c (Accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA. Statistical differences between ages or treatments were analysed with the REST software tool (Pfaffl et al., 2002).

3. Results and discussion

3.1. Datasets description

We constructed four T7-8 transcriptomes corresponding to four physiological situations. N5D3-5: T7-8 from fifth (penultimate) nymphal instar during the peak production of 20E (days 3, 4 and 5) (Cruz et al., 2003). N6D5-7: T7-8 from sixth (last) nymphal instar during the peak production of 20E (days 5, 6 and 7) (Cruz et al., 2003). N6D1C: T7-8 from 1-day-old sixth (last) nymphal instar, when JH production and Kr-h1 expression are no longer present (Lozano and Belles, 2011; Treiblmayr et al., 2006). N6D1JH: T7-8 from 1-day-old sixth (last) nymphal instar, treated with JH when freshly emerged, thus re-inducing the expression of Kr-h1 and inhibiting metamorphosis (Lozano and Belles, 2011) (Fig. 2). The four T7-8 transcriptomes were sequenced; the number of reads and bases obtained before and after the Newbler trimming are shown in Table 1. The table also shows the same information for seven other transcriptomes previously obtained in our laboratory, which were used in the present study for assembly and annotation purposes.

3.2. Reference transcriptome and functional annotation

We assembled *de novo* 11 transcriptomes (Table 1) in order to obtain a robust reference transcriptome of *B. germanica*, following a strategy of first assembling then mapping for transcriptomic analysis when the genome of the species is not available (Nookaew

et al., 2012; Zhao et al., 2011). Quantitative data related to this assembling are detailed in Table 2. A total of 15,624 out of the 32,606 contigs obtained were homologous to known proteins, thus the accession codes of them were assigned to the corresponding contigs. For these, 2922 associated GO-terms were finally obtained. Concerning functional annotation, we also searched functional domains based on the Protein Families Database (PFAM) (Friedberg, 2006). From 9743 different contigs we found a total of 12,071 PFAM matches corresponding to 3636 different PFAM families.

3.3. Differential expression analysis

To obtain the abundance of each sequence characterized in the reference transcriptome in each of the four T7-8 transcriptomes, we mapped the reads of the latter to the reference transcriptome, thus obtaining the table of expected counts and the table of normalized abundances in FPKMs (Trapnell et al., 2010). The "expected counts" are the non-normalized expression values that are given by the expression estimation software RSEM (Li and Dewey, 2011) for each reference sequence taking into account the quality of the reads and their mapping specificity. In our case, as we have single-end reads, the FPKMs (fragments per kilobase of transcript per million fragments mapped) are equivalent to RPKMs (reads per kilobase per million mapped reads) (Mortazavi et al., 2008).

With the normalized abundances for each sequence of the reference transcriptome in our four T7-8 transcriptomes, we performed a differential expression analysis based on two pairwise comparisons: N5D3-5 vs. N6D5-7 on one hand and N6D1C vs. N6D1IH on the other. For the comparisons, we used NoiSeg software and a probability threshold of 90% as recommended by the software authors (Ferrer et al., 2011). Using this approach, a total of 5622 contigs appeared significantly overrepresented in N6D5-7 with respect to N5D3-5, and 2611 overrepresented in N5D3-5 with respect to N6D5-7 (Fig. 3A). Among those overrepresented in N6D5-7, which should include the factors needed to form the tergal gland, we found one corresponding to Nejire, a CREB-binding protein that acts as a transcriptional coactivator by interacting with a large number of TFs involved in development, and which is now under functional study in our laboratory. Nejire (contig4322, 1264 bp) resulted overrepresented in N6D5-7 with a probability of 97.92% and a log2(FC) = 9.91. Measurement of Nejire mRNA levels by qRT-PCR in T7-8 of male nymphs in N5D4 and N6D6 shows that they are significantly higher in N6D6 (Fig. 4A). When comparing N6D1C with N6D1JH, 3074 contigs appeared significantly overrepresented in N6D1C, whereas 2632 were overrepresented in N6D1JH (Fig. 3B). One of these sequences overrepresented in N6D1C corresponds to E93, a TF containing a pipsqueak (Psq) motif that triggers metamorphosis, as shown by our functional studies that followed these transcriptomic data (Belles and Santos, 2014) (see also Ureña et al., 2014). E93 (contig 10947, 703 bp) was underrepresented in N6D1JH with respect to N6D1C with a probability of 97% and a log2(FC) = -9.06. Measurement of E93 mRNA levels in T7-8 of male nymphs in control N6D1 and in N6D1 of specimens that were treated with JH on N6D0, show that they are significantly lower in the specimens treated with JH (Fig. 4B). This validates the observations obtained when comparing N6D1C and N6D1JH transcriptomes. In parallel, mRNA levels of Kr-h1, a JHdependent factor that repress E93 (Belles and Santos, 2014) was significantly upregulated by the JH treatment (Fig. 4B). This validates the observation that Kr-h1 (contig15641, 540 bp) is overrepresented in transcriptome N6D1JH with respect to N6D1C with a probability of 99.5% and a log2(FC) = 12.42, and that the treatment with JH produces the expected effects in molecular terms, as Kr-h1 is a JH-dependent gene (Lozano and Belles, 2011). Other factors emerging from these differential expression analyses are



