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Response of detoxification and immune genes and of transcriptome expression in *Mythimna separata* following chlorantraniliprole exposure

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ABSTRACT

The oriental armyworm *Mythimna separata* is a serious polyphagous pest in China and there are major efforts to control this pest. In the present study, an RNA-Seq method was used to explore transcriptome data of *M. separata* and identify the responses of genes to chlorantraniliprole. Sequencing and *de novo* assembly yielded 134,533 transcripts that were further assembled into 77,628 unigenes with an N50 length of 2165 bp. A total of 76 unigenes encoding insecticide targets were identified. Furthermore, 62 cytochrome P450s, 34 glutathione S-transferase (GSTs)and 64 carboxylesterase (CCEs) were curated to construct phylogenetic trees. In addition, we identified 647 the differentially expressed genes following treatment with chlorantraniliprole. The pathways of calcium signaling was identified as response to the pesticide The transcriptome data we generated represents a comprehensive genomic resource for further studies focused on control of *M. separata*. The response of genes to chlorantraniliprole treatment will elucidate the molecular mechanisms of insecticide resistance and allow for the development of new chemical pesticides to control this pest.

1. Introduction

The oriental armyworm, *Mythimna separata* Walker (Lepidoptera: Noctuidae) is a devastating corn pest in northern China. In addition to corn, *M. separata* is also a documented pest of > 300 crops, such as rice and sugarcane (Sharma et al., 2002; Jiang et al., 2011; Zeng et al., 2013). *M. separata* is a migratory pest, with outbreaks that can result in complete crop loss (Zhang et al., 2012; Jung et al., 2013; Jiang et al., 2014).

To date, control strategies for this insect pest are highly dependent on usage of chemical pesticides, especially those with high selective activity (Jiang et al., 2011; Rashid et al., 2013). Chlorantraniliprole belongs to the class of diamides targeting insect ryanodine receptors (RyRs), and has been widely used to control lepidopteran species (Ebbinghauskintscher et al., 2006; Kato et al., 2009; Li et al., 2013). However, the long-term use of this insecticide causes serious environmental problems and has also led to rapid development of resistance. The diamondback moth *Plutella xylostella* has developed > 1000-fold higher resistance to chlorantraniliprole compared to the susceptible strain (Wang and Wu, 2012). In contrast, several bioassays conducted in *M. separate* showed no resistance or lower resistance to chlorantraniliprole in different parts of China (Dong et al., 2014; Zhao et al., 2017). However, potential outbreaks and insecticide resistance are major problems associated with *M. separata* and both make control of this pest difficult. Hence, understanding the molecular mechanisms of *M. separata* in response to chlorantraniliprole is a key step to studying resistance, and can also be applied in predicting future resistance.

Despite its economic importance, there have been few studies of *M. separata* biology and physiology. The lack of genomic information for this pest hinders the study of gene expression patterns and functions, and thus makes it difficult to explore new methods for management. The transcriptome of *M. separata* was first reported in 2016 (Liu et al., 2016), however, the authors just described the outline of the transcriptome without detailed gene family descriptions, or functional analysis of specific biological processes. Hence, the first aim of the present study was to use next generation sequencing (NGS) methods to

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produce high quality transcriptome data of *M. separata* and identify genes involved in target proteins and potential insecticide detoxification enzymes. In addition, the discovery of differentially expressed genes would provide insights to reveal the molecular mechanism of *M. separata* responses to chlorantraniliprole treatment.

2. Materials and method

2.1. Insect samples

M. separata was originally purchased from Henan Jiyuan Baiyun Industry Company (Jiyuan Henan). Insects were maintained on an artificial diet in the laboratory at 26 ± 1 °C, with 70% relative humidity and a 16:8 (L:D) photoperiod without insecticide exposure for > 10 years.

2.2. Bioassay and sample collection

Twenty-five 3rd instar larvae were selected and starved for 12 h before bioassay using the feeding method. The larvae were fed with an artificial diet with different dosages of chlorantraniliprole. The control group was treated with an artificial diet containing *N, N*-Dimethylformamide (DMF). After 24 h, the mortality rate was recorded and corresponding LC_{20} values were calculated using Probit analysis in SPSS 18.0 (George and Mallery, 1998). The experiment was replicated three times.

Then, thirty larvae were treated with the LC_{20} concentration of chlorantraniliprole. Ten surviving larvae of each treatment 24 h post experiment were collected in one tube and snap-frozen in liquid N_2 and immediately stored at −80 °C until further use. The experiment was replicated three times. Hence three tubes of ten pooled larvae for treatment and three tubes for control were collected for further sequencing.

