Transcriptome analysis of molecular mechanism to pyrethroids resistance in *Spodoptera litura*

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Abstract

Background *Spodoptera litura* is a destructive agricultural pest and had evolved resistance to multiple insecticides, especially pyrethroids. At present, the resistance mechanism to pyrethroids remains unclear. Results Four field-collected populations, namely CZ, LF, NJ and JD, were identified to have high resistance to pyrethroids, and the resistant ratio ranged from 40.8 to 1764.0-fold. To explore pyrethroids resistance mechanism, the transcriptome between pyrethroid-resistant (LF and NJ) and pyrethroid-susceptible (GX) populations were compared by RNA-sequence. Results showed that multiple differential expressed genes were enriched in metabolism-related GO terms and KEGG pathways. 35 up-regulated metabolism-related genes were screened to verify by qRT-PCR. Consistent up-regulation of 13 unigenes, including 3 P450s, 4 GSTs, 1 UGTs, 4 COEs and 1ABC, were verified in the additional pyrethroids resistant populations CZ and JD. The expression level of CYP3 and GST3, which were homologous to CYP321A8 and GST1, respectively, showed good correlation with their pyrethroids resistance level among CZ, LF, NJ and JD populations. While the expression level of CYP12, CYP14, COE4 and ABC5 showed good correlation with their pyrethroids resistance level in at least three populations. UGT5 had the highest expression level among the tested UGT genes in the four pyrethroids resistant populations. Conclusion CYP3, CYP12, CYP14, GST3, COE4, UGT5 and ABC5 play important roles in pyrethroids resistance among the four field-collected populations. Our study provided a valuable resource for further study of pyrethroid resistance mechanisms in *S. litura*.

Background

The common cutworm, *Spodoptera litura* (Fabricius), is a polyphagous agricultural pest worldwide and caused enormous losses to many economical crops like cotton, soybean, tobacco and vegetables. Insecticides resistance developed rapidly in the last decades due to its extensively application. To date, *S. litura* has evolved high resistance to multiple conventional and new insecticides, which led to both failure control of pest and the destruction of natural enemies [1-6].

Insecticides resistance is the consequence of pest evolutionary adaptation to insecticides selection, a genetic phenomenon, which is conferred by target-site-based resistance (TSR) and non-target-based resistance (NTSR) mechanisms [7]. TSR is endowed by gene mutations in target enzymes, which can be easily detected by sequencing. Multiple mutations in sodium channel conferring resistance to pyrethroid insecticides had been identified in insect species, such as L1014F, L1014S, M918V, L925I, E435K, C785R, P1999L, F1538I, A1410V, M1524I, D1549V, T929I and M918T [8-9]. NTSR is achieved by mechanisms reducing insecticide concentration reaching the target-site, including metabolism-based resistance, which enhanced detoxification rate of insecticide by cytochrome P450 monooxygenase (P450), glutathione *S*-transferase (GST), carboxylesterases (COE), UDP-glycosyltransferase (UGT) and ATP-binding cassette transporters (ABC) [10]. Synergism is the primary step to identify the involvement of metabolism-related enzyme by piperonyl butoxide (PBO), diethyl maleate (DEM) and *S,S,S*-tributylphosphorothioate (DEF), which are inhibitors of P450, GST and COE, respectively [7]. The determination of putative gene expression and detoxification enzyme activity is also employed to explore the mechanism of metabolism-

based resistance. However, these methods only establishing the resistance phenotype is metabolic involved, fails to comprehensively identify the individual genes responsible for insecticide resistance.

The transcriptome is the complete set of all transcripts within a cell or tissue, that reflect the mRNA expression levels of different genes in a given sample [11]. RNA-Sequence (RNA-Seq) provided opportunity for the comprehensive analysis of pest transcriptome without reference genome in greater depth than ever before [12]. Although multiple researches have investigated the resistance of *S. litura* to various insecticides, molecular mechanism of metabolic resistance in *S. litura* is still not well defined due to lack of detailed genetic and sequence information [13-14]. In addition, pyrethroid insecticides were widely applied to control *S. litura* and other agricultural pests, for its high efficiency and low toxicity to mammalian animals. Yet excessive usage has imposed pyrethroids resistance the most severe situation [15]. To minimize the evolution of resistance and prolong the life time of insecticide, the molecular mechanisms for pyrethroids resistance in this pest should be declared. In this study, RNA-seq was performed by Illumina HiSeq™ 2000 sequencing platform and the transcriptome of pyrethroid-resistant and pyrethroid-susceptible populations were compared to provide new insights into mechanism of metabolism-based resistance to pyrethroids in *S. litura*.

Results

Toxicity of insecticides to *S. litura*

Eleven insecticides were chosen to test the toxicity to the larvae of GX, LF and NJ populations. High level resistance to fenvalerate, beta-cypermethrin and cyhalothrin were found, which were 809.5-, 141.3-, 1764.0-fold in LF population and 322.0-, 40.8-, 951.0-fold in NJ population, respectively, compared with that in GX population (Table 1-3). LF and NJ populations showed no resistance to phoxim, profenofos, chlorpyrifos and emamectin benzoate, with resistance ratio (RR) lower than 5.0-fold. However, low resistance had been developed to chlorantraniliprole, bromine cyantraniliprole, imidacloprid and methomyl, with RR ranged from 4.0 to 19.8-fold. The results above indicated that field-collect populations of *S. litura* had high level resistance to pyrethroid, which was urgent to be managed.