Fig. 2. Hormonal context of the T7-8 transcriptomes of *Blattella germanica*. The transcriptomes were prepared with RNA extracts of male tergites 7 and 8 from fifth (N5) or sixth (N6) nymphal instar, on day 1 (D1) or during the production of the peak of 20-hydroxyecdysone (20E). N5D3-5 transcriptome correspond to high levels of juvenile hormone (JH), and the subsequent molt would result in new juvenile, flat tergites. N6D5-7 transcriptome correspond to virtual absence of JH, and the subsequent molt result in adult tergites with the tergal gland formed. N6D1C corresponds to the stage where the adult program is being determined, when JH has just decreased. N6D1JH corresponds to the same stage, but using specimens that were treated with JH on N6D0, thus aborting the adult program.

Table 2

Quantitative parameters of the assembling *de novo* of the 11 previous transcriptomes into a *Blattella germanica* reference transcriptome. The empirical per base coverage depth was calculated according to Sims et al. (2014).

Parameter	Quatitative value
Total trimmed reads	1,306,009
Total number of bases (Table 1)	446,021,729
Contigs obtained	32,606
Number of bases contained in the contigs	23,002,710
Empirical per base coverage depth	19.39X
Number of large (more than 500 bp) contigs	17,980
N50 contig size	1165 bp

currently under functional studies in our laboratory.

3.4. GO enrichment analysis

In order to assess enriched functions in the overrepresented sequences of T7-8 transcriptomes, we undertook a Gene-ontology enrichment analysis of Molecular Function Ontology (Ashburner et al., 2000). Table S2 summarizes the analysis of the 5622 contigs overrepresented in N6D5-7 with respect to N5D3-5, and Table S3 that of the 2611 contigs overrepresented in N5D3-5 with respect to N6D5-7. As we aimed at identifying the genes whose expression is upregulated at the metamorphic transition, we

focused on the GO-groups overrepresented in N6D5-7 with respect to N5D3-5. Moreover, we considered those GO-groups having a pvalue < 0.05 and that comprised at least 25 contigs. These were GO: 0003676 ("Nucleic acid binding"), with a p-value of 0.0388 and 49 contigs, and the redundant GO-groups GO: 0043169 ("Cation binding") and GO: 0046872 ("Metal ion binding"), with a p-value of 0.0466 and 57 contigs (highlighted in Table S2).

The GO-term "Nucleic acid binding" refers, among other factors, to gene regulators that act by interacting with DNA, which would include TFs. This GO-term associated to TFs is especially interesting since differences in gene regulation have long been recognized as major contributors to phenotypic diversity (Babu et al., 2004; Levine and Tjian, 2003). "Metal ion binding" is a child term of "Cation binding", and both GO- groups contain the same 57 contigs. These include transcripts of genes corresponding also to TFs (proteins containing zinc finger domains), related to the formation of extracellular matrix (like fibropellin and fibrillin), to membrane organization and trafficking (like annexin), or to multiple homeostatic functions (like E3 ubiquitin-protein ligase). Of these, the most directly related to morphogenetic processes are zinc finger TFs, which are also comprised in the "Nucleic acid binding" GO-group. Interestingly, the GO-groups "Cation binding" (and "Metal ion binding") and "Nucleic acid binding" share a significant correlation, as shown in the "co-occurring terms" tab of QuickGO (Binns et al., 2009).



