Proc. Arthropod. Embryol. Soc. Jpn. 48, 53–54 (2017)

ISSN 1341–1527

Identification of Gene Transcripts Using Whole Transcriptome Shotgun Sequencing (RNA-Seq) in the Sawfly *Athalia rosae ruficornis* (Hymenoptera)*1, *2

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The sawfly, *Athalia rosae ruficornis*, has unique features in the aspects of evolution, development and gene function, such that the larva has remarkable abdominal appendages called prolegs, the general mode of reproduction is haplo-diploid, parthenogenetically developed haploid males produce sperm without reductional divisions (meiosis) and the sex is determined by a single locus-multiple allete system (Naito and Suzuki, 1991; Oishi *et al*., 1993, 1995; Fujiwara *et al*., 2004; Yamamoto *et al*., 2004). We have demonstrated by morphological and gene expression analyses that the abdominal proleg of *A. rosae ruficornis* is homologous to the outgrowth of coxopodite (endite) resembling that of mouthparts and not the serial homologue of thoracic leg (Oka *et al*., 2010). Functional analysis of the genes involved in the regulation of appendicular development, such as Hox genes (Hughes and Kaufman, 2002), will provide evidence to unravel the molecular mechanisms of proleg formation. In *A. rosae ruficornis* as well as other members of Hypmenoptera, the occurrence of meiosis is closely linked to the sex: meiosis takes place in females during oogenesis but not in males during spermatogenesis. It is intriguing what molecular interactions underlie the sex and the regulation of meiosis. Analyses of gene functions related to sex determination and maturation divisions, and their interaction during spermatogenesis will give clues to understand the characteristic mechanisms of Hymenoptera.

One possible approach for gene functional analysis is to induce the misexpression of candidate genes and examine the consequent phenotypes. Gene knockdown by RNA interference (RNAi) is a very useful tool for this purpose in insects, especially in non-model species (Tomoyasu *et al*., 2008; Belles *et al*., 2010; Mito *et al*., 2011). RNAi by injection of double-stranded RNA (dsRNA) of the targeted gene transcript is practicable in most developmental stages of *A. rosae ruficornis* (Sumitani *et al*., 2005; Yoshiyama *et al*., 2010, 2013; Yoshiyama and Hatakeyama, 2012). Nevertheless, isolation of the genes of interests remains laborious by conventional PCR-based cloning methods. We applied whole transcriptome shotgun sequencing (RNA-Seq) for efficient identification and isolation of target genes.

We prepared total RNAs to be sequenced from embryos 12 h-144 h after egg activation to obtain Hox gene homologues and from the testes of males spanning the stages of last instar larva to PCF 10 pupa (right before adult eclosion) to obtain spermatogenesis-related genes. Libraries for sequencing were synthesized using a TruSeq RNA sample prep kit (Illumina) as follows: poly (A)-RNAs were selected from total RNAs, fragmented to about 100 bp, reverse-transcribed to cDNA, adaptors ligated, and PCR-amplified. The libraries were sequenced by a single read method using a high throughput DNA sequencer (HiSeq2000, Illumina). The fragments read were 7.5×10^7 for the embryonic library and 6.3×10^7 for the testicular library. The sequences were assembled and finally gave rise to transcriptomes of about 25,000 transcripts for the embryo and 29,000 transcriptomes for the testis, respectively.

We searched Hox genes of *A. rosae ruficornis* based on the known homologous genes of insects using a Basic local alignment search tool (BLAST) program supplied with GENETYX bioinformatics software (GENETYX Corporation). Complete coding sequences of the homologues of *Hox3/zerknullt* (*zen*), *Deformed* (*Dfd*), *Sex comb reduced* (*Scr*), *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), and partial sequences containing complete homeodomains of *proboscipedia* (*pb*) and *Abdominal-B* (*Abd-B*) were obtained. The *labial* (*lab*) gene homologue was not found unfortunately. The deduced amino acid sequences corresponding to the homeodomain of each Hox gene were well aligned with those of other insects so far identified with only a few amino acid substitutions. *Hox3/zen*, which is responsible for the formation of extraembryonic tissues, is known to be a functionally changed Hox gene in

*1 Abstract of paper read at the 49th Annual Meeting of the Arthropodan Embryological Society of Japan, June 7–8, 2013 (Tsukuba-san, Ibaraki).
*2 This article, which was accepted in 2013 and should have been published in 2014, was printed in 2017 being much delayed due to various circumstances.

insects (Falciani *et al*., 1996; Damen and Tautz, 1998). Less conservation in the homeodomain of *Hox3/zen* with several amino acid substitutions was seen compared to those of other Hox genes.

We also found some sex-determining gene homologues: *Sex lethal* (*Sxl*), *transformer 2* (*tra2*), *doublesex* (*dsx*), *fruitless* (*fru*) and *intersex* (*ix*), although it remains to be examined whether sex-specific splicing occurs in these transcripts. In spite of extensive efforts to isolate *dsx* by degenerate primer PCR, we only found two *dsx*-related transcripts that have conserved DM domains but are not classified as members of insect *dsx* homologues (Lee *et al*., 2005). We succeeded in identifying the authentic *dsx* with less effort by searching the transcriptomes.

Moreover, some isoforms of the *boule* (*bol*) gene transcripts were identified in addition to the *bol* homologues obtained by conventional PCR-based methods. *bol* is essential for meiosis in spermatogenesis in animals and is found in the genomes of hymenopteran species in which meiosis does not take place during sperm production (Schurko *et al*., 2010). It is of great interest to unravel whether *bol* is required for spermatogenesis in Hymenoptera and what is the function of each isoform.

In conclusion, searching a transcriptome makes it efficient and easier to identify and isolate genes of interest, especially in insects with less genomic information. In the next step, we will investigate the functions of these genes with a gene knockdown strategy.

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