(Table 1-3 Insert here)

To determine the involvement of detoxification enzyme for pyrethroids resistance in *S. litura*, the synergists of P450, COE and GST, namely PBO, DEF and DEM, were used before pyrethroids treatment. Results showed that PBO increased pyrethroids toxicity distinctly in both LF and NJ populations, while DEF and DEM increased fenvalerate and cyhalothrin toxicity slightly and had no influence on beta-cypermethrin toxicity (Figure 1).

(Figure 1 Insert here)

Illumina sequencing and reads assembly

RNA-Seq was conducted with three cDNA libraries of GX, LF and NJ populations, respectively. Clean reads ranging from 51429712 to 60901328 per cDNA library were obtained by removing reads containing adapter, ploy-N and low quality reads from raw data (Table 4). According to the sequence analysis, almost 97% raw reads had Phred-like quality score at the Q20 level (an error probability of 1%), with GC content around 47%. Clean reads were used to assemble 140003 transcripts and 82014 unigenes ranging from 201 to 29587 bp (Table 5). Pearson correlation coefficients of different RNA samples in the same population ranged from 0.868 to 0.916, which was higher than 0.525 to 0.648 between different populations (Additional file 1).

(Table 4-5 Insert here)

Functional annotation of unigenes

Seven databases, (NCBI non-redundant protein sequences database (Nr), NCBI non-redundant nucleotide sequences data-base (Nt), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-port, KyotoEncyclopedia of Genes and Genomes (KEGG) and Geneontology (GO) database) were used for unigenes functional annotation. There are 47185 (57.53%) unigenes annotated in at least one databases, and 8974 (10.94%) unigenes annotated in all databases. Among the annotated unigenes, 50.24% hit in Nr database, 39.15% in GO database and 38.87% in Pfam database (Table 6). More than 90% annotated unigenes had nucleotides similarity above 60% (Additional file 2). The results of Nr database annotation indicated that *S. litura* has 41.3% sequence similarity to *Bombyx mori*, 16.2% to *Danaus plexippus*, 14.1% to *Plutella xylostella*, 1.9 % to *Helicoverpa armigera* and 1.6% to *Papilio xuthus* (Additional file 3).

(Table 6 Insert here)

GO assignments were used to predict gene function. Total 82014 unigenes were classified into 54 subgroups, including molecular function (MF), biological process (BP) and cellular component (CC). In BP, cellular process was the largest subgroup, followed by metabolic process and single-organism process. In CC, the largest two subgroups were cell and cell part, respectively. For MF, binding and catalytic activity formed the largest two subgroups (Figure 2).

(Figure 2 Insert here)

KEGG database was used for understanding high-level functions and utilities of the biological system from molecular-level information. 20164 unigenes were divided into five groups, among which 5622 unigenes (27.88%) were involved in organism systems; 5563 unigenes (27.59%) were involved in metabolism; 3023 unigenes (14.99%) were involved in environmental information processing; 3002 unigenes (14.89%) were involved in cellular processes; 2954 unigenes (14.65%) were involved in genetic information processing (Figure 3).

(Figure 3 Insert here)

Differential gene expression among LFvsGX and NJvsGX groups

In order to explore the molecular mechanism of pyrethroid resistance, differentially expressed genes (DEGs) were selected according to q -value <0.05 and $|\log_2(\text{Fold change})|>1$. Compared with the transcriptome of GX population, there are 18043 DEGs (10063 up-regulated and 7980 down-regulated) in LF population and 20370 DEGs (11147 up-regulated and 9223 down-regulated) in NJ population, respectively. And 11739 DEGs were found involved in both LFvsGX and NJvsGX groups, including 5720 up-regulated and 5442 down-regulated genes (Additional file 4).

GO and KEGG enrichment of the DEGs

To further analysis the function of DEGs, GO and KEGG enrichment analysis were conducted. According to GO enrichment, the up-regulated unigenes in LFvsGX group were enriched into multiple GO terms belonging to 20 BP, 5 CC and 20 MF; while 20 BP and 20 MF in NJvsGX group. In BP, the top three largest GO terms were metabolic process, single-organism process and oxidation-reduction process in both LFvsGX and NJvsGX groups. In MF, the top three largest GO terms were catalytic activity, hydrolase activity and oxidoreductase activity in LFvsGX group, while catalytic activity, oxidoreductase activity and peptidase activity in NJvsGX group (Figure 4).

(Figure 4 Insert here)

Much less unigenes were significantly enriched in the down-regulated GO terms in both LFvsGX and NJvsGX groups, including chitin metabolic process and chitin binding, which indicated that different epidermal penetration rate may be involved in pyrethroid resistant *S. litura* populations (Figure 5).

(Figure 5 Insert here)

Among the top-twenty enriched KEGG pathway terms in LFvsGX and NJvsGX groups, fourteen terms were shared in both groups, including metabolism of xenobiotics by cytochrome P450 and drug metabolism-cytochrome P450 pathways (figure 6). The results of GO and KEGG enrichment suggested that

metabolism was the main enriched terms of DEGs and should be a main reason of pyrethroids resistance in *S. litura*.

(Figure 6 Insert here)

Validation of the candidate DEGs

Based on the high reading count and significant high expression level in RNA-Seq, 14 P450, 5 COE, 5 GST, 5 UGT and 6 ABC up-regulated unigenes were screened out and validated by qRT-PCR. Among the 35 selected DEGs, 30 unigenes (including 14 P450, 5 GST, 5 UGT, 4 COE, and 1 ABC) were both significantly up-regulated by qRT-PCR validation in LFvsGX and NJvsGX groups (Table 7). CYP12 (annotated as CYP6AE43) had the highest expression level among all the P450 unigenes, with 358.27-fold in LF and 237.95-fold in NJ population. UGT5 (annotated as UGT33T2) had the highest expression level among all the UGT unigenes, with 309.49-fold in LF and 3236.54-fold in NJ population.