Fig. 3. Pairwise comparison of contigs representation. The representation values are in logarithmic scale and colored dots means significant differences (probability > 90%). (A) Contig representation in transcriptome N6D5-7 versus N5D3-5. The 5622 red dots represent the contigs significantly overrepresented in N6D5-7, whereas the 2611 blue dots, those significantly overrepresented in N5D3-5. (B) Contig representation in transcriptome N6D1C versus N6D1JH. The 3074 red dots represent the contigs significantly overrepresented in N6D5-5, whereas the 2632 blue dots those significantly overrepresented in N6D1C, whereas the 2632 blue dots those significantly overrepresented in N6D1JH.



Fig. 4. Validation by qRT-PCR of transcriptomic data after the differential expression analysis. A: mRNA levels of Nejire in N5D4 and N6D6. B: mRNA levels of E93 and Kr-h1 in N6D1 in specimens treated with acetone on N6D0 (N6D1C) and in specimens treated with JH on N6D0 (N6D1JH). Measurements were carried out in tergites 7 and 8 of male nymphs; each point represents 3 biological replicates; results represent copies of the given transcript per 1000 copies of BgActin-5c mRNA and are expressed as the mean \pm SEM; the asterisk indicates statistically significant differences (p < 0.05), according to the REST software tool (Pfaffl et al., 2002).



Fig. 5. Venn diagram showing the co-occurrence of TF-pfam-motifs in the four T7-8 transcriptomes of *Blattella germanica*.

The comparison of N6D1C with N6D1JH gave 3074 contigs overrepresented in N6D1C (GO terms analysis in Table S4), and that of N6D1JH with N6D1C gave 2632 contigs overrepresented in N6D1JH (GO terms analysis in Table S5). Again, as we were interested in the genes influencing tergal gland (and adult) morphogenesis, we examined in detail the genes overexpressed in N6D1C with respect to N6D1JH. However, there were no GO-groups with a p-value < 0.05 and comprising at least 25 contigs (Table S4). The GO-group with the lowest p-value and with more than 25 contigs is just "Nucleic acid binding", but the data (26 contigs found versus 23.4 expected, p-value = 0.2805) indicate that this GO-group is not significantly enriched in N6D1C with respect to N6D1JH.

After obtaining the results of the differential expression and GO enrichment analyses, and considering that TFs could play a major role in tergal gland formation, we examined in more detail this group of contigs. Firstly, we obtained the proportion of counts corresponding to them in each T7-8 transcriptome (expected counts in the sequences with a TF-Pfam-motif divided by total expected counts in the transcriptome). The proportions obtained were as follows: 0.0179 in the N6D5-7 transcriptome; 0.0106 in N5D3-5; 0.015 in N6D1C; and 0.0198 in N6D1JH. Statistic tests comparing the proportions of TFs in N5D3-5 vs. N6D5-7 and in N6D1C vs. N6D1JH gave significant differences (p-value < 0.05) in both comparisons.

3.5. Diversity of transcription factors analyzed through Pfam motifs

Finally, we approached the study of the TFs from a qualitative point of view and distinguishing the different Pfam motifs. Thus, we compared the occurrence of TF-Pfam-motifs in the four T7-8 transcriptomes using the criterion of presence or absence of a given TF-Pfam-motif in each of them. To avoid a bias due to the different number of total reads between the four T7-8 transcriptomes, we randomly subsampled each of them in order to have the same number of reads in all them (Huson et al., 2009). Then, we mapped the subsample reads against the reference transcriptome and for each TF-Pfam-domain type we checked for their presence (at least one count) or absence in each T7-8 transcriptome. For this analysis, we used the PFAM-domains directly related to TF activity reported by de Mendoza et al. (2013).

