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Comparative transcriptome analysis of ATP-binding cassette (ABC) transporter genes in eri-silkworm, *Samia cynthia ricini* in response to 1-deoxynojirimycin

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ABSTRACT

1-Deoxynojirimycin (DNJ) as a kind of alkaloid has been confirmed that could modulate glycometabolism and has toxicity for the eri-silkworm in our previous research. On the contrary, what is the potentially defensive mechanism when the DNJ enters the eri-silkworm. Based on comparative transcriptome sequencing, we found that ATP-binding cassette (ABC) transporter genes could be induced significantly by DNJ. In this study, a total of 16 putative ABC transporter genes were identified, which can be classified into seven subfamilies, namely one ABCA, four ABCBs, three ABCCs, two ABCDs, one ABCE, three ABCFs, and two ABCGs. Phylogenetic analysis revealed that ScABCs had strong conservation with *Bombyx mori*. Reverse transcription quantitative PCR (RT-qPCR) suggested that 6 ABC transporters had a strong positive correlation between RT-qPCR and transcriptome data. Additionally, *S. c. ricini* ABC transporter C-subfamily 4 (*ScABCC4*), *S. c. ricini* ABC transporter G-subfamily 4 (*ScABCG4*), *S. c. ricini* ABC transporter A-subfamily 3 (*ScABC*A3) and *S. c. ricini* ABC transporter C-subfamily 10 (*ScABCC10*) showed different expression pattern in two feed dose (1% and 2% DNJ) and three time points (6h, 12 h, 48 h). This study provides the first study on identification, characterization and expression patterns of ABC transporter genes in *S. c. ricini* response to DNJ, and lays a foundation for further understanding of their physiological roles response to the alkaloid.

Introduction

The ATP-binding cassette (ABC) transporters as one of the largest transporter families that are ubiquitously in all living organisms including ions, sugars, amino acids, vitamins, peptides, lipids, hormones and alkaloids (Hock et al., 2000; Yazaki, 2006). It belongs to the multidomain integral membrane protein that can utilize the energy produced by ATP hydrolysis to transport solutes across cellular membrane (Jones and George, 2004). ABC full-length transporters consist of two cytosolic nucleotide-binding domains (NBDs) and two integral transmembrane domains (TMDs), while half-length ABC transporters contain only one NBD and one TMD (Deeley et al., 2006; Jeong et al., 2017). The NBDs are highly conserved and contain characteristic sequence motifs, including the Walker A, Walker B, A-loop, D-loop, H-loop and Q-loop. In addition, it also contains the signature C motif, which is diagnostic of the ABC superfamily (George and Jones, 2013). TMDs have five to six membrane-spanning helices that offer substrate specificity and are involved in the transport of substrates across the membrane (Davidson et al., 2008). To date, ABC transporters have been identified in many species from human to invertebrate based on whole genome analysis. Broehan et al. identified 73 ABC transporters from *Tribolium castaneum*, which could be classified into eight subfamilies (A-H) (Broehan et al., 2013). In *Tetranychus urticae*, 103 ABC transporters have been found, which are the highest numbers discovered in a metazoan species (Dermauw et al., 2013). Due to the importance of ABC transport, several members of the ABC transporter families have been extensively researched in several model insects, including *Drosophila melanogaster* and *Bombyx mori* (Hock et al., 2000; Liu et al., 2011). Based on their functions, the ABC transporters can be divided into importers, exporters and non-transport proteins (Saurin et al., 1999). Importers and exporters are mainly associated with the transport of a wide variety of substances, while non-transport proteins are responsible for cellular processes such as DNA repair, translation and the regulation of gene expression. Interestingly, in recent years, ABC transporters were

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identified that could be interacted with alkaloids (Tajima et al., 2014). As described above, members of the ABC transporter gene superfamily have been identified in various species. However, no studies are reported to investigate the *S. c. ricini* ABC transporter genes, especially with regard to the way in which ABC transporters interact with DNJ.

DNJ is a glucose analogue with an NH group replacing the oxygen atom of the pyranose ring in mulberry latex, which is mainly distributed in mulberry latex (Kimura et al., 2004; Tsuduki et al., 2013). In recent years, DNJ has been reported to improve diabetic conditions by inhibiting the activity of α-glucosidase. Therefore, it has been used as a medical food to control postprandial blood glucose, thereby preventing diabetes mellitus (Lee et al., 1994; Mingrone et al., 1999). The eri-silkworm, *S. c. ricini*, belongs to the lepidopteran family Bombycidae and is a good model organism for biochemistry research (Kimura et al., 2004; Asai et al., 2011). In our previous research, DNJ had a positive impact on reverse glycometabolism by modulating glycometabolism and inhibiting glucogenesis and energy metabolism (Deng et al., 2015; Wen et al., 2016). On the contrary, what is the potentially defensive mechanism when the DNJ enters the eri-silkworm. In *D. melanogaster*, multiple transporters were up-regulated in response to dietary exposure to methotrexate (MTX). Increased expression levels of transporter genes might enhance the elimination of toxic compounds such as MTX or its metabolites (Chahine and O'Donnell, 2009). Ahmed et al. (1993) revealed that the *Bacillus subtilis* multidrug transporter Bmr effluxes structurally diverse toxic compounds out of bacterial cells. In addition, ABC transporters were found that play important roles in increasing efficacy and delaying resistance to Bt toxins (Heckel, 2012). Therefore, we also speculated that *S. c. ricini* ABC transporters might play a critical role in transporting DNJ from the intracellular to extracellular spaces.