(Table 7 Insert here)

The expression of candidate DEGs in additional two field populations

CZ and JD are another two field populations with high resistance level to pyrethroid insecticides. Compared with GX population, CZ showed 84.5-fold resistance to fenvalerate, 11.5-fold resistance to beta cypermethrin and 678.5-fold resistance to cyhalothrin (Table 8), while JD showed 9123.5-fold resistance to fenvalerate, 642.0-fold resistance to beta cypermethrin and 6986.0-fold resistance to cyhalothrin (Table 9).

(Table 8-9 Insert here)

Based on the RR values, resistance level of JD>LF>NJ>CZ>GX could be concluded. To further validate candidate DEGs, the expression level of the up-regulated DEGs in LF and NJ populations were also determined by qRT-PCR in CZ and JD populations. Results indicated that 13 unigenes (CYP3, CYP12, CYP14, GST1, GST2, GST3, GST4, UGT5, COE1, COE2, COE4, COE5 and ABC5) were also significantly higher expressed in the two additional populations. UGT5 had the highest expression level among all the tested UGT genes in the four pyrethroids resistant populations. The expression level of CYP3 and GST3 among the four field-collected populations conformed well with their pyrethroid resistance level. While the expression level of CYP12, CYP14, COE4 and ABC5, showed good correlation with their pyrethroids resistant level in at least three field-collected populations (Table 10).

(Table 10 Insert here)

Discussion

Insecticides continue to be key weapon for insect control in agriculture. However, the evolution of insecticides resistance due to strong selection imposed by widespread insecticides usage threatens the weapons' availability. Studying the molecular mechanism of insecticides resistance, especially for metabolism-based resistance, provides valuable insights into how genetic, physiological and biochemical changes in insects and opens windows for improving future pest control. In the present study, the transcriptome of GX, NJ and LF populations were sequenced by Illumina platform with high criteria of data quality (Table 4), enabling assembling of 140003 transcripts and 82014 unigenes (Table 5-6). GO and KEGG enrichment analysis of DEGs indicated that LF and NJ populations have relative high ability in oxidation-reduction activity, hydrolase activity and P450 involved metabolic process than GX population (Figure 4, 6). Recently, Cheng et al. [16] conducted the genome sequencing of *S. litura*, and 138 P450, 47 GST, 110 COE and 54 ABC genes were annotated, which was consistent with our transcriptome results (Table 11). In addition, the validation of genes expression level showed that RNA-Seq results were well coincident with that of qRT-PCR (Table 10). Therefore, the RNA-Seq results were reliable and this information will facilitate further researches on pyrethroids resistance and other physiological processes in *S. litura*.

(Table 11 Insert here)

Bioassay result in LF and NJ populations of *S. litura* showed high level resistance to fenvalerate, beta cypermethrin and cyhalothrin (Table 1-3). However, resistance to beta cypermethrin was much lower than that to fenvalerate and cyhalothrin in both LF and NJ populations, which might result from different sets of detoxification enzymes to each insecticide. No cross-resistance between organophosphates and pyrethroids was found in LF or NJ populations, which is similar with the result reported by Huang et al [13]. While Tong et al. found high level resistance to both organophosphates and pyrethroids in five field *S. litura* populations during 2010 to 2012 [14]. This opposite phenomenon could be explained by metabolic-based activation and passivation of insecticides. Multiple studies had revealed a direct link between elevated P450 activities and organophosphate and pyrethroids resistances in insects [17]. However, some P450s can also be involved in the activation of insecticides, chemically modifying insecticides to biologically active forms, as is the case for some organophosphorus insecticides [7]. For example, chlorpyrifos can be converted to chlorpyrifos-oxon by P450 and chlorpyrifos-oxon is 100-fold toxicity than chlorpyrifos [18-19]. Thus different sets of up-regulated P450s in insects could cause cross resistance or negative cross resistance between organophosphates and pyrethroids.

Cytochrome P450 monooxygenases can mediate resistance to most insecticides for their genetic diversity, broad substrate specificity and catalytic versatility. GO enrichment of up-regulated DEGs in LFvsGX and NJvsGX groups showed that oxidation-reduction process and metabolic process were significantly enriched (Figure 4). Furthermore, P450s involved metabolism of xenobiotics and drug metabolism pathways were also enriched in KEGG enrichment analysis (Figure 6). Besides, P450s inhibitor (PBO) significantly increased the toxicity to pyrethroids in LF and NJ populations (Figure 1). Therefore, up-regulated P450s should be the major reason for pyrethroids resistance in LF and NJ populations. The qRT-PCR results indicated that most selected P450 genes were significantly over

expressed in the four resistant populations, especially for CYP2, CYP3, CYP10, CYP12 and CYP14 (Table 10). Furthermore, the expression level of CYP3, CYP12 and CYP14, which were annotated as CYP321A8, CYP6AE43 and CYP6AB31, respectively, showed good correlation with pyrethroids resistance level among GX, CZ, NJ, LF and JD populations. *CYP321A8* and *CYP6AB31* could be induced by deltamethrin, lambda-cyhalothrin and lambda-cyhalothrin, respectively, and had high expression level in midgut and fat body [20-22]. *CYP6AE43* of *S. frugiperda* could only be detected in the midgut and malpighian tubules [23]. However, there is building evidence that the key tissues for the metabolism of most compounds are midgut, malpighian tubules and fat body [23-24]. Therefore, P450 genes that had higher expression levels in these tissues may be involved in xenobiotic detoxification. Hence, most up-regulated P450s might be the set of pyrethroids detoxified genes in *S. litura*, especially for CYP3, CYP12 and CYP14 in consideration of the higher expression level and detoxify function.