The results (Fig. 5 and Table S6) indicate that N6D5-7 is the

Table 3

Characteristics of the contigs corresponding to each one of the seven Pfam types present only in the N6 (see Table S5), either in transcriptome N6D1C or in transcriptome N6D5-7 or in both. "Presence" is indicated by the number of reads.

Pfam type	Contig	Contig length	Presence/absence		Best BLAST hit (Genbank accession number)	Best hit E-value
			N6D5-7	N6D1C		
PF02045 CBFB_NFYA	Contig5531	1104 nt	27.28	_	Nuclear transcription factor Y subunit alpha isoform X3, Orussus abietinus (XP_012274605.1)	1E-79
PF03615 GCM	Contig12872	627 nt	149.67	-	Transcription factor glial cells missing, Zootermopsis nevadensis (KDR23998.1)	3E-103
PF02376 CUT	Contig15402	551 nt	210.92	_	Cut, Blattella germanica (CCX34994.1)	3E-114
PF02376 CUT	Contig17060	516 nt	129.22	_	Cut, Blattella germanica (CCX34994.1)	2E-70
PF10545 MADF_DNA_bdg	Contig15654	544 nt	_	146.47	Dihydrouridine synthase domain containing protein, Danaus plexippus (EHJ74574.1)	0.085
PF02135 zf-TAZ	Contig2170	1724 nt	30.02	-	Histone acetyltransferase p300 isoform X3, Ceratitis capitata (XP_012155272.1)	6E-011
PF02135 zf-TAZ	Contig4322	1264 nt	67.57	90.2	CREB-binding protein, Zootermopsis nevadensis (KDR19833.1)	5E-127
PF00352 TBP	Contig875	2368 nt	30.69	-	TATA box-binding protein-like protein 1, Zootermopsis nevadensis (KDR19315.1)	6E-157
PF00352 TBP	Contig6835	972 nt	33.05	44.1	TATA-box-binding protein, <i>Athalia rosae</i> (XP_012257098.1)	2E-148
PF05920 Homeobox_KN	Contig23016	348 nt	-	571.23	Homeobox protein homothorax, Cerapachys biroi (XP_011342704.1)	5E-055
PF05920 Homeobox_KN	Contig2649	1587 nt	99.98	44.5	Homeobox protein araucan, Zootermopsis nevadensis (KDR19022.1)	1E-119

transcriptome with the highest TF diversity, with 29 different types of TF-Pfam-motifs, while N6D1JH exhibited the lowest diversity, with 21 different types. N5D3-5 and N6D1C transcriptomes had intermediate diversity, with 24 and 25 types, respectively. Concerning specific diversity in each T7-8 transcriptome, a total of 17 TF-Pfam-domain types are shared by the four transcriptomes. N6D5-7 and N6D1C are the only transcriptomes with exclusive types, 3 and 1 respectively, and they share just three more between them. These seven types found exclusively in the preimaginal (N6) stage are detailed in Table 3, which also shows the number of contigs having the corresponding motif, the length of each contig and the closest homolog of them found in public databases. Table S1 shows the respective sequences of these contigs. Those present in transcriptome N6D5-7 but not in N5D3-5 are: Nuclear



Fig. 6. Validation by qRT-PCR of transcriptomic data after TF-Pfam-motifs analysis. A: Factors present in the transcriptome N6D5-7 but not in N5D3-5; these are: Nuclear transcription factor Y subunit alpha isoform X3 (NTFY), Glial cells missing (GCM), Cut, Histone acetyltransferase p300 isoform X3 (HAT), CREB-binding protein (Nejire), TATA box-binding protein-like protein 1 (TAB-L), TATA-box-binding protein (TAB) and Araucan (ARA). B: Factors present in the transcriptome N6D1C but not in N6D1]H; these are: Dihydrouridine synthase domain containing protein (DHUP), Nejire, TAB, Homothorax (HTH) and ARA. Measurements were carried out in tergites 7 and 8 of male nymphs of the indicated ages and treatments; each point represents 3 biological replicates; results represent copies of the given transcript per 1000 copies of BgActin-5c mRNA and are expressed as the mean \pm SEM; the asterisk indicates statistically significant differences (p < 0.05), according to the REST software tool (Pfaffl et al., 2002).