Over the past several years, the next-generation sequencing technologies have made it possible to carry out genome-wide study of transcriptomes in a highly cost-effective way. They have been widely used to explore gene structure and expression profiling in model organisms (Wilhelm et al., 2003). The Trinity software was employed for the *de novo* assembly of full-length transcripts and evaluated it in some samples, whose reference genome was not yet available (Haas et al., 2013). Hence, transcriptome sequencing is an optimal method to discover genes in the eri-silkworm.

In this study, we identified 16 putative ABC transporters based on transcriptome sequencing, which were assigned to seven subfamilies (ABCA-H). We further characterised the gene motifs, and performed phylogenetic analysis using *B. mori*, *D. melanogaster* and *T. castaneum* to understand the evolutionary relationships between the seven subfamilies. In addition, we carried out RT-qPCR to analyse the expression patterns of the differentially expressed ABC transporter genes in response to DNJ. Our findings will provide useful information about the potential defence mechanism of *S. c. ricini* in response to DNJ.

Materials and methods

Eri-silkworm rearing and samples preparation

The eri-silkworm (*S. c. ricini*) B7 strain was obtained from the Sericultural Research Institute of Chinese Academy of Agricultural Sciences, Zhenjiang, China. The larvae were reared with fresh caster leaves at 27 ˚C under a relative humidity of 75% and a photoperiod of a 12:12 (L:D). Firstly, 90 newly moulted fifth-instar larvae were divided into three groups, and then fed with 5 μL 0.2% DNJ (J&K Chemicals), 0.1% DNJ and sterile water (Millipore, America). Ultimately, 500 μL larvae hemolymph were collected from control and different treatment groups at 6, 12 and 48 h, respectively. The freshly collected hemolymph was treated with a small amount of thiourea to prevent melanization, as well as 500 μL TRIzol (Invitrogen, Grand Island, NY, USA). All samples were stored at − 80 ˚C.

RNA extraction, library preparation, Illumina sequencing

Total RNA was extracted from 2% DNJ-treated and sterile water-treated eri-silkworm hemolymph for 12 h using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The $A_{260/A280}$ ratio and RNA concentration of all samples were detected using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, New York, NY) and confirmed using 1% agarose gel electrophoresis.

Fragment interruption, cDNA synthesis, the addition of adapters, PCR amplification and Illumina sequencing were completed by Beijing Novogene Bioinformatics Technology Co., Ltd. In total, 3 μg RNA per sample was used to construct cDNA library by a NEBNext® Ultra™ RNA Library Prep Kit from Illumina® (NEB, Ipswich, MA, USA) following the manufacturer's instructions, and index codes were added to attribute sequences in each sample. The library quality was assessed using an Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed with a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) based on the manufacturer's instructions. The libraries were sequenced using an Illumina HiSeq™ 2000 platform and generated 100 bp paired-end reads. The raw reads in fastq format were processed with in-house Perl scripts. Clean reads were obtained by removing reads containing adapters, poly-N, and low quality reads from the raw data. The Q20 (The percentage of bases with a Phred value > 20), Q30 (The percentage of bases with a Phred value > 30), and GC (base G and C) content of the clean data were calculated. Three biological replicates were performed to minimize sample differences.

Reads assembly and functional annotation

The reads assembly were performed according to the previous instructions (Haas et al., 2013). All these reads were concatenated into two files after paired-end sequencing. The left files (read1 files) from all samples were pooled into one large left.fq file, and right files (read2 files) were pooled into one large right.fq file. Transcriptome assembly was accomplished according to the left.fq and right.fq files by using Trinity software (Grabherr et al., 2011). The min_kmer_cov was set at 2 and all other parameters were set at default. Gene functions were annotated to the following databases: NR (NCBI non-redundant protein sequences); GO (Gene Ontology); KEGG (Kyoto Encyclopaedia of Genes and Genomes) and COG (Clusters of Orthologous Groups of proteins). All searches were performed with an E-value < 10^{-5}. Fragments per kilobase of transcript per million fragments mapped (FPKM) were calculated to represent the expression level of the unigenes.

Identification of ABC transporter genes and Bioinformatics analysis in S. c. ricini

In order to identify the ABC transporter genes from the *S. c. ricini* transcriptome database, a systematic BLASTP search was performed using arthropod ABC transport protein sequences available from NCBI (National Center for Biotechnology Information), with the cut-off set at E-value < e^{-20}. The open reading frames (ORFs) of the ABC genes were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Finally, the candidate ABC transporter genes were submitted to the NCBI protein database to search for ABC transporter domains. We used the program Pfam (http://pfam.xfam.org/) to identify the NBD and TMD structure (Punta et al., 2008). Clustering analysis of the ABC transporter genes was performed by the Genesis software provided by the Genesis team of Institute for Genomics and Bioinformatics, Graz University of Technology (Graz, Austria) (http://genome.tugraz.at/) (Sturn et al., 2002). We used the MEME software (http://meme.sdsc.edu) to identify the conserved motifs in the *S. c. ricini* ABC transporters using the following parameters: number of repetitions = any, maximum number of motifs = 10, and optimum motif width = 3 to 10 residues.