COE were classified as Phase I detoxification enzymes and perform hydrolysis reaction at the first step of detoxification. It has been demonstrated that COE were involved in insecticide resistance, especially for organophosphates, pyrethroids and carbamates [25]. For instance, the over-expression of carboxylesterase E4 in *Myaues persicae* enhanced degradation and sequestration of pyrethroids, organophosphates and carbamates [26]. According to GO enrichment analysis of up-regulated DEGs, hydrolase activity was enriched in LF and NJ populations (Figure 4). Validation in qRT-PCR revealed that the 4 selected COEs were higher expressed in resistance populations, especially for COE4 (Table 10). Although only slight synergism to fenvalerate and cyhalothrin by DEF was observed (Figure 1), COE may also play an important role in pyrethroids resistance in consideration of the enrichment analysis and high expression level in the four resistance populations.

The products of Phase I reactions often become substrates for Phase II enzymes, such as GST and UGT, that add glutathione and glycosyl groups, respectively. Insect GSTs are a superfamily of detoxification enzymes and had been reported to be associated with resistance to pyrethroids, organophosphorus and organochlorine insecticides [27]. In contrast to organochlorine and organophosphorus resistance, GSTs are not involved in the direct metabolism of pyrethroids but rather are involved in the sequestration of pyrethroids and/or the detoxification of lipid peroxidation products induced by pyrethroids [28-29]. Functional study of GSTs in *Nilaparvata lugens* showed that *NIGSTD1* had peroxidase activity and confers pyrethroids resistance via gene amplification [29]. Two *S. litura* GST (*Slgste2* and *Slgste3*) were reported to be induced by carbaryl, Bt and DDT, which suggested that *Slgste2* and *Slgste3* may play roles in detoxifying various insecticides in *S. litura* [30]. Here, total 32 GSTs were annotated and mapped in *S. litura*, and 23 GST genes had high expression level in both LF and NJ populations. GST3, which was annotated as *GST1* and showed good correlation with the resistance level among the four resistant populations, was reported to possess high binding activities to deltamethrin [31], indicating the role of higher expression levels of GSTs in pyrethroids resistance in *S. litura*.

UGT mediated glycoside conjugation is an important metabolic pathway for the biotransformation of multiple lipophilic endogenous and exogenous compounds, including insecticides. Kaplanoglu et al. demonstrated that silencing of *CYP4Q3* and *UGT2* significantly increased susceptibility of resistant

beetles to imidacloprid, indicating that over-expression of these two genes contributes to imidacloprid resistance [32]. UGT5, annotated as UGT33T2, was significantly over expressed and had the highest expression level among the tested UGT genes in the four pyrethroids resistant populations. The involvement of UGT5 in pyrethroids resistance needs to be further studied.

Majority ABC proteins function as primary-active transporters that bind and hydrolyze ATP while transporting a large diversity of substrates across lipid membranes. Bariami et al. showed that ABCB4 was up-regulated in a pyrethroids resistant strain of *A. aegypti* [33]. RNA-seq analysis and qRT-PCR revealed that four ABC transporters were up-regulated in a deltamethrin resistant strain of *Cimex lectularius* and RNAi mediated knockdown of these ABC genes resulted in an increased susceptibility to betacyfluthrin in deltamethrin resistant female bed bugs [34]. Among the 6 selected ABC unigenes, only ABC5 (annotated as ABCB3) was significantly up-regulated in the four pyrethroids resistant populations, which indicated a possible role of ABC5 in pyrethroids resistance.

Decreased penetration rate was well documented to be involved in insecticides resistance [35]. Here, it was also cued by our GO enrichment results, where the down-regulated DEGs was enriched in chitin metabolism process, chitin binding, and structure constituent of cuticle term (Figure 5, Additional file 5).

Conclusions

A comprehensive comparison of metabolism related genes expression profiles between pyrethroid-resistant and pyrethroid-susceptible populations had been conducted by RNA-Seq and qRT-PCR. Metabolism-based resistance to pyrethroids was in a serious situation in *S. litura* and over-expressed P450s was the major reason for pyrethroids resistance based on the synergism experiments and enrichment analysis of DEGs. Other metabolism related genes like GSTs, COEs, UGTs and ABC transporters were also involved in pyrethroids resistance for their higher expression level in several resistant populations. Besides, different insecticide penetration may also account for pyrethroids resistance in *S. litura* for the down-regulated DEGs enriched in chitin metabolism process. And CYP3, CYP12, CYP14, GST3, UGT5, COE4 and ABC5 were the most valuable genes for further functional identification as their expression level showed good correlation with pyrethroids resistant level among the tested populations and the high expression level. Constitutive over-expression of multiple CYPs, COEs, GSTs, UGTs, and ABC transporters is associated with neonicotinoid resistance in the whitefly, *Bemisia tabaci* [36], *Lygus lineolaris* [37], and *Aphis gossypii* [38] as well as pyrethroids resistance in the house fly, *Musca domestica* [39]. Our study provides a comprehensive understanding that CYPs, COEs, GSTs, UGTs, and ABC transporters may associated with pyrethroids resistance in *S. litura*, which will greatly extend our understanding of metabolism-based insecticide resistance mechanism, and facilitate the further identification of pyrethroids resistance involved genes.