2.3. RNA isolation, library construction and Illumina sequencing

RNA isolation, cDNA synthesis, library construction and Illumina sequencing were all performed at Novogene Technology Ltd. (Beijing, China). Total RNA for each sample was isolated using the RNeasy micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For each sample, RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used for examining RNA integrity. The RIN value of treatment and control were 7.20 ± 0.45 and 9.03 ± 1.02, which reached the criterion for quality control. A total of 1.5 μg RNA was used to generate one RNA library per sample using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's instructions. The final products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2500 and paired-end reads were generated.

2.4. De novo assembly of sequencing reads and bioinformatics analysis

The low quality reads were removed with following standard: base with Phred quality score (Q_{phred}) < −20 occupied > 50% in total read. Processing of raw data (raw reads) started with removing reads containing adapters, poly-N and reads of low quality. Then the files from all libraries on the left file were pooled into one big left.fq file, and right files into one big right.fq file. Transcriptome assembly was accomplished based on the left.fq and right.fq using Trinity (Grabherr et al., 2011) with min_kmer_cov set to 2 by default and all other parameters set default.

All *de novo* assembled unigenes were annotated based on the following databases: Nr (NCBI non-redundant protein sequences) by diamond v0.8.22, with e-value = $1e^{-5}$, Nt (NCBI non-redundant nucleotide sequences) by NCBI blast 2.2.28+, with e-value = $1e^{-5}$, Pfam (Protein family) by HMMER 3.0 package, with hmmscan e-value = 0.01, KOG/COG (Clusters of Orthologous Groups of proteins) by diamond v0.8.22, with e-value = $1e^{-3}$, Swiss-Prot (A manually annotated and reviewed protein sequence database) diamond v0.8.22, with e-value = $1e^{-5}$, KO (KEGG Ortholog database)by KAAS, with KEGG Automatic Annotation Server e-value = 1e-10, and GO (Gene Ontology) by Blast2GO v2.5with e-value = $1e^{-6}$.

Gene expression levels were estimated by RSEM (RNA-Seq by Expectation-Maximization). Differential expression analysis was performed using the DESeq R package (1.10.1) in Trinity software. The resulting *P* values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P*-value < 0.05 found by DESeq were assigned as differentially expressed.

2.5. Analysis of insecticide target site and detoxification genes

Unigenes were curated against the Nr database with a cut-off *E*-value < $1.0\ E^{-5}$. The unigenes found in the same BLAST results or that shared high homologies to other unigenes were regarded as allelic variants or as different parts of the same gene. The gene families of cytochrome P450s, glutathione S-transferase (GSTs) and carboxylesterase (CCEs) were aligned at the amino acid level using the default settings in ClustalW 2.0 (Thompson et al., 2002). Proteins longer than 200 amino acids were used for further phylogenetic analysis in MEGA 6.0 (Tamura et al., 2011) with the neighbor-joining method and p-distance under default parameters. Bootstrap analysis with 1000 replications was performed to evaluate the branch strength of each tree.

2.6. Experimental validation

Sixteen candidate genes with different expression trends were selected randomly to test sequencing accuracy. Primers used for amplification are listed in Table S1, and the PCR procedure was conducted as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s (changed based on annealing temperature for each set of primers) and 72 °C for 1 min, then with a final extension step at 72 °C for 10 min. The RT-PCR amplicons of were eluted from the low melting agarose gel using QIAquick® Gel Extraction Kit (QIAGENE, USA). The purified PCR fragments were cloned in to pMD19-T vector (TaKaRa, China) and then transformed into competent cells of *Escherichia coli* DH5α. The primers sequences were exclusive from fragment sequences obtained in each isolates in this study, and then aligned with transcriptome data sequence with DNAMAN (Woffelman, 2004) and a BLAST search of the Nr database was conducted to double check the sequence. After confirmation of those gene sequences, qRT-PCR was performed to confirm the expression level. Total RNA was extracted using the same method mentioned in Section 2.2 before, and then 1 μg RNA was used to synthesize cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China) according to the kit instructions. Real-time PCR was conducted using the Takara SYBR Ex Taq premix reagent with the 'shuttle PCR' program and the primer sets shown in Table S1. At the end of the PCR reaction, a melt curve was generated to rule out the possibility of undesirable side products. The housekeeping gene *β-actin* was used as the reference gene. The fold change of target genes was calculated using the relative quantitative method ($2^{-\triangle\triangle Ct}$). Statistical analysis was performed using Duncan's Multiple Range Test for significance in SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).