transcription factor Y subunit alpha isoform X3 (NTFY), Glial cells missing (GCM), Cut, Histone acetyltransferase p300 isoform X3 (HAT), CREB-binding protein (Nejire), TATA box-binding proteinlike protein 1 (TAB-L), TATA-box-binding protein (TAB) and Araucan (ARA). qt-RT-PCR measurements of mRNA levels of these factors in T7-8 by comparing N6D6 and N5D4 show that in most cases mRNA levels are higher in N6D6 (Fig. 6A), which is in agreement with transcriptomic data. Only in the case of Cut and HAT levels in both ages were not significantly different. Those present in N6D1C but not in N6D1JH are: Dihydrouridine synthase domain containing protein (DHUP), Nejire, TAB, Homothorax (HTH) and ARA. qt-RT-PCR measurements of mRNA levels of these factors in T7-8 by comparing N6D1C and N6D1 treated with JH show that in the case of Nejire, TAB and ARA, mRNA levels are lower in the JHtreated insects, as expected, whereas this was not the case in DHUP and HTH measurements (Fig. 6B).

4. Conclusions

For species where an annotated genome is not available, such as *B. germanica*, a *de novo* assembly of sequence data from different transcriptomes followed by assembling a reference transcriptome and then mapping of reads against it has shown to be an appropriate approach for obtaining useful genomic information.

The results from differential expression analyses have afforded a preliminary idea about which genes may be of interest to proceed with functional studies. In the present study, for example, they revealed that the transcription factors E93 and Nejire may be important in the transition to the adult stage. However, these results must be considered with caution since the transcriptomes compared had no replicates, thus they cannot be used to test hypotheses (Anders et al., 2013). Thus, qRT-PCR measurements to validate the results obtained in the most promising candidates are recommended before approaching the study of them from a functional point of view.

The GO enrichment analyses showed that the N6D5-7 transcriptome, which represents the moment at which the imaginal molt is determined, is especially enriched in the GO-term "nucleic acid binding", which includes TFs. The complementary approach of examining the presence or absence of the different TF Pfam types in the four T7-8 transcriptomes confirmed that the N6D5-7 transcriptome contained the highest diversity of TF types. The analysis of the contigs contained in each TF Pfam type led to the identification of 8 TFs that are present in N6D5-7 but not in N5D3-5. gRT-PCR measurements confirmed that 6 of them are significantly more expressed in N6D6 than in N5D4, whereas two of them showed no significant differences, and that there was no correlation between the number of reads when the factor was present, and the difference found in qRT-PCR measurements. This suggests that transcriptome comparisons can serve as a first step to select a short list of promising factors, but that transcriptomic differences must be confirmed with qRT-PCR measurements. In the comparison of N6D1JH with N6D1C, there were five TFs only present in the latter, and qRT-PCR measurements confirmed that three of them were significantly less expressed in specimens treated with JH than in controls. This suggests again than validation by qRT-PCR must be an associated step to this kind of analysis.

The whole data suggest that the formation of the tergal gland requires more diverse TFs than those required for the formation of ordinary juvenile tergites. However, it must be taken into account that there are overrepresented contigs in N6 transcriptomes that correspond to TFs than might not be directly related to the formation of the tergal gland, but with other proteins that are typical of the adult stage. An example of this can be those factors related with the expression of adult cuticular proteins (Willis, 2010), which are expressed in all tergites in the adult stage, but not in nymphs. Along a similar line of reasoning, we must also consider the possibility that a number of differentially expressed genes are not TFs involved in different physiological processes, for example with the biosynthesis of chemicals associated with tergal gland rather than the formation of the gland itself. However, most of the seven TF types overrepresented exclusively in the sixth nymphal instar look rather related to morphogenetic processes, a relationship that should be confirmed with the functional studies that are already in progress in our laboratory.

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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.2015.06.008.

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