Methods

Insect culture and bioassay

GX population of *S. litura* was kindly provided by Guangxi Tianyuan Biochemistry Corp., Ltd and served as susceptible population. It had been reared on artificial diet in the laboratory without exposure to any insecticide for more than 70 generations. LF, NJ, CZ and JD populations were collected from Langfang, Hebei province, Nanjing, Changzhou, Jiangsu province, and Shanghai respectively. The rear condition was $26\pm 1^{\circ}\text{C}$ and 60-70% relative humidity with a 14:10 h of light:dark photoperiod. Insecticides were diluted to 7 concentrations and 36 larvae were included in each concentration. One microlitre insecticide in acetone was applied on the thoracic dorsum of the third instars larvae by Hamilton syringe. For synergism experiments, PBO, DEF, and DEM were applied 1 h before insecticide treatment at $10\text{ }\mu\text{g larva}^{-1}$ (dissolved in acetone), respectively, and pyrethroid insecticides were applied at the tenth of LD_{50} . Control groups were applied with PBO, DEF, DEM alone. Larvae were held individually in a 12-well tissue culture plates with artificial diet and the mortality was checked 48 or 72 h after treatment.

RNA extraction and quality determination

Total RNA was extracted from the third instars larvae (18 larvae per group) using TaKaRa MiniBEST Universal RNA Extraction Kit (Takara) according to the manufacturer's instructions. The integrity and quality of the total RNA were examined by RNA Nano 6000 Assay Kit, Agilent 2100 and 1% agarose gels. Three biological replicates were prepared in each population.

Library preparation for transcriptome sequencing

Total 3 μg RNA per sample was used as input material for RNA sample preparations. Sequencing libraries were generated using NEBNext[®] Ultra[™] RNA Library Prep Kit for Illumina[®] (NEB, USA) following manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H⁻). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. To select cDNA fragments of preferentially 150-200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The 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, nine libraries (GX1, GX2, GX3, LF1, LF2, LF3, NJ1, NJ2, NJ3) preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated (Novogene, China).

Quality control, Transcriptome assembly and Gene functional annotation

Clean data were obtained by removing reads containing adapter, reads containing poly-N and low quality reads from raw data. Q20, Q30, GC-content and sequence duplication level of the clean data were calculated and all the downstream analyses were conducted based on clean data with high quality. Transcriptome assembly was accomplished using Trinity [40] with min_kmer_cov set to 2 by default and all other parameters set default. Gene function was annotated based on Nr, Nt, Pfam, Swiss-Prot database and KOG/COG, with a significant threshold of $E\text{-value} \leq 10^{-5}$. The GO terms for functional categorization were analyzed using Blast2go software with the $E\text{-value}$ threshold $\leq 10^{-6}$. The pathway assignments were carried out by sequence searches against the KEGG database, with the $E\text{-value}$ threshold $\leq 10^{-10}$.

Differential expression analysis of unigenes

Differential expression analysis between the three populations was performed using the DESeq R package (1.10.1). DESeq provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted $p\text{-value} < 0.05$ found by DESeq were assigned as differentially expressed.

GO enrichment analysis of the DEGs was implemented by the Goseq R packages based on Wallenius non-central hyper-geometric distribution [41]. KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways [42].

Validation of the RNA-Seq DEGs

To validate the transcriptional results from RNA-Seq and computational analysis, 35 unigenes were selected for quantitative RT-PCR (qRT-PCR). *EF1a* and *RPL 10* were used as housekeeping genes to normalize the expression of each gene [43]. First strand cDNA was synthesized from 1 μg total RNA using FastQuant RT Kit (With gDNase) (TIANGEN, Beijing, China) according to the manufacturer's protocol. Primers were designed based on the measured mRNA sequences using Primer Premier 5.0 software and are summarized in Additional file 6. SuperReal PreMix Plus (SYBR Green, TIANGEN, Beijing, China) was used in a 20 μl system with 2 $\times$ Super real SYBR Mix (10 μl), cDNA (1 μl), 50 $\times$ ROX (0.4 μl) and each primer (0.6 μL , 10 μM) by ABI Prism 7500 Real-Time PCR System (Applied Biosystems by Life Technologies, Foster, CA, USA). Thermal cycling was run at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 60°C for 32 s. A melting curve was added as a final step to make sure the PCR product was unique and specific. Each test was repeated with 3 independent mRNA samples and each reaction was carried out in triplicate.

Statistical analysis

The data of bioassay was analyzed with SPSS 16.0 (SPSS, Chicago, IL, USA). The results of qRT-PCR were calculated with the Ct value on 7500 software v2.3 (Applied Biosystems by Life Technologies, Foster, CA, USA) according to the $2^{-\Delta\Delta C_t}$ method. Student t-test was performed to analyze the statistical difference between means. A p -value<0.05 was considered statistically significant.

Abbreviations

ABC, ATP-binding cassette transporters; BP, biologic process; CC, cellular component; DEF, S,S,S-tributylphosphorothioate; DEGs, differentially expressed genes; DEM, diethyl maleate; COE, carboxylesterases; GO, gene ontology; GST, glutathione S-transferase; KEGG, Kyoto encyclopedia of genes and genomes; KOG/COG, clusters of orthologous groups of proteins; MF, molecular function; NCBI, National Center for Biotechnology Information; Nr, NCBI non-redundant protein sequences database; Nt, NCBI non-redundant nucleotide sequences database; NTSR, non-target-site based resistance; P450, cytochrome P450 monooxygenase; PBO, piperonyl butoxide; Pfam, protein family; RR, resistance ratio; RNA-Seq, RNA-sequence; SRA, sequence read archive; TSR: target-based resistance; UGT, UDP-glycosyltransferase.

Declarations

Acknowledgements

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The raw Illumina sequence reads have been deposited in the NCBI Sequence Read Archive (SRA) database with accession number PRJNA541123.

Authors' contributions

This study was designed and performed by DZL and LX. DZL analyzed the sequencing data, performed qRT-PCR validations and wrote the manuscript. The collection of insect materials was conducted by YM and JHW. XLC and CJW provided helpful suggestion in data analysis and manuscript revision. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This research did not involve any human subjects, human material, or human data. *S. litura* in current research did not belong to the endangered or protected species.

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Tables

Table 1. Toxicity of different insecticides to GX population

Insecticides	GX			
	Slope (SE)	LD ₅₀ (95% FI) (µg/larva)	R ²	RR
Fenvalerate	2.592 (0.289)	0.004 (0.003-0.005)	0.951	1.0
Beta cypermethrin	3.238 (0.364)	0.004 (0.002-0.011)	0.833	1.0
Cyhalothrin	2.346 (0.274)	0.002 (0.001-0.002)	0.957	1.0
Phoxim	2.238 (0.334)	0.219 (0.122-0.540)	0.879	1.0
Profenofos	3.226 (0.508)	0.065 (0.052-0.080)	0.979	1.0
Chlorpyrifos	1.617 (0.286)	0.243 (0.175-0.349)	0.948	1.0
Chlorantraniliprole	3.848 (0.586)	0.001 (0.001-0.002)	0.970	1.0
Bromine cyantraniliprole	1.064 (0.157)	0.002 (0.001-0.003)	0.938	1.0
Imidacloprid	1.882 (0.273)	0.573 (0.425-0.784)	0.965	1.0
Methomyl	1.743 (0.367)	0.010 (0.004-0.015)	0.853	1.0
Emamectin benzoate	1.524 (0.440)	0.011 (0.004-0.016)	0.798	1.0

Resistance ratio (RR) = LD₅₀ value of field population/ LD₅₀ value of GX population

Table 2. Toxicity of different insecticides to LF population

Insecticides	LF			
	Slope (SE)	LD ₅₀ (95% FI) (µg/larva)	R ²	RR
Fenvalerate	1.312 (0.302)	3.238 (1.933-4.804)	0.925	809.5
Beta cypermethrin	1.962 (0.290)	0.565 (0.410-0.753)	0.929	141.3
Cyhalothrin	1.063 (0.223)	3.528 (2.129-5.941)	0.971	1764.0
Phoxim	1.752 (0.298)	0.092 (0.058-0.128)	0.978	0.4
Profenofos	4.018 (0.659)	0.118 (0.096-0.143)	0.999	1.8
Chlorpyrifos	1.879 (0.375)	0.086 (0.039-0.129)	0.933	0.4
Chlorantraniliprole	1.777 (0.267)	0.004 (0.003-0.005)	0.960	4.0
Bromine cyantraniliprole	1.583 (0.261)	0.011 (0.008-0.017)	0.997	5.5
Imidacloprid	3.120 (0.487)	3.298 (2.604-4.126)	0.991	5.8
Methomyl	1.250 (0.178)	0.048 (0.008-0.168)	0.830	4.8
Emamectin benzoate	1.090 (0.253)	0.028 (0.008-0.052)	0.981	2.6

Table 3. Toxicity of different insecticides to NJ population

Insecticides	NJ			
	Slope (SE)	LD ₅₀ (95% FI) (µg/larva)	R ²	RR
Fenvalerate	1.330 (0.339)	1.288 (0.379-2.172)	0.981	322.0
Beta cypermethrin	1.679 (0.351)	0.163 (0.073-0.249)	0.901	40.8
Cyhalothrin	1.347 (0.264)	1.902 (1.176-2.927)	0.990	951.0
Phoxim	2.911 (0.410)	0.121 (0.095-0.151)	0.986	0.6
Profenofos	2.120 (0.451)	0.253 (0.172-0.343)	0.969	3.9
Chlorpyrifos	2.125 (0.297)	0.095 (0.072-0.126)	0.957	0.4
Chlorantraniliprole	1.662 (0.254)	0.005 (0.003-0.007)	0.978	5.0
Bromine cyantraniliprole	1.329 (0.236)	0.017 (0.011-0.027)	0.898	8.5
Imidacloprid	1.210 (0.462)	11.33 (5.887-66.473)	0.920	19.8
Methomyl	1.985 (0.326)	0.077 (0.057-0.115)	0.962	7.7
Emamectin benzoate	1.952 (0.293)	0.030 (0.021-0.041)	0.976	2.7

Table 4. The sequencing data quality of nine *S. litura* samples

Sample	Raw Reads	Clean Reads	Clean Bases (G)	Error (%)	Q20 ^a (%)	Q30 ^b (%)	GC Content (%)
GX1	62007376	59467694	8.92	0.02	96.74	91.94	47.37
GX2	63447084	60901328	9.14	0.02	96.83	92.10	47.14
GX3	54466924	52267042	7.84	0.02	96.84	92.11	47.23
LF1	61103822	58580638	8.79	0.02	96.68	91.78	46.39
LF2	59399040	57018846	8.55	0.02	96.88	92.21	46.44
LF3	55142308	52908196	7.94	0.02	96.78	91.98	46.23
NJ1	53662738	51429712	7.71	0.02	96.58	91.56	46.54
NJ2	53682236	51474730	7.72	0.02	96.73	91.88	46.51
NJ3	61665510	59119284	8.87	0.02	96.90	92.22	46.34

^aQ20: the percentage of bases with a Phred value > 20

^bQ30: the percentage of bases with a Phred value > 30

Table 5. Summary of assembly quality of *S. litura* RNA-seq

Assembly quality parameters	Transcript	Unigene
Length 200-500bp	79168	22583
Length 500-1kbp	23312	21928
Length 1-2kbp	16865	16846
Length ≥2kbp	20658	20657
Min length	201	201
Median length	419	876
Mean length	1039	1571
Max length	29587	29587
N50 value ^a	2377	2790
N90 value ^b	340	637
Total number	140003	82014
Total nucleotides	145522193	128847016

^aN50, 50 % of the assembled bases were incorporated into sequences with length of N50 or longer

^bN90, 90 % of the assembled bases were incorporated into sequences with length of N90 or longer

Table 6. Gene annotation by BLAST-searching against public databases

Public database	Number of unigenes	Percentage (%)
Annotated in NR	41212	50.24
Annotated in NT	22566	27.51
Annotated in KO	17722	21.60
Annotated in SwissPort	30369	37.02
Annotated in PFAM	31887	38.87
Annotated in GO	32111	39.15
Annotated in KOG	22893	27.91
Annotated in all databases	8974	10.94
Annotated in at least one database	47185	57.53
Total unigenes	82014	100.00

Table 7. qRT-PCR validation of pyrethroids resistance related candidate DEGs in LF/GX and NJ/GX groups

Access Number (NR)	Function annotation	Species	Expression fold of LF/GX		Expression fold of NJ/GX	
			RNA-seq	Validation	RNA-seq	Validation
AKH15488.1	CYP9A40	<i>Spodoptera litura</i>	663.43	1.82*	76.13	1.72*
ADA68173.1	CYP6B29V1	<i>S. litura</i>	11.59	20.65*	18.86	30.16*
AGO62006.1	CYP321A8	<i>Spodoptera frugiperda</i>	53.41	41.51*	24.31	32.71*
AFP20588.1	CYP6AE47	<i>Spodoptera littoralis</i>	8.68	5.80*	3.01	1.99*
AID54863.1	CYP340G1	<i>Helicoverpa armigera</i>	14.20	15.15*	5.10	5.85*
ADA68175.1	CYP321B1	<i>S. litura</i>	5.39	6.43*	4.94	9.59*
AID54877.1	CYP4G9	<i>H. armigera</i>	217.34	2.03*	550.81	2.27*
AID54870.1	CYP354A4	<i>H. armigera</i>	408.08	2.67*	395.66	1.86*
BAM73813.1	CYP6AB4	<i>Bombyx mori</i>	3.06	1.73*	3.67	1.62*
AGO62000.1	CYP4G74	<i>S. frugiperda</i>	226.10	11.28*	590.83	44.46*
AFP20600.1	CYP4S8V1	<i>S. littoralis</i>	2.73	1.92*	3.96	2.37*
AID55427.1	CYP6AE43	<i>S. frugiperda</i>	11.26	358.27*	7.10	237.95*
AAL48300.1	CYP4L4	<i>Mamestra brassicae</i>	3.16	2.73*	3.99	3.50*
AFP20591.1	CYP6AB31	<i>S. littoralis</i>	5.42	2.66*	6.25	2.77*
AIH07591.1	Sigma 4	<i>S. litura</i>	8.64	7.24*	9.36	10.70*
AIH07594.1	Delta 1	<i>S. litura</i>	4.82	4.86*	3.93	5.72*
AAS79891.1	GST1	<i>S. litura</i>	44.61	67.31*	22.61	48.24*
AIH07601.1	Omega 2	<i>S. litura</i>	2.52	2.49*	3.01	2.48*
AIH07597.1	Delta 4	<i>S. litura</i>	2.30	1.94*	1.83	1.68*
AEW43126.1	UGT40F2	<i>H. armigera</i>	17.93	20.49*	5.62	9.16*
AHY99686.1	UGT40R3	<i>S. littoralis</i>	6.50	4.39*	3.79	2.37*
AHY99688.1	UGT41D2	<i>S. littoralis</i>	4.80	4.52*	6.49	6.81*
AHY99682.1	UGT33J2	<i>S. littoralis</i>	7.32	4.31*	13.63	11.10*
AHY99683.1	UGT33T2	<i>S. littoralis</i>	15.85	309.49*	53.45	3236.54*
ADR64697.1	CXE13	<i>S. litura</i>	5.95	4.21*	2.77	2.33*
AFI64313.1	Acidic lipase	<i>H. armigera</i>	7.60	7.56*	4.89	5.75*
ADF43475.1	CCE014a	<i>H. armigera</i>	107.96	0.32	111.99	0.47
AFI64313.1	Acidic lipase	<i>H. armigera</i>	53.93	87.57*	13.56	27.89*
XP_004924046.1	Arylsulfatase B-like	<i>B. mori</i>	12.30	6.51*	2.45	2.13*
AKC34899.1	ABCC1-like	<i>S. litura</i>	2.67	1.03	3.13	1.30
AKC34055.1	ABCC3	<i>S. litura</i>	1.77	1.07	2.47	0.94
AIB06821.1	ABCC2	<i>Spodoptera exigua</i>	1.51	1.09	2.63	2.00*
XP_012549772.1	MDR	<i>B. mori</i>	1.79	1.21	1.94	1.38
ADV76538.1	ABCB3	<i>T. ni</i>	4.73	6.30*	10.53	16.84*
XP_012544694.1	ABCCX2	<i>B. mori</i>	1.98	1.01	1.68	1.11

Table 8. Toxicity of pyrethroids insecticide to CZ population

Insecticides	CZ			
	Slope (SE)	LD ₅₀ (95% FI) (µg/larva)	R ²	RR
Fenvalerate	1.119 (0.266)	0.338 (0.101-0.609)	0.952	84.5
Beta cypermethrin	1.462 (0.333)	0.046 (0.014-0.083)	0.991	11.5
Cyhalothrin	1.006 (0.254)	1.357 (0.619-2.581)	0.966	678.5

Table 9. Toxicity of pyrethroids insecticide to JD population

Insecticides	JD			
	Slope (SE)	LD ₅₀ (95% FI) (µg/larva)	R ²	RR
Fenvalerate	1.603(0.253)	36.496 (25.858-51.746)	0.956	9123.5
Beta cypermethrin	1.745(0.265)	2.598 (1.898-3.702)	0.982	642.0
Cyhalothrin	1.213(0.239)	13.972 (7.025-21.737)	0.951	6986.0

Table 10. Relative expression level of candidate DEGs among GX, CZ, LF, NJ and JD populations.

Gene name	Relative expression level				
	GX	CZ	NJ	LF	JD
CYP1	1.00	1.32	1.72	1.82	1.28
CYP2	1.00	0.80	30.16	20.65	12.41
CYP3	1.00	2.31	32.71	41.51	66.14
CYP4	1.00	1.20	1.99	5.80	5.51
CYP5	1.00	2.15	5.85	15.15	1.90
CYP6	1.00	1.43	9.59	6.43	3.60
CYP7	1.00	0.19	2.27	2.03	0.57
CYP8	1.00	2.14	1.86	2.67	1.28
CYP9	1.00	1.79	1.62	1.73	1.41
CYP10	1.00	1.34	44.46	11.28	5.01
CYP11	1.00	1.95	2.37	1.92	1.08
CYP12	1.00	118.32	237.95	358.27	270.86
CYP13	1.00	1.56	3.50	2.73	1.10
CYP14	1.00	2.60	2.77	2.66	3.02
GST1	1.00	2.84	10.70	7.24	5.26
GST2	1.00	3.55	5.72	4.86	3.40
GST3	1.00	5.57	48.24	67.31	86.90
GST4	1.00	2.01	2.48	2.49	2.32
GST5	1.00	1.85	1.68	1.94	1.86
UGT1	1.00	1.12	9.16	20.49	11.95
UGT2	1.00	1.21	2.37	4.39	3.62
UGT3	1.00	0.75	6.81	4.52	2.55
UGT4	1.00	0.37	11.102	4.313	4.464
UGT5	1.00	255.66	3236.54	309.49	4.32
COE1	1.00	2.63	2.32	4.21	2.12
COE2	1.00	5.96	5.75	7.56	5.84
COE3	1.00	1.68	2.80	1.82	0.74
COE4	1.00	20.96	27.89	87.57	13.92
COE5	1.00	3.67	2.13	6.51	3.48
ABC1	1.00	1.84	1.30	1.03	1.38
ABC2	1.00	1.74	0.94	1.07	1.88
ABC3	1.00	0.84	2.00	1.09	0.61
ABC4	1.00	3.98	1.38	1.21	1.68
ABC5	1.00	2.54	16.84	6.30	6.75
ABC6	1.00	0.98	1.11	1.01	1.00

Table 11. The number and expression level of metabolism related genes among GX, LF and NJ populations

Classes of Enzyme	Total Number	Expression level of LF/GX $\pm$ 1	Expression level of NJ/GX $\pm$ 1	Expressed higher both in LF and NJ	
P450	139	101		97	65
GST	32	27		29	23
COE	77	50		46	35
UGT	38	32		33	25
ABC	60	47		45	35

Additional File Legend

- Additional file 1: Pearson correlation between susceptible (GX) and resistant populations (LF and NJ). (PDF 132 kb)
- Additional file 2: Similarity distribution of sequence comparison by BLAST search. (PDF 67 kb)
- Additional file 3: Species that *Spodoptera litura* unigenes were annotated by BLAST search in Nr database. (PDF 72 kb)
- Additional file 4: The Venn diagram of differential expression genes between LFvsGX and NJvsGX groups. (A) The Venn diagram of up-regulated genes between LFvsGX and NJvsGX groups; (B) The Venn diagram of down-regulated genes between LFvsGX and NJvsGX groups. (PDF 71 kb)
- Additional file 5: Directed acyclic graph of down-regulated differential expression genes enriched GO terms. (A) Directed acyclic graph of down-regulated differential expression genes enriched GO terms in LFvsGX group; (B) Directed acyclic graph of down-regulated differential expression genes enriched GO terms in NJvsGX group. (PDF 200 kb)
- Additional file 6: Primers used for qRT-PCR validation. (XLSX 16 kb)

Figures

Figure 1

Synergists effect of PBO, DEF and DEM to pyrethroid insecticides in LF(A) and NJ(B) populations.

Figure 2

Gene ontology (GO) classification of unigenes function. The unigenes were summarized in subgroups of biological process, cellular component and molecular function.

KEGG Classification

Figure 3

Pathway classified by KEGG annotation. The y-axis represented KEGG pathways. The x-axis indicated the percentage of a specific category of genes in each main classification. According to participation in KEGG pathways, genes were divided into five groups: A, cellular processes; B, environmental information processing; C, genetic information processing; D, metabolism; E, organism systems.

Figure 4

GO enrichment of up-regulated unigenes in LFvsGX (A) and NJvsGX (B) groups.

Figure 5

GO enrichment of down-regulated unigenes in LFvsGX (A) and NJvsGX (B) groups.

Figure 6

KEGG enrichment of up-regulated unigenes in LFvsGX (A) and NJvsGX (B) groups. The arrows indicate xenobiotic compounds metabolism related pathways.

Supplementary Files

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